



In vitro synthesis of phospholipids with yeast phospholipase B, a phospholipid deacylating enzyme

Yasuo Watanabe*, Itsuki Kobayashi, Takanori Ohnaka, Seiya Watanabe

Department of Bioscience, Graduate School of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama, Ehime, 790-8566, Japan

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ABSTRACT

The gene encoding the *Saccharomyces cerevisiae* phospholipid deacylation enzyme, phospholipase B (*ScPLB1*), was successfully expressed in *E. coli*. The enzyme (*Scplb1p*) was engineered to have a histidine-tag at the C-terminal end and was purified by metal (Ni) affinity chromatography. Enzymatic properties, optimal pH, and substrate specificity were similar to those reported previously. For example, deacylation activity was observed in acidic pH in the absence of Ca^{2+} and was additive in neutral pH in the presence of Ca^{2+} , and the enzyme had the same substrate priority as reported previously, with the exception of PE, suggesting that yeast phospholipase B could be produced in its native structure in bacterial cells. *Scplb1p* retained transacylation activity in aqueous medium, and esterified lysophosphatidylcholine with free fatty acid to form phosphatidylcholine in a non-aqueous, glycerin medium. We propose that phospholipase B could serve as an additional tool for *in vitro* enzyme-mediated phospholipid synthesis.

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1. Introduction

Enzymes that hydrolyze the ester linkage of phospholipids are referred to as phospholipases, and are classified into types A-D depending on the site they hydrolyze (Fig. 1A). Type A enzymes hydrolyze one of two fatty acid esters. When they hydrolyze phospholipids at the *sn*-1 site, they are referred to as A_1 enzymes, and when they hydrolyze at the *sn*-2 site, they are referred to as A_2 enzymes. The type B enzyme, PLB, simultaneously hydrolyzes two acyl-ester bonds in phospholipids, without accumulation of lysophospholipids.

Saito [1] previously made the following observations about PLB: 1) PLB completely deacylates phospholipids, and then remodels their fatty acids, 2) the fate of liberated *sn*-2 fatty acids is well known, but that of *sn*-1 fatty acids is unclear, and 3) PLB does not produce lysophospholipids, which are thought to be cytotoxic in general.

The presence of PLB in rat intestinal mucosa (Protein ID: BAA23813.1) and bovine pancreas (Protein ID: DAA24488.1) [1]

Abbreviations: HPLC, high-pressure liquid chromatography; ELSD, evaporated light scattering detector; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; CL, cardiolipin; PA, phosphatidic acid; LPC, lysophosphatidylcholine; FFA, free fatty acid; PLB, phospholipase B; PLA₂, phospholipase A₂; EDTA, ethylenediaminetetraacetic acid; DHA, docosahexaenoic acid.

* Corresponding author.

E-mail address: watanabe.yasuo.mg@ehime-u.ac.jp (Y. Watanabe).

suggests that the enzyme digests phospholipids in higher mammals. While the role of the enzyme in yeast is largely unknown, it was reported that the pathogenicity of pathogenic yeast (e.g., *Candida albicans*, *Cryptococcus neoformans*, and *Paracoccidioides brasiliensis*) is eliminated in the absence of the *PLB1* gene [2–4]. Thus, the *in vivo* role of PLB may involve “fatty acid-remodeling” of phospholipids, since remodeling can be achieved with a combination of phospholipid deacylation by PLB and esterification of fatty acyl residues with another enzyme, acyltransferase (Fig. 1B), or by PLB itself.

Certain phospholipases have been used for the *in vitro* synthesis of phospholipids, including PLA₂ and lipase [5,6]. Since only food compatible substances are used in methods based on PLA₂, its application in food and medical fields holds promise [5]. Preparation of phospholipids by transacylation with lipase has also been reported, although this method has less position specificity for phospholipid transacylation [6].

We have conducted numerous studies on properties of the yeast *PLB* gene from species such as *Torulaspota delbrueckii* [7–11], *Schizosaccharomyces pombe* [12], *Kluyveromyces lactis* [13], and *Candida utilis* [14]. We previously cloned DNA encoding *PLB* genes and elucidated their primary structure [10,11,13,14]. The first yeast genome project [15] revealed the presence of three *PLB* genes in *Saccharomyces cerevisiae*, including *PLB1* (ORF name: YMR008C), *PLB2* (YMR006C), and *PLB3* (YOL011W). Gene descriptions in the *Saccharomyces* GENOME DATABASE provide hints on the properties of these phospholipases. Moreover, Merkel et al. reported on the

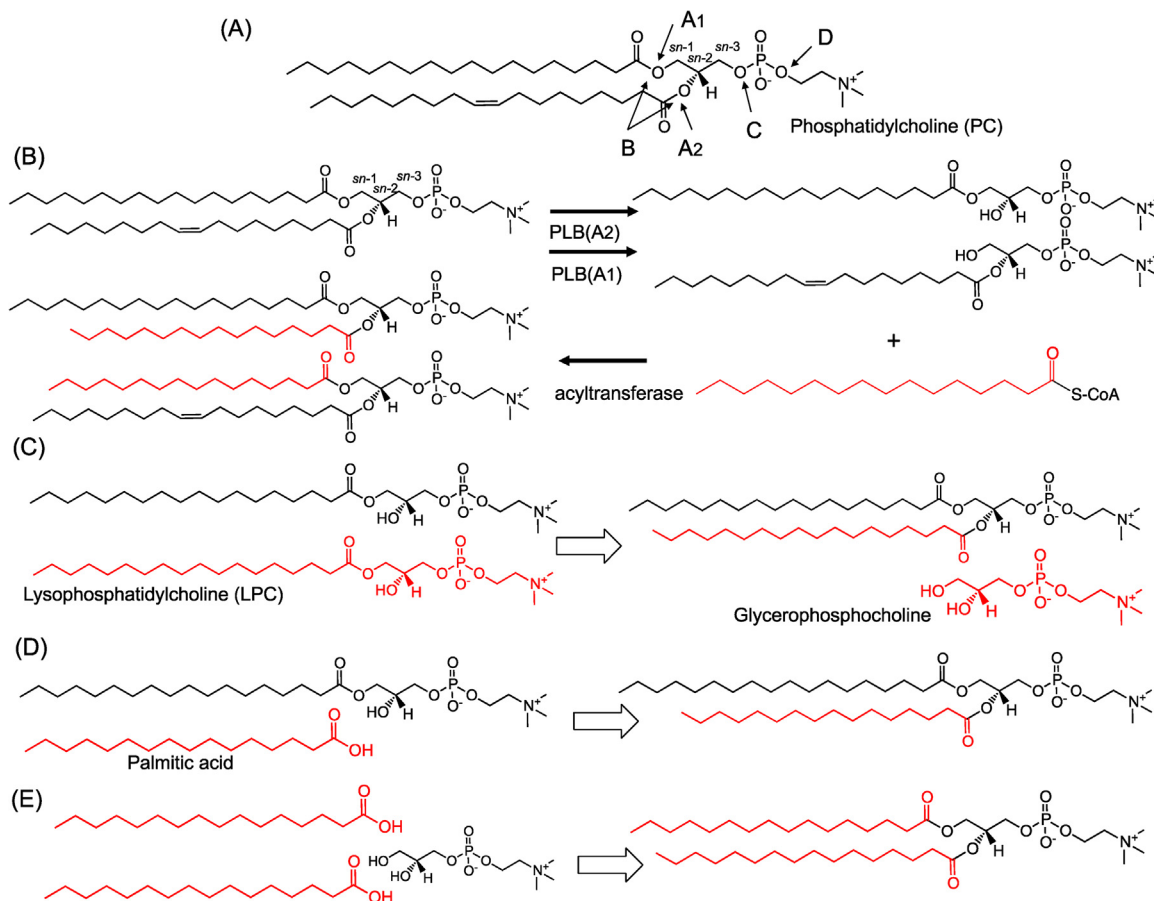


Fig. 1. Hydrolysis sites of various phospholipases, fatty acid-remodeling, and schemes of phospholipid synthesis mechanisms. (A) Sites hydrolyzed by phospholipase A1, A2, B, C, and D. (B) Speculative fatty acid-remodeling mechanism of phospholipids. (C) Scheme of acyltransferase activity. (D) Scheme of (single) esterase activity. (E) Scheme of (double) esterase activity.

enzymatic properties of these three phospholipases and found that Scplb1p had the highest activity [16,17].

In this study, we expressed the *S. cerevisiae* *PLB1* gene (*ScPLB1*) in *E. coli*, purified the enzyme (Scplb1p), and elucidated its enzymatic properties. Our findings have implications in the *in vitro* synthesis of phospholipids using yeast PLB proteins.

2. Materials and methods

2.1. Materials

LB broth was prepared according to the recipe of Nacalai Tesque (Japan). Highly pure phospholipids were obtained from Sigma-Aldrich (Japan). Other chemicals used in the study were ultra-pure grade.

2.2. Cloning of *S. cerevisiae* *PLB1* (*ScPLB1*) gene into *pET-21d* vector

A DNA fragment encoding the *PLB1* gene was prepared with the following two primers using *S. cerevisiae* genomic DNA as the template: sense primer 5'-CGACTCCATGGGCATGAAGTTGCA-GAGTTTGTGGG-3' and anti-sense primer 5'-GCAGTCTCGAGAAT-TAGACCGAAGACGGCAC-3' (underline indicates the two restriction enzyme sites, i.e., NcoI at the 5' terminus and XhoI at the 3' terminus). After cutting the DNA and *pET-21d* vector (Novagen-Merck, Germany) with NcoI and XhoI, the DNA and vector were ligated. The resulting recombinant vector was transformed into JM109 cells (Takara Bio, Japan). The nucleotide sequence of the vector (*ScPLB1*-*pET-21d*) and lack of substitutions were confirmed

by sequencing. We also confirmed that the C-terminus of Scplb1p had a histidine tag.

2.3. Preparation of Scplb1p

The *ScPLB1*-*pET-21d* plasmid was transformed into the Rosetta gami strain of *E. coli* (Novagen-Merck). Transformants were used to produce Scplb1p under the following conditions: culture temperature of 30° C, IPTG concentration of 0.4 mM, and culture period of 4 h. Obtained cells were subjected to freeze-thawing and disruption by ultrasound. After centrifugation, the clear supernatant was collected.

Purification of histidine-tagged Scplb1p was carried out with an automatic Ni-affinity chromatographic apparatus (Profinia; Bio-Rad Ltd, USA) according to the manufacturer's recommended protocol. We obtained about 0.6 mg of Scplb1p from 1000 mL of culture. Protein was concentrated by freeze-drying or ultrafiltration (Amicon Ultra 3KDa, Millipore-Merck, Germany). Protein was quantitated using Coomassie Brilliant Blue G-250 (Nacalai Tesque) [18], using bovine serum albumin as a standard.

2.4. Quantitation of phospholipids

Separation and detection of phospholipid species were carried out by HPLC (Shimadzu Ltd., Japan) combined with an ELSD (ELSD LTII, Shimadzu Ltd.). The HPLC system Prominence consisted of LC-20AD (X2), SIL-20AD, CBM-20A, DGU-20A3, and CTO-20AC. The column YMC-Pack Diol-120-NP (YMC-Pack Diol-NP, 250 × 4.6 mm, S-5 μm, 12 nm, YMC Ltd, Japan) was used at 35° C. Mobile phase A

was acetone-*n*-hexane-acetic acid-triethylamine (1000/70/7.5/5, v/v) and mobile phase B was ethanol-acetic acid-triethylamine (1000/7.6/5, v/v). Elution was carried out with the following gradient: 0% to 15% of B from 0 min to 16.5 min, 15% to 50% of B from 16.5 min to 28 min, 50% of B from 28 min to 32 min, 50% to 0% of B from 32 min to 35 min, and 0% of B from 35 min to 46 min. Flow rate was 1 mL/min. ELSD LTII was used with the following settings: filter, 4 s; detector temperature, 40° C; N₂ nebulizer-gas pressure, 330–350 KPa; and gain, 6.

2.5. Construction of standard curves for phospholipids

To estimate the effects of Scplb1p on phospholipid degradation and synthesis, standard curves were generated by quantitation with the HPLC-ELSD system for the following highly pure phospholipids (99% purity; Sigma-Aldrich): PC, LPC, PI, PE, and PS, and palmitic acid (FFA).

2.6. Measurement of PLB activity

An exemplary reaction mixture consisted of 0.44 mg PC, 0.48 mL buffer solution, 0.06 mL 50 mM EDTA solution, and 0.06 mL 40 mM taurocholate (mol ratio of detergent to phospholipid = 0.2). Scplb1p solution (0.02 mL) was added to 0.6 mL substrate solution and the mixture was incubated at 30° C by mixing with a rotator. Chloroform-methanol mixture (1.5 mL; 1:2, v/v) was added to the reaction mixture (0.4 mL), mixed well, and allowed to stand for 10 min. Water (0.5 mL) and chloroform (0.5 mL) were then added. After mixing well, the mixture was centrifuged at 1000 × *g* for 5 min. Most of the lower chloroform phase was collected and dried *in vacuo*. After dissolving in an appropriate volume of chloroform-methanol mixture (2:1, v/v), phospholipids were analyzed using the HPLC-ELSD system. Control reactions were carried out without enzyme, and PLB activity was estimated as the difference relative to the amount of phospholipids in the control reaction. Results represent the average of at least three experiments.

2.7. Measurement of acyltransferase activity

A reaction mixture for measuring acyltransferase activity (Fig. 1C) consisted of 0.288 mg LPC, 0.48 mL 50 mM glycine-HCl buffer solution (pH 3.0), 0.06 mL of 50 mM CaCl₂ solution, and 0.0001 mL Triton X-100. After adding Scplb1p to the solution (0.02 mL), the mixture was incubated at 25° C by gentle mixing with a rotator. Chloroform-methanol (0.375 mL; 1:2, v/v) was added to the reactant (0.1 mL) and mixed well. Water (0.125 mL) and chloroform (0.125 mL) were then added, and after mixing well, the mixture was centrifuged at 1000 × *g* for 5 min. The remaining steps were similar to those described above for measuring phospholipase activity.

2.8. Phospholipid synthesis

Synthesis of phospholipids from LPC and FFA (Fig. 1D) was carried out as described by Tanaka *et al.* [5]. Briefly, 1 mg LPC, 1 mg FFA, 1 mg glycine, 1 mg alanine, and 1 g glycerin were mixed and evaporated *in vacuo*. Scplb1p (0.2 mg) and 0.3 M CaCl₂ solution (0.01 mL) were added to the mixture. With continuous evaporation *in vacuo*, the mixture was incubated at 50° C for 24 h. To this reaction mixture, chloroform-methanol (4 mL, 1:2, v/v) was added and allowed to stand for 10 min. This was followed by the addition of chloroform (0.5 mL). Water was then added until two phases formed. After centrifugation at 1000 × *g* for 5 min, the chloroform phase was collected and dried *in vacuo*. Phospholipid formation was analyzed with the HPLC-ELSD system.

3. Results and discussion

3.1. Expression of PLB1 in *E. coli*

Although we attempted to purify Scplb1p from *S. cerevisiae* cells harboring the *PLB1* gene (Yeast ORF Collection, purchased from Dharmacon, a Horizon Discovery Group Co., UK, formerly Open Biosystems, Ltd), sufficient amounts of the enzyme with deacylation activity could not be obtained (data not shown). Accordingly,

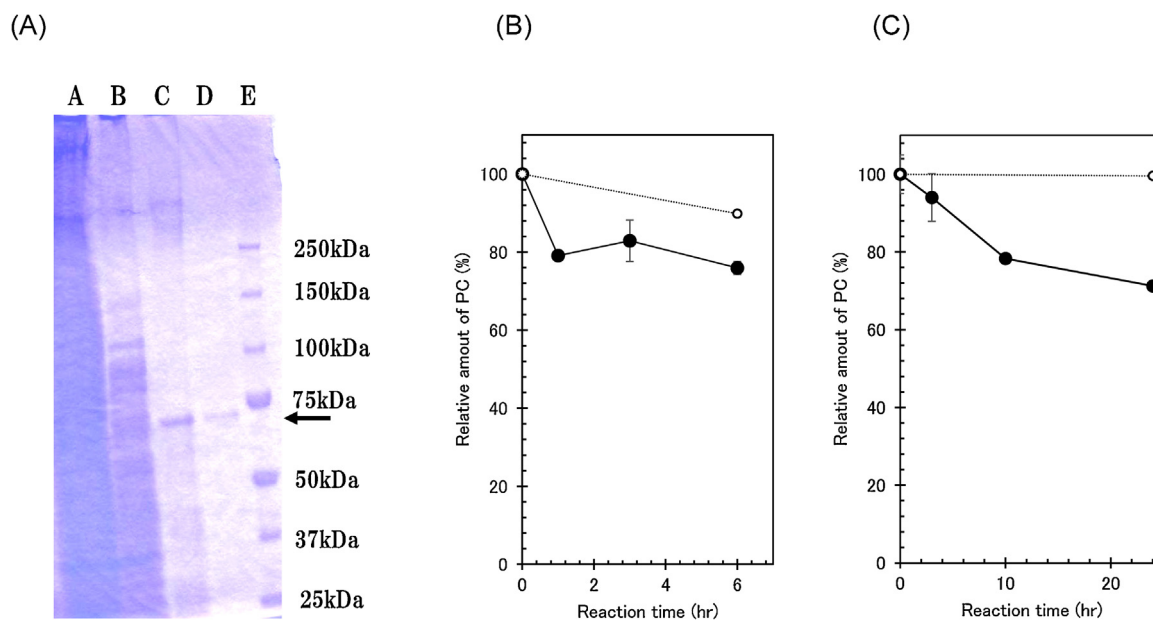


Fig. 2. Purification of Scplb1p and its PLB activity. (A) SDS-PAGE of protein samples of Scplb1p at each step of purification. Column A: precipitate after centrifugation of cell homogenate, Column B: supernatant after centrifugation of cell homogenate, Column C: protein sample concentrated by ultrafiltration of sample from Column D. Column D: proteins bound to Ni column of Profinia. Column E: molecular weight markers. Arrow corresponds to the expected MW of Scplb1p. (B) Time course of PLB activity of freeze-dried Scplb1p sample. The error bars represent SD (n = 3). (C) Time course of PLB activity of ultrafiltration-concentrated Scplb1p sample. n = 3.

we switched to the *E. coli* expression system, which could potentially produce mass quantities of the enzyme while retaining its biological properties. Moreover, given a previous study [16] reporting that, of the three PLB enzymes in yeast, Scplb1p had the highest activity, we expressed the *PLB1* gene in *E. coli* (Rosetta Gami strain cells). Scplb1p expression was carried out according to the procedures described above. Since Scplb1p contains a C-terminal histidine tag, purification was performed with Ni-column chromatography, and samples were concentrated by freeze-drying or ultrafiltration. Each step of the purification process was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2A). The calculated molecular weight of Scplb1p is 71,667 Da (Scplb1p) + 841 Da ($6 \times \text{His}$) = 72,490 Da. The molecular weight of main protein band (arrow) was similar to this value. The purified Scplb1p sample (Fig. 2A-lane D) was essentially pure, but a few other proteins were observed when concentrated by about 10-fold with ultrafiltration (Fig. 2A-lane C). However, these proteins are likely to be non-specific Ni-binding proteins derived from *E. coli* cells, and so Scplb1p was of high purity.

3.2. Enzymatic properties of Scplb1p

To assess the ability of Scplb1p to degrade phospholipids, we developed a method to analyze phospholipid species using the HPLC column, YMC-Pack Diol-120-NP, which can separate phospholipid species. After separation by HPLC, none of the lipids of interest had special structures that required analysis with a detector other than the refractive index detector, and thus we used an ELSD, which detects a larger variety of chemical substances. With ELSD, the mobile solvent is first evaporated, and then chemical substances that elute from the column are made into microparticles. The level of light scattering due to the microparticles is then measured. Many substances with a low boiling temperature can be detected, and substances undetectable with absorbance detectors (e.g., hydrocarbons, alcohols, lipids, fatty acids, detergents, and terpenoids) can be detected by ELSD. ELSD can also be used in mobile solvent-gradient analysis. The HPLC pattern of separated phospholipid species was previously reported [19].

The degradation curve of PC by Scplb1p is shown in Fig. 2B and C. LPC could not be detected during the degradation of PC, and we

confirmed the pattern of phospholipid degradation to be type B. Only small differences in the amount of PC were observed before and after the reaction in the absence of Scplb1p, suggesting that PC degradation is minimal in the absence of enzyme. The specific activity of Scplb1p was calculated to be 0.05 $\mu\text{mol}/\text{min}/\text{mg}$ protein in the case of freeze-dried Scplb1p (Fig. 2B), and 0.011 $\mu\text{mol}/\text{min}/\text{mg}$ protein in the case of ultrafiltration-concentrated Scplb1p (Fig. 2C), which were much lower than those reported for Plb1p purified from other types of yeast (e.g., *T. delbrueckii*, *S. pombe*, *K. lactis*, and *C. utilis*) (0.85–28.7 $\mu\text{mol}/\text{min}/\text{mg}$ protein) [7,12–14]. This could be due to misfolding of the protein during synthesis in *E. coli*, steric hindrance, or the addition of positive charges (His^+) from the histidine tag. Although, as mentioned above, the purified Scplb1p sample contained a small amount of proteins other than Scplb1p, they are unlikely to have influenced PLB activity. Moreover, due to the low activity of purified Scplb1p at this stage, further purification procedures likely would have reduced the activity even further. Thus, we used this Scplb1p preparation to elucidate the enzymatic properties of the enzyme.

The optimal pH and PLB activity of Scplb1p when PC was used as the substrate are shown in Fig. 3A and B. Activity was observed at acidic pH, and activity increased at weak acidic and neutral pHs when Ca^{2+} was added to the reaction mixture (Fig. 3B). These results are consistent with those reported by Melkel et al. [16].

Substrate specificities of some PLB enzymes have been reported previously, as follows: Scplb1p (by Melkel et al.), $\text{PS} > \text{PI} > \text{PC} > \text{PE}$ [16], and corresponding enzymes from yeast *S. pombe*, $\text{PI} > \text{PC} > \text{PS} > \text{PE}$ [12]; from *T. delbrueckii*, $\text{PI} > \text{PA} > \text{PC} > \text{PS} > \text{PE}$ [7]; from *K. lactis*, $\text{PS} > \text{PC} > \text{PI} > \text{PA} > \text{PE}$ [13]; from *C. utilis*, $\text{PC} > \text{PA} > \text{PI} > \text{PS} > \text{PE}$ [14]; and from the fungus *Penicillium notatum*, $\text{PS} > \text{PI} > \text{PA} > \text{PC} > \text{PE}$ [1]. Thus, the substrate specificity of PLB differs across species of yeast and other organisms. In the present study, we found the substrate specificity of Scplb1p to be $\text{PE} > \text{PS} > \text{PI} > \text{PC}$ under conditions of acidic pH and no Ca^{2+} (Fig. 3C). Although our purified Scplb1p showed high activity towards PE, the substrate priority was similar to that reported by Melkel et al. [16], with the exception of PE. In addition, as described above, the optimal pH of Scplb1p was the same as that of PLB purified from *S. cerevisiae*. These results suggest that Scplb1p prepared by expressing the *ScPLB1* gene in *E. coli* retained the

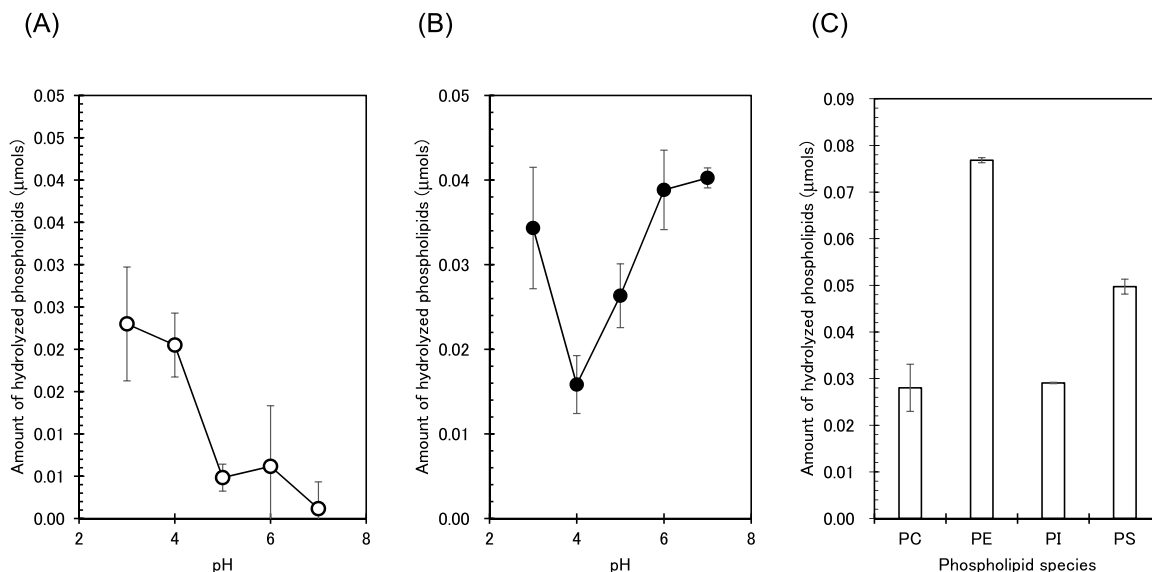


Fig. 3. Properties of purified Scplb1p. (A) Optimal pH of PLB activity without Ca^{2+} using phosphatidylcholine (PC) as substrate. The error bars represent SD ($n = 4$). (B) Optimal pH of PLB activity with Ca^{2+} using PC as substrate. $n = 4$. (C) Substrate specificity of Scplb1p using PC, phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS). $n = 3$.

protein structure and enzyme function of Scplb1p expressed in *S. cerevisiae*.

3.3. Phospholipid synthesis

With respect to the enzymatic synthesis of phospholipids, a previous study reported on the acyltransferase activity of PLB enzyme proteins (Scplb1p and Scplb2p) purified from *S. cerevisiae* [20]. In aqueous buffer solution used to measure PLB activity (deacylation), the enzyme hydrolyzes an acyl residue of LPC at the *sn*-1 site and transfers the *sn*-2 residue to another LPC (Fig. 1C). Scplb1p prepared from *E. coli* produced PC in the reaction solution containing only LPC (Fig. 4A and B), but only in long-term reactions (i.e., 25 h). Although transacylation using PLB enzyme purified from yeast, such as *S. pombe* [11], *K. lactis* [12], and *C. utilis* [13], was reported to occur in a short reaction period (e.g., 1 h), our results differed. One potential reason longer reaction periods were needed may relate to the low specific activity of our Scplb1p preparation.

Although transacylation by PLB is carried out in an aqueous environment, it can be inferred that the reverse reaction (i.e., diacylation of phospholipids) proceeds under non-aqueous or low-water (hydrophobic) conditions. PLA2 (lethnase) has been reported to synthesize PC in the presence of LPC and FFA (Fig. 1D) [5]. Accordingly, we examined the possibility that Scplb1p may share similar activity.

Reaction conditions were similar to those reported by Tanaka *et al.* [5] and are described in the Materials and Methods. Briefly, Scplb1p (0.2 mg) was added to glycerin solution (4 mL), and the mixture was incubated at 50 °C for 15 *h* *in vacuo*. Lipids from the reaction mixture were extracted and analyzed with the HPLC-ELSD system. In the presence of both LPC and FFA, PC was detected together with LPC and FFA (Fig. 4C-2). In a control reaction lacking

FFA, only LPC was detected (Fig. 4C-1), suggesting the lack of transacylase activity in the glycerin medium. This suggests that Scplb1p can catalyze esterification (i.e., LPC + FFA to PC) in a similar manner to PLA2. In the experiment with PLA2 by Tanaka *et al.* [5], the substrate used was a mixture of LPC (ca. 70%), lysophosphatidylethanolamine, lysophosphatidylinositol, and lysophosphatidic acid, but there was no evidence that PLA2 esterified lysophospholipids other than LPC. Since Scplb1p purified in our study showed a high specificity for PE, it will be interesting to determine whether it can esterify lysophosphatidylethanolamine in future studies.

Our results suggest that a PLB enzyme derived from yeast cells and expressed in bacteria can synthesize phospholipids through *in vitro* esterification. This indicates that, in addition to PLA2, PLB can synthesize phospholipids. Tanaka *et al.* [5] reported that esterification of LPC having an acyl residue at the *sn*-1 site with polyunsaturated fatty acid (e.g., DHA and eicosapentaenoic acid) was achieved with only food compatible substances. These polyunsaturated fats are known to have positive effects on several organs, including the brain. It was recently reported that translocation of DHA into the brain occurs when this fatty acid is attached to lysophospholipids (LPC), but not triacylglycerols [21,22]. In addition, DHA attached to phospholipids was highly resistant to oxidation [23]. Considering these features and the low abundance of phospholipids in comparison with oils in nature, *in vitro* phospholipid synthesis technology, e.g., using PLB, may enjoy wide use in the future. Although we could not determine the optimal conditions for phospholipid synthesis with Scplb1p, further efforts aimed at improving methods of purifying Scplb1p having higher specific activity would contribute to this goal, in particular, achieving esterification of two acyl residues to glycerophosphocholine (Fig. 1E), the reverse of the reaction

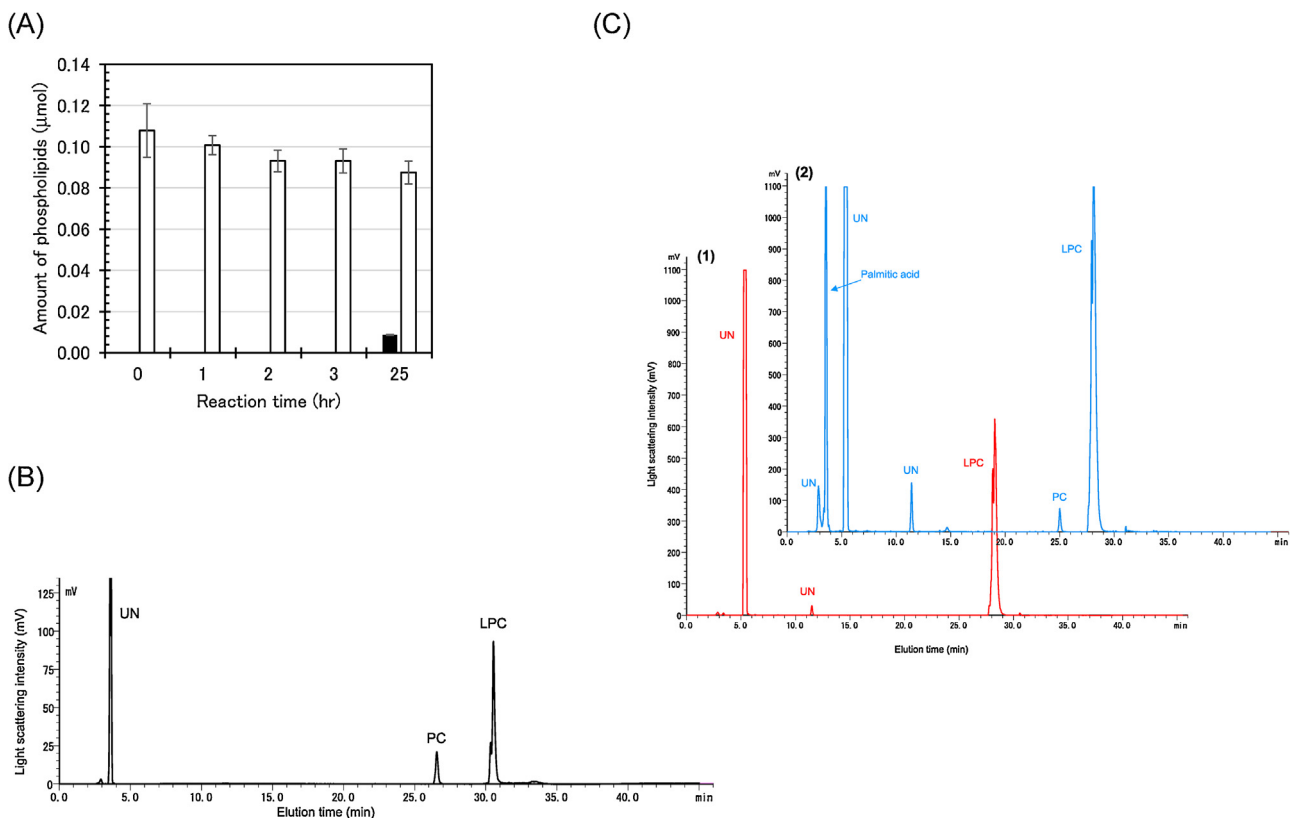


Fig. 4. Phospholipid (PC) synthesis by purified Scplb1p. (A) Acyltransferase activity of Scplb1p. White bars: without Scplb1p; black bars: with Scplb1p. The error bars represent SD (n = 3). (B) HPLC-ELSD for analysis of acyltransferase activity at 25 h reaction times. (C) Esterase activity. HPLC-ELSD for analysis of esterase activity without free fatty acid (palmitic acid; 1, red-line) and with palmitic acid (2, blue-line). UN, unknown; PC, phosphatidylcholine; LPC, lysophosphatidylcholine.

catalyzed by PLB. In principle, this so-called double esterification reaction is unlikely to be catalyzed by PLA2 if we consider the reverse reaction catalyzed by the enzyme. Future efforts will be aimed at purifying another yeast PLB (e.g., from *T. delbrueckii* or *S. pombe*) to obtain an enzyme with higher activity and applying it to phospholipid synthesis.

Taken together, we have shown that PLB is a new tool for phospholipid synthesis. The activity of the enzyme we used was weak and its concentration was low, thus, we achieved only a low level of PC production based on esterification of LPC with FFA. Determining the optimal conditions for obtaining high levels of high activity enzyme in *E. coli* could lead to the achievement of double esterification, i.e., the formation of glycerophosphocholine from two acyl residues.

Author agreement

All authors agreed to submit this manuscript to *Biotechnology Reports*, and this work has not been published or submitted to other journals.

Contributors

Y.W. planned, designed, and performed the experiments, analyzed the data, and wrote the manuscript. I.K. and T.O. performed the experiments and analyzed the data. S.W. contributed to writing the manuscript.

Conflict of interest

None.

Ethical considerations

This article does not contain any studies with human participants or animals.

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