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Isolation of Phospholipase D Mutants Having Phosphatidylinositol-Synthesizing Activity with Positional Specificity on *myo*-Inositol

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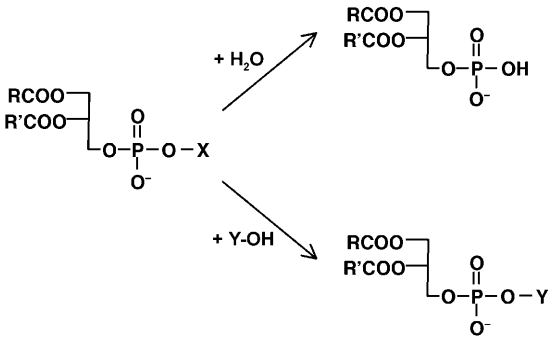
Phospholipase D (PLD) mutants that have phosphatidylinositol (PI)-synthesizing activity with positional selectivity towards 1- or 3-OH groups of myo*-inositol have been isolated. A mutant PLD library, in which site-directed saturation mutations were introduced in vitro at positions 187, 191, and 385 of the wild-type PLD of* Streptomyces antibioticus*, was screened for PI-synthesizing mutants. TLC and HPLC analyses of the PI synthesized by the isolated mutant PLDs revealed that three mutants, namely 187D/191Y/385R (DYR), 187A/191Y/385R (AYR), and 187M/191Y/385R (MYR), selectively generated 1- or 3-PI among the other possible PI positional isomers. Taking into account the consensus sequence of the three mutants, a series of mutants, 187X/191Y/385R (XYR), was constructed and analyzed. Almost all the XYR mutants generated 1(3)-PI selectively, thus suggesting that the Y385R mutation contributed to the selectivity for the 1(3)-PI synthesis. The XYR mutants showed similar phosphatidylcholine-hydrolyzing activity among the mutants, but the PI-synthesizing activities were different depending on the amino acid at position 187. In particular, aromatic amino acids at position 187 greatly reduced the PI-synthesizing activity. The ratios of 1-PI versus 3-PI in the PIs synthesized with the XYR mutants were analyzed by selective hydrolysis with PI-specific phospholipase C. It was found that 187H/191Y/385R (HYR) generated 1-PI more than 3-PI (ratio = 7:3), whereas 187T/191Y/385R (TYR) generated 1-PI less than 3-PI (ratio = 2:8). This confirmed that the amino acid at position 187 determined the selectivity between 1-PI and 3-PI formation.*

Introduction

Phosphatidylinositol (PI) is a kind of naturally occurring phospholipid found mainly in eukaryotes. PI is now attracting attention because of its biological effects, such as increasing high-density lipoprotein–cholesterol levels in humans and decreasing triacylglycerol levels in the serum and liver of mice.[1] Currently, PI is produced by extraction from natural sources such as soybean lecithin. However, the PI contents of such natural sources are not very high (approximately 15% in soybean phospholipids),[2] and the extraction process requires a large amount of solvents. Furthermore, the PI extracted from natural sources is a heterogeneous mixture of PI molecular species with different fatty acid residues; this can be problematic if the PI is intended for use as fine chemicals.

An alternative for supplying PI is the use of phospholipase D (PLD, E.C. 3.1.4.4.). This enzyme hydrolyzes phospholipids into phosphatidic acid (PA) and the corresponding alcohols, but also catalyzes transphosphatidylation, in which the polar head groups of phospholipids are changed into coexisting alcohols (Scheme 1). PLDs from microorganisms such as *Streptomyces* are widely used for industrial phospholipid syntheses, because of their broad substrate specificity towards the alcohol compounds. Most natural types of phospholipid can be synthesized from commercially available lecithin or phosphatidylcholine (PC) and appropriate acceptor alcohols by using *Streptomyces* PLD.[3]

Until recently, no *Streptomyces* PLD was capable of synthesizing PI, possibly because of steric hindrance towards the bulky inositol molecule in the substrate binding pocket, and also because of relatively lower activity towards secondary alcohols than towards primary alcohols.



Scheme 1. Reactions catalyzed by PLD.

In our previous study,[4] we successfully isolated mutant PLDs with PI-synthesizing activity. This work was performed by introducing site-directed saturation (random) mutagenesis into

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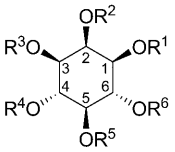
the amino acid residues that might be involved in substrate recognition (namely W187, Y191, and Y385) to construct a mutant enzyme library. Then the library was screened for PI-synthesizing activity, and mutant PLDs with the desired activity were isolated.

Although the isolated mutant PLDs were promising for enzymatic PI synthesis, there was a drawback in that the mutant enzymes had low positional specificity towards the hydroxy groups of *myo*-inositol. Since *myo*-inositol has six chemically nonequivalent hydroxy groups, there were potentially six structures in the enzymatically synthesized PI (Scheme 2). In fact, the isolated mutant enzymes generated mixtures of several PI isomers. In application of the mutant PLDs for PI synthesis, it is desirable to isolate other mutant PLDs that can generate particular PI isomers specifically.



	R^1	R^2	R^3	R^4	R^5	R^6
1-PI	Ptd	H	H	H	H	H
2-PI	H	Ptd	H	H	H	H
3-PI	H	H	Ptd	H	H	H
4-PI	H	H	H	Ptd	H	H
5-PI	H	H	H	H	Ptd	H
6-PI	H	H	H	H	H	Ptd

Scheme 2. The structure of PI isomers. "Ptd" denotes the phosphatidyl group.

In this paper, as a continuation of the previous work, we focused on isolating mutant PLDs with higher positional specificity towards the 1- or 3-position of the *myo*-inositol ring.

Results

Screening for PI-synthesizing mutants with higher positional specificity

In the previous study,[4] 25 PI-synthesizing PLD mutants were identified by screening approximately 10000 recombinant *Escherichia coli* clones from the mutant PLD library. The mutants were able to synthesize PI, but most of them formed mixtures of PI positional isomers. As the library was constructed by introducing saturation mutagenesis into three amino acid residues (W187, Y191, and Y385) of wild-type (WT)-PLD, it contained 8000 ($=20^3$) clones. Hence, theoretically, at least 23964 colonies should be screened to cover 95% of this library size.[5] Thus, we expected that further screening the library would isolate new PI-synthesizing mutants with enhanced positional specificity.

High-throughput screening of approximately 18000 colonies for PI-synthesizing mutants identified 375 positive clones. Crude enzyme from each of the clones was tested by TLC for PI-synthesizing activity by using dioleoyl-PC (DOPC) and *myo*-inositol as the substrates to exclude false positives. After DNA sequencing of the resulting positives to reduce redundancy, we identified different 65 clones. Together with the previous 25 mutants, the final lineup contained 90 mutants.

TLC analysis of the PI synthesized with each of the mutant PLDs revealed that three mutants, namely 187D/191Y/385R (DYR), 187A/191Y/385R (AYR), and 187M/191Y/385R (MYR), generated specifically 1-PI or 3-PI (Figure 1A). Figure 1A also

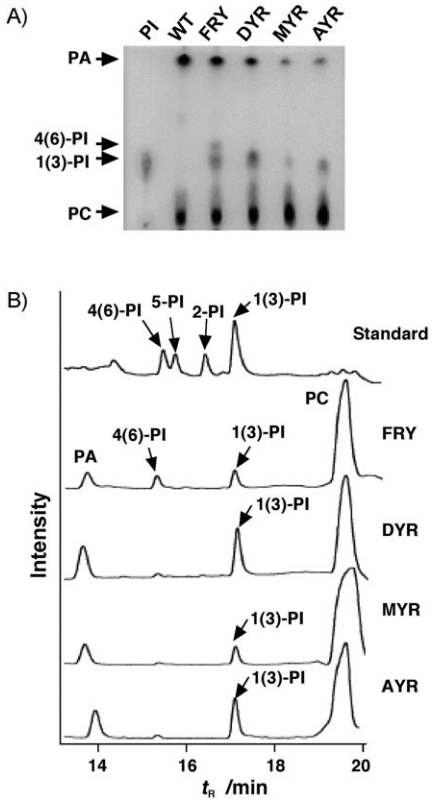


Figure 1. Analysis of the PI isomers synthesized by the mutant enzymes. A) TLC analysis of the phospholipids generated by WT-PLD and the mutants. PI denotes the soybean PI as the standard. B) HPLC analysis of the PI standard isomers and the products generated by the PLD mutants (FRY, DYR, MYR and AYR).

shows that WT-PLD did not generate PI at all, and that 187F/191R/385Y (FRY, one of the previously isolated mutants) formed two spots of 1(3)-PI and 4(6)-PI. The selectivity of the three mutants was confirmed more quantitatively by HPLC analysis (Figure 1B). The 1(3)-PI/4(6)-PI ratio of the three mutants calculated from the peak areas were 95:5 for DYR and AYR, and 99:1 for MYR. None of these enzymes generated 2-PI or 5-PI. A common feature in these three mutants was the mutation "Y385R", thus indicating that this mutation might contribute to the selectivity for 1(3)-PI synthesis.

Saturation mutagenesis at position 187

Although the Y385R mutation might play a role in determining the specificity of 1(3)-PI synthesis, the role of the mutation at the position 187 remained unclear. To evaluate its role, saturation mutagenesis was carried out to prepare 187X/191Y/385R or "XYR" mutants (X denotes any amino acid).

The PI-synthesizing and PC-hydrolyzing activities of these XYR variants were tested by using crude enzymes. Almost all

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the XYR mutants formed selectively 1(3)-PI (Supporting Information). This result again indicated that the Y385R mutation contributed to the selective 1(3)-PI formation. An exception was 187W/191Y/385R (WYR); in this case PI could not be detected by TLC. No significant difference in PC-hydrolyzing activity was observed among the XYR variants; in contrast, their PI-synthesizing activities were considerably different (Figure 2). In particular, those with aromatic residues at position 187 that is, WYR, FYR and YYR, showed low PI-synthesizing activity; this indicated that these bulky residues might interfere with the entry of the *myo*-inositol molecule into the active site.

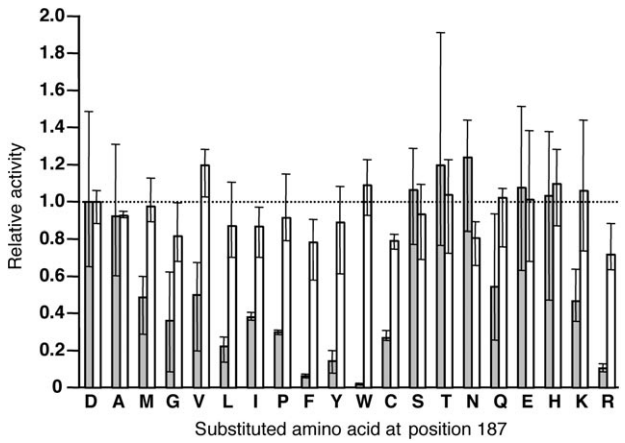

Figure 2. Comparison of the enzymatic activities of XYR variants. The PI-synthesizing (gray columns) and PC-hydrolyzing (white columns) activities of the culture supernatants of the corresponding recombinant strains were assayed. The activities relative to that of DYR (indicated with a dotted line) are shown. The data are averages of three independent experiments. The mutants are indicated by single letters denoting the substituted amino acid at position 187.

Among the 20 XYR variants, we chose seven that showed high PI-synthesizing activity (DYR, AYR, SYR, TYR, NYR, EYR, and HYR) for further characterization.

Characterization of the seven XYR variants

The seven XYR mutant enzymes were purified from recombinant *E. coli* to homogeneity and used for kinetic analysis. The PI-synthesizing and PC-hydrolyzing activities of the variants are summarized in Table 1. DYR showed the highest PI-synthesizing potential (1.08 ± 0.11 μmol min^{-1} mg^{-1}). Similar PI-synthesizing activity was also observed in AYR, NYR, EYR, and HYR. SYR and TYR showed lower activity than the others. The PI/PA ratio is a parameter for selectivity of two competing reactions that is, transphosphatidylation and hydrolysis. The seven variants showed similar PI/PA values, though EYR showed a slightly higher value. The PC-hydrolyzing activity of the XYR variants was considerably lower than that of WT-PLD, as observed in the previous work for the FRY mutant.[4]

To date, two plant PLDs have been reported to catalyze PI-synthesis.[6] The PI-synthesizing rate of PLD from poppy seedlings is reported to be 66 ± 7.2 nmol min^{-1} mg^{-1},[6a] that is, 16 times lower than that of DYR. Therefore, we may mention that, although their PC-hydrolyzing activity was lower than that of WT-PLD, our mutant PLDs have the highest PI-synthesizing activity recognized so far.

Table 1. Kinetic parameters of XYR variants.[a]

Enzyme	PI synthesis v [μmol min^{-1} mg^{-1}]	PI/PA	PC hydrolysis V_{max} [μmol min^{-1} mg^{-1}]	K_M [mM]	V_{max}/K_M
WT	n.d.[b]	n.d.[b]	4700 ± 90	1.5 ± 0.1	3.1×10^3
DYR	1.08 ± 0.11	0.83	3.9 ± 0.8	4.6 ± 0.5	0.85
AYR	0.76 ± 0.04	0.99	1.1 ± 0.02	3.9 ± 0.1	0.28
SYR	0.59 ± 0.14	0.87	1.4 ± 0.1	1.9 ± 0.2	0.74
TYR	0.29 ± 0.03	0.81	0.6 ± 0.02	4.3 ± 0.3	0.14
NYR	0.85 ± 0.12	0.86	0.8 ± 0.04	4.6 ± 0.5	0.17
EYR	0.85 ± 0.05	1.13	2.1 ± 0.05	4.3 ± 0.3	0.49
HYR	0.88 ± 0.06	0.83	1.5 ± 0.1	4.3 ± 0.2	0.35

[a] The data are taken from three independent experiments. The values are shown as the mean ± s.d. [b] Not determined because the WT enzyme showed no PI-synthesizing activity (Figure 1).

Ratio of 1-PI to 3-PI synthesized by XYR variants

1-PI and 3-PI could not be distinguished by HPLC analysis alone, because they were not separated. Since 1-PI and 3-PI (and also 4-PI and 6-PI) have a chiral center at the *sn*-2 position of the glycerol moiety with *R* configuration, they are diastereomers. Therefore, one may expect that these diastereomers could be separated by conventional chromatographic techniques. However, the diastereomer separation is not possible if the two chiral centers are more than four chemical bonds apart,[7] as in the cases of 1-PI and 3-PI. Thus, to estimate the ratio of 1-PI versus 3-PI in the synthesized PI, we used PI-specific phospholipase C (PI-PLC) from *S. antibioticus* (Sa-PLC1).[8] The principle of the PI-PLC-mediated analysis is that the enzyme hydrolyzes 1-PI but not 3-PI. Thus, comparing the amount of 1(3)-PI before and after PI-PLC treatment by HPLC allows us to estimate the ratio of 1-PI to 3-PI.

Figure 3 A and B verifies the analytical method itself. When chemically synthesized 1-PI or 3-PI was treated with Sa-PLC1, only 1-PI was hydrolyzed (Figure 3 A). HPLC analysis with dioleoyl-phosphatidylethanolamine (DOPE), which is not a substrate for Sa-PLC1, as the internal standard enabled quantitative analysis and showed again the selective hydrolysis of 1-PI (Figure 3 B). From these results, we concluded that PI-PLC-mediated hydrolysis followed by HPLC quantification of PI could be applied to estimate the 1-PI/3-PI ratio.

Next, the 1-PI/3-PI ratio of the PI products synthesized by the XYR variants was analyzed. The results are summarized in Figure 3 C. Among the seven variant tested, DYR, AYR, NYR, EYR, and HYR showed more or less 1-PI preference over 3-PI, with HYR showing the strongest preference (ratio of 7:3). SYR exhibited almost no preference (5:5). Interestingly, the selectivity of TYR was opposite to that of HYR, generating preferentially 3-PI over 1-PI (ratio of 2:8). The results indicated that a difference in only one amino acid residue at position 187 of the

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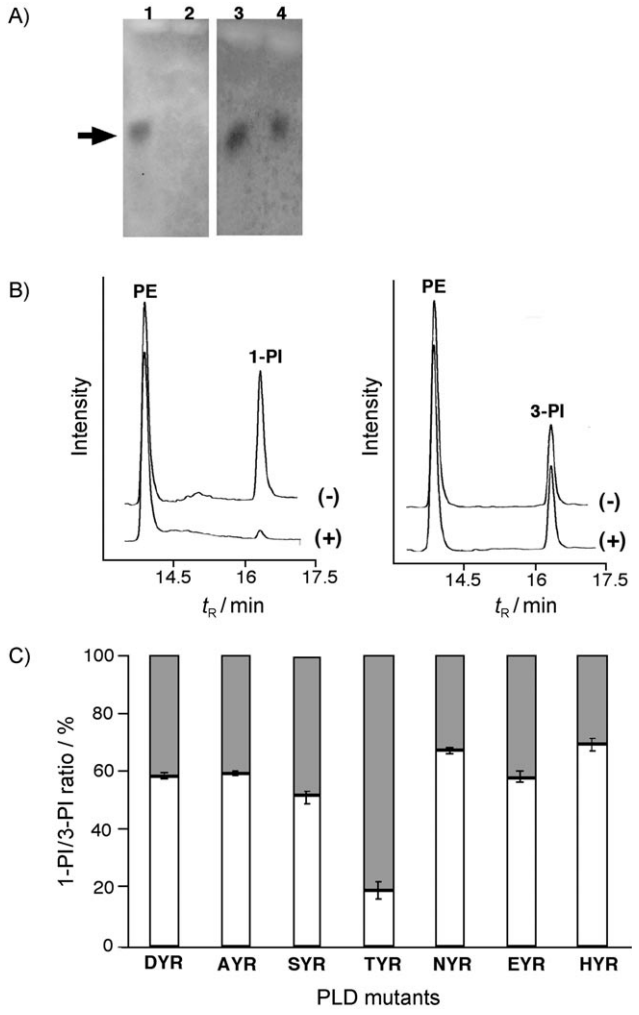

Figure 3. Analysis of the 1-PI/3-PI ratio in the PI synthesized by the mutant PLDs. A) TLC analysis of standard 1-PI (lanes 1 and 2) and 3-PI (lane 3 and 4) with (lanes 2 and 4) or without (lanes 1 and 3) Sa-PLC1-treatment. The position of 1(3)-PI is indicated by an arrow. B) HPLC analysis of standard 1-PI (left) and 3-PI (right) with DOPE as internal standard and with (+) or without (−) Sa-PLC1 treatment. C) The ratio of 1-PI (□) versus 3-PI (■) in the PI generated by the XYR mutants. The data were obtained from two independently synthesized PI samples ($n = 2$), each of which was analyzed in at least duplicate.

XYR variants might determine the selectivity between 1-PI and 3-PI. The results also suggested that the most suitable enzymes for the selective synthesis of 1-PI and 3-PI were HYR and TYR, respectively.

Discussion

Our previous study demonstrated the first example of PI-synthesizing *Streptomyces* PLD mutants generated by saturation mutagenesis. However, the mutant PLDs formed a mixture of at least two PI isomers, 1(3)-PI and 4(6)-PI.[4] In this study, we further screened the PLD mutant library and isolated new mutants, DYR, AYR, and MYR, that selectively synthesized 1(3)-PI.

In our library, three amino acids (W187/Y191/Y385) were selected for alteration of substrate specificity because these residues have bulky side chains and are oriented in the space that was expected to accommodate the acceptor compound in the substrate binding pocket. This strategy is similar to the so-called combinatorial active-site test (CAST) proposed by Reetz's research group.[9]

In the FRY mutant, (which generates both 1(3)- and 4(6)-PI), the residue Y385 was not substituted, thus suggesting that the other positions (W187F and Y191R) were involved in substrate recognition, and possibly in the low positional specificity.[4] On the other hand, the three mutants DYR, AYR, and MYR conserved the common amino acid substitution "Y385R". Therefore, the substitution Y385R should be important for specific 1(3)-PI synthesis. This is also supported by the fact that almost all the XYR variants selectively synthesized 1(3)-PI (Supporting Information).

The various XYR variants showed similar activity for PC hydrolysis, whereas their PI-synthesis activities were considerably different (Figure 2). The mutants FYR, YYR, and WYR with aromatic resides at the position 187, showed markedly lower activity in PI synthesis. This result is consistent with our speculation that bulky amino acids might interfere with the entry of *myo*-inositol and thereby with PI synthesis. Of the 90 positive clones, only two retained the Trp residue at position 187. The results suggested that aromatic residues at position 187 especially hinder the substrate *myo*-inositol from entering into the substrate binding pocket.

Composition analysis of 1-PI versus 3-PI was achieved by HPLC in combination with Sa-PLC1-mediated selective hydrolysis, as it was not possible by HPLC alone. Bacterial PI-PLC requires an axial 2-OH group on the inositol ring of PI as a nucleophile to attack the phosphorus atom connected to the 1-OH during the hydrolysis.[10] The 2-OH needs to be deprotonated by a His residue from a certain direction in the active site.[10b] Therefore, 1-PI can be a substrate for PI-PLC, while 3-PI, its diastereomer can not. Direct experimental evidence for the stereospecificity of *Bacillus cereus* PI-PLC (Bc-PI-PLC) by using synthetic D-PI (= 1-PI) and L-PI (= 3-PI) has been reported.[10c] Although Sa-PLC1 is different in the primary sequence from Bc-PI-PLC, its catalytic His residues are conserved.[8b] Therefore Sa-PLC1 might employ a similar reaction mechanism to Bc-PI-PLC. This is why Sa-PLC1 selectively hydrolyzed 1-PI but not 3-PI (Figure 3).

It is interesting that the XYR variants have different 1-PI/3-PI specificity despite only differing at position 187. This means that the residue at the position 187 determines the 1-PI/3-PI selectivity. The difference in selectivity might depend on which OH group of the *myo*-inositol reacts. In other words, the selectivity might come from the orientation of the *myo*-inositol in the active site, that is, how the enzyme recognizes the molecule. At the moment, however, we can only speculate that the residue at position 187 is involved in the orientation of the *myo*-inositol, possibly by forming hydrogen bonds directly or indirectly. X-ray crystallographic analysis of the mutant enzymes will provide useful information on this matter.

All the mutant PLDs significantly lost PC-hydrolyzing activity as they acquired PI-synthesizing activity (Table 1). But the PI-synthesizing activities of our enzymes are higher than that of

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the known PI-synthesizing PLD. The higher transphosphatidylation potential makes our mutant enzymes useful for the preparation of PI.

The great advantage of the protein engineering strategy is that it leads to improvements in enzymes' properties without affecting the initial activity.[11] But in some cases, mutations for improvements in the properties seem to decrease the initial activity.[12] In our selection strategy for the PI-synthesizing PLD mutants, the selection pressure allowed the isolation of enzymes with PI-synthesizing activity, but it does not guarantee high activity. Hence, further engineering of the mutant PLDs by other strategies such as structure-based rational design, should provide mutants with improved activity.

Experimental Section

Chemicals: *myo*-Inositol was from Wako (Osaka, Japan). DOPC and DOPE were from NOF Corporation (Tokyo, Japan). Soybean PI (99% purity) was from Sigma.

PLD-catalyzed PI-synthesis: A mixture containing DOPC (1 mg, 1.27 μmol) dissolved in ethyl acetate (100 μL), *myo*-inositol (18 mg, 0.1 mmol) in sodium acetate buffer (50 mM, pH 5.6, 90 μL), and PLD solution (either the culture supernatant of the mutant *E. coli* or purified enzymes, 10 μL) was incubated at 37 °C with shaking for 16 h. Aqueous HCl (1 M, 10 μL) and chloroform/methanol (2:1, *v/v*, 200 μL) were then added in order to stop the reaction. After centrifugation, the lower, lipid-containing layer was recovered. Approximately 5 μL of this lipid solution was spotted on a silica gel TLC plate and chromatographed with chloroform/petroleum ether/methanol/acetic acid (4:3:2:1, *v/v/v/v*). The phospholipids were visualized by spraying with Dittmer–Lester reagent.[13]

Generation of XYR variants: Saturation mutagenesis at position 187 was performed by overlap extension using PCR. Saturation mutagenesis is a general concept whereby particular amino acids in the target protein are replaced with all other 19 amino acids. The *pld* gene was amplified by PCR using specific primers and DYR-expressing plasmid as the template. The primers used to construct the XYR variants are given in the Supporting Information. The presence of the mutation and the fidelity of the mutagenesis were confirmed by DNA sequencing.

Quantification of PI-synthesizing activity: A mixture containing DOPC (0.9 mg, 50 μmol) dissolved in ethyl acetate (50 μL), *myo*-inositol (0.5 mg, 0.64 mmol) in sodium acetate buffer (50 mM, pH 5.6, 40 μL), and purified PLD enzyme (0.1 μg μL^{-1}, 10 μL) was incubated at 37 °C with shaking for 20 min. HCl (1 M, 5 μL) and chloroform/methanol (2:1, *v/v*, 95 μL) were then added in order to stop the reaction. After centrifugation, the lower, lipid-containing layer was recovered. This lipid solution was subjected to HPLC analysis. To calculate the PI/PA value, the phospholipids were separated by TLC and visualized with Dittmer–Lester reagent, followed by composition analysis using NIH imaging software.

Sa-PLC1-mediated analysis of the 1-PI/3-PI ratio: A solution of lipid mixture containing DOPE (40 μg) and PI isomers (chemically synthesized 1- and 3-PI, or PI synthesized by PLD mutants, approximately 50–100 μg) in chloroform/methanol (2:1, *v/v*) was evaporated. The lipids were redissolved in sodium deoxycholate solution (0.2%, 20 μL), and mixed with Tris-maleate-NaOH buffer (40 mM, pH 7.0, 20 μL) containing Sa-PLC1 (3.6 μg). The resulting mixture was incubated at 37 °C for 2.5 h. Another batch of the lipid mixture was incubated similarly but without Sa-PLC1. The lipids were extracted with chloroform/methanol (2:1, *v/v*, 80 μL) and analyzed by HPLC (or TLC). The ratio of 1-PI to 3-PI in the sample was calculated by comparison of the peak areas of 1(3)-PI with PLC treatment to that without treatment. The peak area of PI was normalized by using that of the internal standard DOPE, which is not a substrate for Sa-PLC1.

HPLC analysis of the phospholipids: HPLC analysis of the phospholipids was performed in principle as described previously[4] by using a normal-phase silica gel column, and a binary gradient system with chloroform/methanol/formic acid-triethylamine buffer. The phospholipids in the effluent were detected with a charged aerosol detector (Corona, ESA Inc, Chelmsford, MA, USA) at a N_2 flow of 34 psi and a detection range of 100 pA. The linearity of the detector response against the amount of the analytes was confirmed by using soybean PI and DOPE in the range of 0.1 to 5 μg (for PI), and 0.5 to 5 μg (for DOPE) on column.

Other methods: High-throughput screening of the mutant PLD library for PI-synthesizing mutants was performed as described previously.[4] Standard 1-PI and 3-PI were chemically synthesized as described,[4] and purified by preparative HPLC. Mutant PLDs were prepared (and purified when necessary) from the culture supernatants of recombinant *E. coli*.[14] The hydrolytic activity of PLD was assayed by using DOPC as the substrate.[14] Sa-PLC1 was purified from the culture supernatant of a recombinant strain of *Streptomyces lividans* as reported.[8]

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Keywords: enzymes · high-throughput screening · phosphatidylinositol · phospholipases · regioselectivity

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