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

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ABSTRACT

The gene encoding the *Saccharomyces cerevisiae* phospholipid deacylation enzyme, phospholipase B (Sc*PLB1*), 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.

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] 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 PLA_2 and lipase [5,6]. Since only food compatible substances are used in methods based on PLA_2, 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 *Torulaspora 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

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.

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Saccharomyces GENOME DATABASE provide hints on the properties of these phospholipases. Moreover, Merkel et al. reported on the 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′-CGACTCCATGGGCATGAAGTTGCAGAGTTTGTTGGG-3′ and anti-sense primer 5′-GCAGTCTCGAGAATTAGACCGAAGACGGCAC-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; N_2 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 $CaCl_2$ 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 $CaCl_2$ 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

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