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Efficient Biosynthesis of Phosphatidylserine in a Biphasic System through Parameter Optimization

Bishan Guo [1], Juntan Wang [1], Mengxue Zhang [1], Huiyi Shang [1], Rui Du [1], Fayun Wang [1], Hui Wang [2], Jun Xu [3,*] and Haihua Zhu [1,*]

[1] Institute of Business Scientific, Henan Academy of Sciences, 87 Wenhua Road, Zhengzhou 450002, China; bishanguo@163.com (B.G.); 18625778668@163.com (J.W.); yzmx1216@163.com (M.Z.); shy_scu@hotmail.com (H.S.); durui1949@163.com (R.D.); wangfayun262@sohu.com (F.W.)
[2] School of Medicine, Shanghai Jiao Tong University, Shanghai 200025, China; huiwang@shsmu.edu.cn
[3] College of Life Sciences, Henan Agricultural University, Zhengzhou 450002, China
* Correspondence: xujun0828@126.com (J.X.); haihuazhu1982@sohu.com (H.Z.); Tel.: +86-13849196619 (J.X.); +86-18638112870 (H.Z.)

Abstract: Phosphatidylserine (PS) has significant biological and nutritional effects and finds wide applications in the food, pharmaceutical, and chemical industries. To produce high-value PS efficiently, phospholipase D (PLD)-induced transphosphatidylation of low-value phosphatidylcholine (PC) with L-serine has been explored. In this research, we purified recombinant PLD from *Streptomyces antibioticus* SK-3 using ion exchange chromatography and gel filtration chromatography. Subsequently, we thoroughly characterized the purified enzyme and optimized the transphosphatidylation conditions to identify the most favorable settings for synthesizing PS in a biphasic system. The purified recombinant PLD displayed a robust transphosphatidylation function, facilitating efficient catalysis in the synthesis of PS. Under the optimal conditions (butyl acetate/enzyme solution 1:1, L-serine 160 mg/mL, soybean lecithin 2 mg/mL, and $MgCl_2$ 15 mM, at 50 °C for 2.5 h with shaking), we achieved a conversion rate of 91.35% and a productivity of 0.73 g/L/h. These results demonstrate the applicability of the process optimization strategy for using the candidate enzyme in the efficient synthesis of PS. Overall, this study presents a novel and scalable approach for the efficient large-scale synthesis of PS.

Keywords: phosphatidylserine; phospholipase D; phosphatidylcholine; transphosphatidylation; optimization

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1. Introduction

Phosphatidylserine (PS) is a phospholipid that plays a vital role in regulating membrane protein function. It occurs naturally in bovine brains, soybeans, and egg yolks, being the primary acidic phospholipid found on human cell membranes, where it plays a crucial role in maintaining normal myelin and neuron membranes. PS has been shown to have positive effects on brain activity, memory, senile dementia [1,2], depression, and muscle soreness. Consequently, it is considered an emerging "intelligent nutrient" and the "brain gold" docosahexaenoic acid (DHA) [3], with potential applications in pharmaceuticals [4] and functional foods [5]. However, the low amount of PS in animals, plants, and microorganisms has limited its use in industries [6]. Therefore, meeting the increasing demand for PS necessitates enhancing its production. One effective method for PS production is enzymatic transphosphatidylation using phospholipase D (PLD). This approach employs L-serine and phosphatidylcholine (PC) as substrates. To improve PS yield, substantial efforts have been devoted to tasks such as screening novel microbial strains that can produce the enzyme with a high transphosphatidylation activity from the different samples [7,8], exploring various immobilization strategies [9], and optimizing the catalytic system [10,11].

Among these various efforts, the optimization of reaction parameters for the system (temperature, pH, enzyme concentration, two-phase volumetric ratios, etc.) has gained much attention due to the high efficiency of this approach and the wide availability, along with cost-effectiveness of the involved substrates, PC and L-serine [12–14].

PLD (EC 3.1.4.4) is an extracellular enzyme that belongs to the PLD superfamily [15]. PLD is used in the production of PS, and this production method is considered highly valuable [16,17]. Numerous PLDs have been identified as being capable of catalyzing the following two reactions: (1) hydrolysis of the phosphodiester bonds in PC to release choline and generate phosphatidic acid (PA) [18]; and (2) catalysis of the phospholipid (generally PC) transphosphatidylation for the formation of PS [19]. There are many factors affecting PLD transphosphatidylation activity, such as pH, temperature, metal ions, and others. Most previous studies have shown that Ca^{2+} has a significant effect on enzymatic activity [10]. Apart from this, Mg^{2+}, Mn^{2+}, and Fe^{2+} also influences PLD activity. PLDs are distributed widely in mammals [20], plants [21], yeast [22], and bacteria [23,24]. Cabbage PLD has the highest abundance in nature, although its transphosphatidylation activity is extremely low [25]. On the contrary, microbial PLDs, particularly those obtained from the *Streptomyces* strains, exhibit higher transphosphatidylation activity, wider substrate specificity, and higher stability against organic solvents and heat [15,26], compared to those obtained from other organisms.

In addition, PLDs have become an important tool for the synthesis and modification of other different, scarce PLs [27–29], such as phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and novel artificial phospholipids. In recent years, PLDs have been employed in numerous applications in the forms of immobilized enzymes [30] and free enzymes [11], and they will be used for large-scale industrial applications in the near future.

Currently, the synthesis of PS via PLD is conducted mainly in an aqueous system [31], the water-organic solvent two-phase system [11], the aqueous-solid system [32], and the biobased ionic liquids [33]. Undeniably, PS production in an aqueous system or an aqueous-solid system has greater environmental friendliness. However, problems such as an increase in the hydrolysis by-products, a longer reaction duration, and a further complex product recovery process are encountered. Mao et al. [31] reported that it could take up to 24 h to reach a PS yield of 65%. Additionally, Li et al. [32] reported that the product requires elution with chloroform, repeated multiple times. Bio-based solvents, such as γ-valerolactone [34] and 2-methyltetrahydrofuran [35], have been used as alternatives, and while the approach has been successful, to the best of the authors' knowledge, it has not been deployed so far in any commercial production. Currently, numerous organic solvents (such as diethyl ether, ethyl acetate, and chloroform) are used for transphosphatidylation under the catalysis of free or immobilized PLD in a two-phase system for achieving maximal PS production [35]. Biphasic systems could promote the product and hydrophobic substrate solubility [36], allowing for a potential reaction equilibrium shift [37]. Moreover, biphasic systems could decrease the product separation difficulty encountered since the products and catalyst are present in the different liquid phases [38]. Most notably, PLDs from *Streptomyces* have achieved higher conversion rates with shorter reaction durations in two-phase systems [11]. This enables the reduction in production costs to a great extent, thereby encouraging large-scale production and industrial application and, consequently, being considered the best choice from the economic perspective.

In a previous study by our research group, a recombinant *Streptomyces antibioticus* (*S. antibioticus*) strain SK-3 was constructed, which realized a stable secretion and efficient expression of PLD [39]. The present work further purified the extracted PLD, then characterized and verified its enzymatic activity and performance in the synthesis of PS. The properties of this enzyme were characterized by comparing its transphosphatidylation activity with its hydrolytic activity. In addition, the critical conditions of transphosphatidylation were progressively optimized in a two-phase butyl acetate/water system. The

highest conversion rate and productivity of PS could reach up to 91.35% and 0.73 g/L/h, respectively.

2. Materials and Methods

2.1. Microbial Strain and Chemicals

The encoding gene of PLD was from *S. antibioticus* (GenBank ID BAA03913.1). The recombinant strain SK-3 had realized the stable secretion and efficient expression of PLD in a previous study [39] by our research group. It was, therefore, used in the present study to produce the enzyme. Choline oxidase and peroxidase were purchased from Sigma-Aldrich Trading Co., Ltd. (Shanghai, China). Soybean lecithin (PC content $\geq$ 98%) was bought from Taiwei Pharmaceutical Co., Ltd. (Shanghai, China). Standard PLs, including PA, PC, and PS, were provided by Sigma-Aldrich Trading Co., Ltd. (Shanghai, China). L-Serine and a high-precision and ready-to-use dialysis bag (YA1040) were procured from Solarbio Tech (Beijing, China). The BCA Protein Assay kit was from Beyotime (Shanghai, China). The reagents n-hexane, methanol, isopropanol, and glacial acetic acid were of pure chromatographic grade. All chemicals (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) applied in the present study were of pure analytical grade.

The seed medium (pH 7.0) was as follows: glucose 10 g/L, yeast extract 20 g/L, peptone 5 g/L, $K_2HPO_4 \cdot 3H_2O$ 2 g/L, and $MgSO_4 \cdot 7H_2O$ 0.5 g/L. The fermentation medium (pH 7.0) consisted of glucose 10 g/L, beef extract 5 g/L, peptone 5 g/L, $K_2HPO_4 \cdot 3H_2O$ 2 g/L, and $MgSO_4 \cdot 7H_2O$ 0.5 g/L. All media used in this study were sterilized at 115 °C for 30 min (glucose was added after separate preparation and sterilization).

2.2. Crude Enzyme Preparation

The recombinant strain SK-3 was inoculated and cultured in a test-tube slant under 30 °C for 7 days. A single colony from the slant was picked using an inoculating loop and then inoculated in the seed medium (50 mL) in a 250 mL flask. The flask was then maintained under 30 °C inside a rotatory shaker incubator (ZQZY-78CN, 200 rpm) for 2 days. Subsequently, the seed culture was inoculated in the fermentation medium (50 mL) using an inoculum of 4%, followed by a 7-day culture under 30 °C. The fermentation broth was centrifuged (Himac CF16RN) at 6000 rpm for 30 min. The resulting supernatant was harvested and filtered using a 0.45 μm filter membrane to obtain the crude enzyme.

2.3. Protein Purification

To obtain the purified enzyme, experiments were carried out according to the method of Li et al. [40]. Ammonium sulfate was added to the crude enzyme solution until the system saturation was 65%. After overnight equilibration and centrifugation at 16,000 rpm for 30 min, the precipitate was re-solubilized in a citric acid–sodium citrate buffer. Then, the suspension was placed into a dialysis bag (Mw 15 kDa), where both ends of the bag were sealed by clipping, and the bag was immersed into 500 mL of the same buffer solution for 8 h with two buffer changes. The dialyzed solution was centrifuged to remove the insoluble materials. The supernatant was applied to a Hitrap SP HP cation exchange column (GE Healthcare, Buckingham, England) for gradient elution. The elution peak with PLD activity was collected and concentrated in ultrafiltration centrifuge tubes. The concentrated solution was then sampled onto a Superdex 200 10/300 GL gel chromatography column and eluted with a citric acid–sodium citrate buffer (50 mmol/L, pH 5.0) containing 0.5 mol/L of NaCl. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to identify the protein molecular mass of the active fraction, followed by gel staining with Coomassie Brilliant Blue G-250 for visualization of the protein bands.

2.4. Hydrolytic Activity Assay

PLD hydrolytic activity was typically measured with a spectrophotometric assay using PC as the substrate. The activity in this study was determined according to the method of Zhou et al. [15] with minor modifications. The reaction mixture (total volume, 500 μL)

consisted of 4 mg/mL of egg yolk lecithin, 40 mM of Tris–HCl (pH 5.5), 0.1% (*v*/*v*) Triton X-100, 10 mM of $CaCl_2$,, and 100 µL of an enzyme sample. After incubation at 37 °C for 10 min, the reaction was terminated by the addition of a 50 µL solution containing 50 mM of EDTA and 100 mM of Tris–HCl (pH8.0), and the PLD enzyme was immediately denatured by heating at 100 °C for 5 min [41]. After cooling the reaction mixture to room temperature for 5 min, 3 mL of the choline identification solution containing 100 mM of Tris–HCl (pH 8.0), 1 mg of phenol, 0.3 mg of 4-aminoantipyrine, 2 U/mL of choline oxidase, and 2 U/mL of horseradish peroxidase was added. After incubation at 37 °C for 30 min, the absorbance of the reaction mixture was measured at 500 nm. The calibration curve was obtained using a standard choline chloride solution instead of the enzyme solution. One unit (U) of hydrolytic activity of PLD was defined as the amount of enzyme that produced 1 mM of choline per min. All assays were performed in triplicate.

2.5. Enzyme Preparation and HPLC Analysis of PS

In this study, PC was converted into PS using a two-phase system with the following conditions: 10 mL of diethyl ether containing 2 g/L of soybean PC served as the organic phase, while 5 mL of the acetate buffer (0.2 M) containing 0.4 g of L-serine along with 6.2 mU of the enzyme was used as the aqueous phase. The reaction was conducted in a sealed flask at 30 °C for 2 h under shaking conditions at 180 rpm. Once the reaction was complete, 0.5 mL of the sample was collected from the organic phase, evaporated in a well-ventilated fume hood, and then reconstituted in 0.5 mL of methanol/chloroform (2:1 *v*/*v*). The lipid compositions were subsequently analyzed using high-performance liquid chromatography coupled with evaporative light scattering detection (HPLC-ELSD) after passing the sample through a 0.22 µm organic filter membrane.

The chromatographic conditions included: phospholipid separation was completed using the Sepax HP-Silica column (5 µm, 250 × 4.6 mm); 5 µL of the sample was loaded onto the column; mobile phase A–methanol/H_2O/acetic acid/triethylamine (85:15:0.45:0.05, *v*/*v*/*v*/*v*) and mobile phase B–n-hexane/isopropanol/mobile phase A (20:48:32, *v*/*v*/*v*); flow rate of 1.0 mL/min; column temperature of 40 °C; nitrogen gas flow of 2 mL/min; and drift tube temperature 60 °C during ELSD. Calibration solutions containing relevant phospholipids were utilized to determine diverse phospholipids based on their specific retention times, while their contents within the samples were determined based on the peak areas calculated through integration. The PS yield (%) was determined by calculating the PS percentage relative to the original PC content. Table 1 presents the gradient elution program employed.

Table 1. The gradient elution program of the mobile phase.

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	10	90
20	30	70
35	95	5
36	10	90
41	10	90

2.6. Biochemical Characterization of the Recombinant Enzyme

An enzymatic hydrolysis reaction was conducted under different temperatures (25–60 °C) to determine the optimal temperature for PLD. A Tris–HCl buffer (0.2 M) with diverse pH levels (4.0–7.0) was adopted to determine the optimal pH for the enzyme. The highest PLD activity was used as a reference (100% relative activity). To determine the functions of the different metal ions in PLD activity, various metal ions, containing Zn^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Fe^{3+}, Na^{+}, and K^{+} were introduced into the reaction mixture at a concentration of 10 mM. The relative activity of the control sample was considered to be 100%. The initial reaction mixtures were prepared without the addition of the metal ions.

The mixtures were incubated at diverse temperatures (30, 40, 50, 55, 60, 65, 70, and 80 °C) to determine the optimal temperature for the transphosphatidylation activity. In addition, acetic acid–sodium acetate-based buffers with different pH values ranging between 4.0 and 8.0 were utilized to determine the optimal pH for PLD. The effect of the metal ions was determined with the same metal ions listed above at the same concentration. In addition, 1 mM and 10 mM working solutions were used to evaluate the effects of these metal ions. The metal ion-free enzyme reaction was used as a control.

2.7. Single Factor Optimization of PS Synthesis

To achieve maximum PS production, reaction condition optimization was conducted using the univariate experimental design (in which the variable to be analyzed was varied within a range while the other variables remained unchanged). The study investigated the impact of various organic solvents (petroleum, n-hexane, cyclohexane, ether, dichloromethane, ethyl acetate, butyl acetate, and chloroform), buffers (acetic acid/sodium acetate, citric acid/sodium citrate, and disodium hydrogen phosphate/potassium dihydrogen phosphate), and different organic solvent-to-water volume ratios (1:4, 1:3, 1:2, 1:1, 2:1, 3:1, 4:1, and 5:1), and the mass ratio between substrates L-Ser and PC (10:1, 20:1, 30:1, 40:1, 50:1, 60:1, 70:1, 80:1, 90:1, and 100:1). These mixtures were subjected to vigorous stirring for 2 h at 50 °C. Mg^{2+} is a critical regulatory factor for PLD activity, and it regulates enzyme catalytic activity and enzyme stability. Therefore, the effect of $MgCl_2 \bullet 6H_2O$ at different doses (0, 5, 10, 15, 20, 25, and 30 mM) on the transesterification activity of the enzyme was also evaluated.

2.8. Statistical Analysis

Each assay was conducted in triplicate. Microsoft Excel and GraphPad Prism 9 were employed for statistical analysis. The results were expressed to be mean ± standard deviation (SD).

3. Results

3.1. Expression and Purification of the Protein

The desired protein showed a predicted isoelectric point (PI) of 5.34 by using the online ExPASy software (https://web.expasy.org/compute_pi/, accessed on 5 June 2022), but the actual value may be higher. Thus, the protein can be bound on a cation-exchange column using a buffer with a pH below the pI value. The fractional purification of PLD is shown in Figure 1a. We harvested 143.6 mg of total protein after the initial purification with ammonium sulfate and dialysis. Hitrap SP HP cation exchange chromatography removed a large amount of heteroproteins from the solution, achieving 7.93 mg of total protein. The final purification via Superdex 200 10/300 GL gel chromatography and the subsequent concentration in ultrafiltration centrifuge tubes gave an enzyme concentration of 3.13 mg/mL. Figure 1b shows that the enzyme solution was essentially electrophoretically pure via fractional purification, with a relative molecular mass of approximately 54 kDa, which conformed to the determined molecular mass of 53,861 Da for the protein.

3.2. Recombinant PLD Characterization

Enzymatic PS synthesis based on soybean lecithin involves the transphosphatidylation reaction, in which PLD is the catalyst, PC is the phosphatidyl residue donor, and L-serine is the acceptor. The transphosphatidylation activity can be enhanced; meanwhile, the hydrolytic activity was depressed via selecting the optimum conditions for transphosphatidylation.

The reaction temperature is a critical factor influencing both the thermodynamic equilibrium of the reaction and the enzyme stability and performance during enzyme catalysis. Figure 2a,b illustrates the dependence of PLD on the reaction temperature. The enzyme achieved the optimal hydrolytic performance at 45 °C, while the optimal temperature for the transphosphatidylation reaction was determined to be 50 °C. The enzyme activity of PLD dropped rapidly when the temperature was above 45 °C for the hydrolysis reaction, while the

activity decreased slightly at higher temperatures and was maintained as high as 89% and even at 80 °C for the transphosphatidylation reaction. Therefore, it was revealed that PLD exhibits better thermostability, a broader temperature range, and a lower loss of activity in the transphosphatidylation reaction. Considering the differences in the optimum temperature in different reactions, selecting the right temperature in the enzymatic preparation of PS could reduce the hydrolytic activity of the enzyme and increase the transesterification activity, thereby improving the PS yield ultimately.

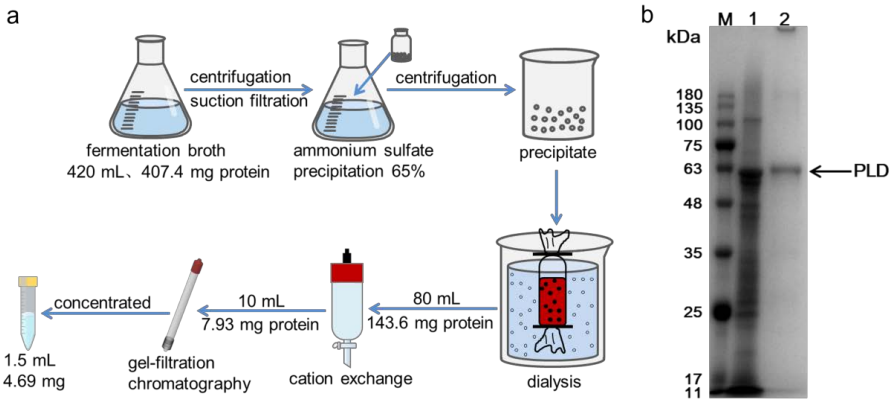

Figure 1. Purification and detection of the target protein. (**a**) The schematic workflow of fractional purification; and (**b**) SDS-PAGE analysis of purified PLD. M: Protein marker; 1: crude enzyme; 2: purified protein.

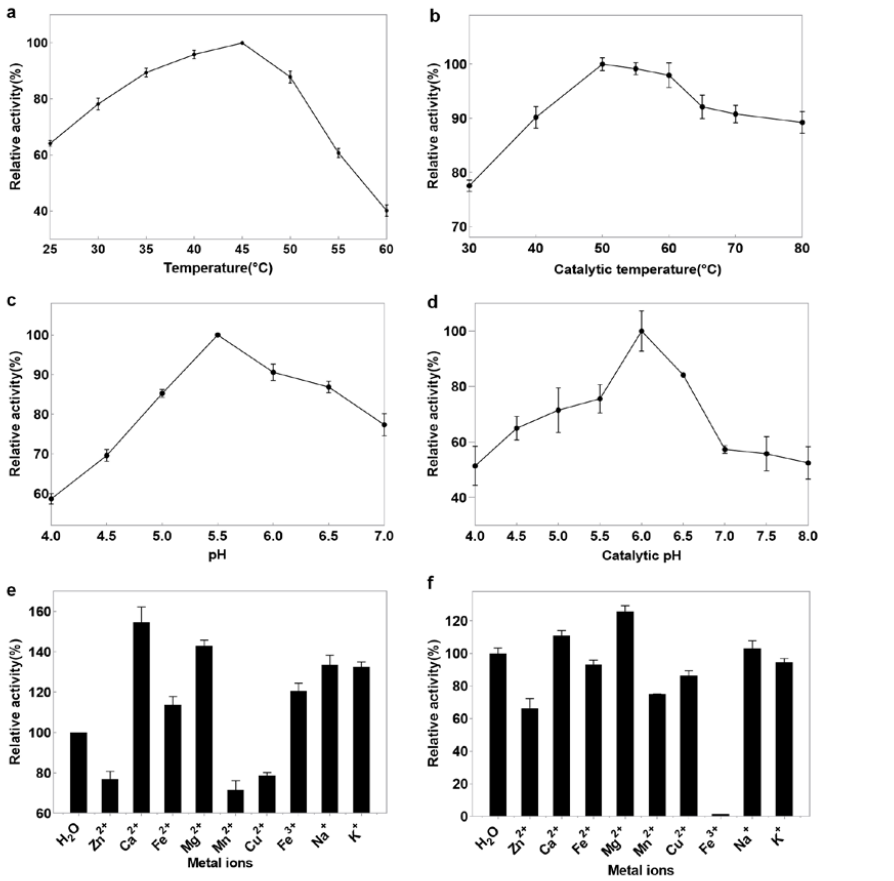

Figure 2. The characterization of the recombinant PLD. (**a**) The role of temperature at which the hydrolysis reaction is conducted; (**b**) the role of catalytic temperature; (**c**) the role of hydrolytic pH; (**d**) the role of catalytic pH; (**e**) the role of the metal ions in the hydrolytic reaction; and (**f**) the role of the metal ions in the transphosphatidylation reaction.

The effect of the aqueous solution pH was studied by conducting the reaction at varying pH levels and at optimal temperature. The findings are presented in Figure 2c,d. The optimal pH for the hydrolysis reaction was determined to be 5.5, while the optimal pH for the transphosphatidylation reaction was determined to be 6.0. Similar results have been reported in a previous study [42]. Moreover, the hydrolytic activity was observed to decline rapidly until the pH < 5.5, and a further increased rate of decline was observed when the pH values were higher than 5.5. At pH 7.0, the enzyme retained over 70% of its original activity. On the contrary, the transphosphatidylation activity increased gradually with the increase in the pH and then decreased sharply when the pH was above 6.0. These results indicated that the recombinant PLD exhibited a greater loss of activity under neutral and alkaline conditions in the transphosphatidylation reaction. Therefore, subsequent experiments were conducted under mildly acidic conditions (pH 6.0).

Some specific metal ions are known to have significant effects on maintaining PLD activity [43]. As depicted in Figure 2e,f, the transphosphatidylation activity of PLD was completely inhibited with the addition of Fe^{3+}, while the hydrolytic activity of the enzyme increased. On the other hand, the addition of Zn^{2+}, Mn^{2+}, and Cu^{2+} resulted in a reduction in both hydrolytic and transphosphatidylation activities, but the enzyme still retained over 60% of its original activities in each case. The hydrolytic activity and the transphosphatidylation activity were increased upon the addition of Mg^{2+} and Ca^{2+}, which could be due to the role of these metal ions as activators to promote the enzymatic activity. However, Ca^{2+} exerted the greatest promoting effect on the hydrolytic activity (154.6%), while Mg^{2+} made the most significant impact on the transphosphatidylation activity (125.9%). The hydrolytic activity was increased upon the addition of Fe^{2+}, Na^{+}, and K^{+}, with the addition of Na^{+} and K^{+} enhancing the hydrolytic activity by 33.4% and 32.5%, respectively. The transphosphatidylation activity was slightly inhibited upon the addition of Fe^{2+}, Na^{+}, and K^{+}.

3.3. Optimization of the PS Preparation Process

The above results indicated the impacts of temperature, pH, and metal ions on the transphosphatidylation activity. Therefore, 50 °C, pH 6.0, and Mg^{2+} were selected as optimal reaction conditions for the subsequent experiments.

As presented in Figure 3a,b, the best transphosphatidylation activity was achieved using butyl acetate as the organic phase and sodium acetate/acetic acid as the buffer. The polarity of the organic solvents did not significantly affect the transphosphatidylation of PC. Butyl acetate, dichloromethane, and diethyl ether led to the highest PS yield (17–31%). Since diethyl ether and dichloromethane have low boiling points (34.5 °C and 39.8 °C), butyl acetate was selected as the optimal organic solvent, conforming to the findings reported by Chen et al. [12]. The sodium acetate/acetic acid buffer (0.2 M, pH 6.0) was selected for the bioconversion experiments, conforming to most studies reported previously [26,30]. The Mg^{2+} content was varied in the range of 5–30 mM to evaluate its impact on the reaction. As visible in Figure 3c, the addition of 15 mM Mg^{2+} led to the greatest activity and a PS yield of 45.71%.

The substrate mass ratio significantly impacted the reaction equilibrium shift to the preferred product during the reversible reaction [44]. Therefore, it was inferred that a sufficient concentration of substrate is an essential condition for improving the efficiency of the transphosphatidylation reaction. Figure 3d illustrates the effect of the substrate mass ratio on PS production. The L-serine-to-PC mass ratio varied from 10:1 to 100:1, which caused the PS concentration and yield to show an increasing trend as the L-serine content was raised, reaching a plateau at a L-serine-to-PC mass ratio of 80:1. At this ratio, the highest PS yield of 81.43% and a PS content of 1.63 g/L were achieved. However, when the L-serine-to-PC mass ratio was further increased beyond 80:1, the PS yield decreased by 7.49% in the range of 80:1 to 100:1. These findings suggest that an appropriate L-serine-to-PC ratio favors the generation of PS, in line with the previous reports [30]. Thus, the optimal L-serine-to-PC ratio of 80:1 was selected for subsequent system optimization experiments.

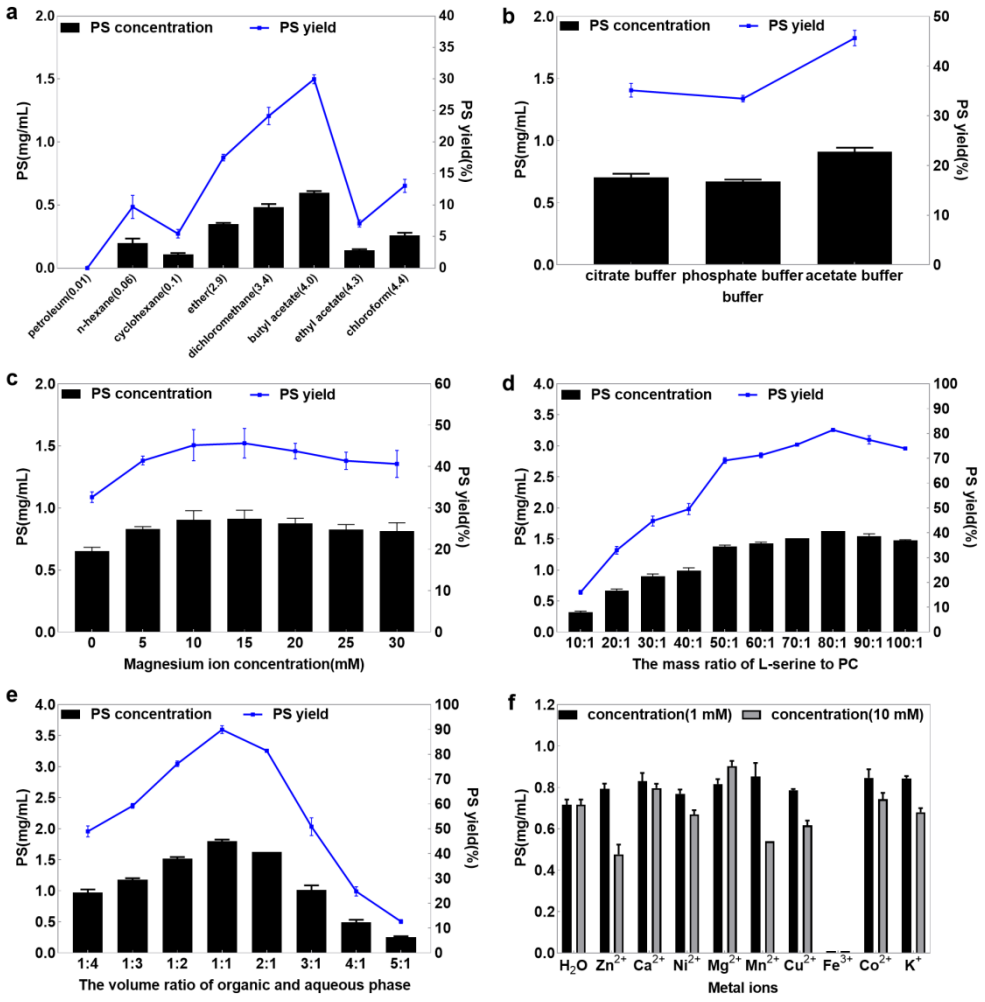

Figure 3. Optimization of the parameters in the transphosphatidylation reaction system. (**a**) The effects of different organic solvents on the conversion rate; (**b**) the effects of different buffers on the conversion rate; (**c**) the effects of the magnesium ions on the PS conversion rate; (**d**) the effect of the L-ser/PC substrate mass ratio on the conversion rate; (**e**) the effect of the volumetric ratio (organic phase to aqueous phase) on the conversion rate; and (**f**) the influence of the addition of the metal ions at different concentrations (1 mM and 10 mM) on the conversion rate.

Excessive water reportedly induces PC hydrolysis and PA accumulation in the PS biphasic bioconversions based on PLD [45]. Therefore, to obtain the maximum PS yield, the reaction was conducted by adjusting the organic-to-aqueous phase volumetric ratio (Figure 3e). When the water level decreased from 80% to 50% (the organic/water phase volume ratio changed from 1:4 to 1:1), the transphosphatidylation rate gradually increased until it remained stable. Moreover, a PS yield of 89.94% was retained when the organic-to-aqueous phase volumetric ratio was 1:1. After this point, the transphosphatidylation rate decreased rapidly with a further decrease in the water content and a further increase in the butyl acetate content. The PS yield decreased evidently to 12.6% when the water level declined to 16.7% (the organic/water phase volumetric ratio was 5:1). This possibly occurred due to the reduced PLD activity upon the lowering of the water content because of the butyl acetate strips of the essential enzyme-surrounding water layer. Therefore, the butyl acetate-to-buffer volumetric ratio of 1:1 was selected as the optimal ratio.

The effect of the metal ions on the transphosphatidylation activity was also analyzed, and the ion concentrations used were 1 mM and 10 mM (Figure 3f). The transphosphatidylation activity was completely inhibited in the presence of Fe^{3+} irrespective of the concentration used (1 mM or 10 mM). The other evaluated metal ions (Zn^{2+}, Ca^{2+}, Ni^{2+},

Mg^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, and K^{+}) promoted the activity of the enzyme to varying degrees when added at the concentration of 1 mM. At the same time, the concentration of 10 mM of Zn^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, and K^{+} suppressed the enzymatic activity and that of Zn^{2+}, Mn^{2+}, and Cu^{2+} evidently inhibited the enzyme activity. These results indicated the diverse effects of different metal ion contents on enzyme activity. Under various experimental conditions containing different metal ion concentrations, enzyme conformation and enzyme activity were possibly impacted due to the interactions between the enzyme and the added metal ions.

3.4. Transphosphatidylation Time Course

It is usually important to investigate the reaction duration as it assists in determining the minimum duration required to obtain the greatest product yield and, thereby, reduce the production costs. Figure 4a illustrates the PC-to-PS transphosphatidylation time course under the action of PLD. The reaction was performed under optimal conditions: catalytic temperature of 50 °C; catalytic pH of 6.0; metal ion concentration of 15 mM Mg^{2+}; butyl acetate/acetate buffer 1:1; L-serine 160 mg/mL; soybean lecithin 2 mg/mL. The reaction was carried out for a total of 4 h, with 0.5 h allocated for each sample group to measure the quantities of PS, PA, and PC. The results showed that PC was completely disintegrated within 2.5 h, while PS and PA reached a plateau 2.5 h later. The PS conversion rate reached 91.35%, and the accumulated PS concentration was 1.83 g/L, with a PS productivity of 0.73 g/L/h. As depicted in Figure 4b, the optimized system demonstrated a reasonable performance, exhibiting an extremely high transphosphatidylation rate.

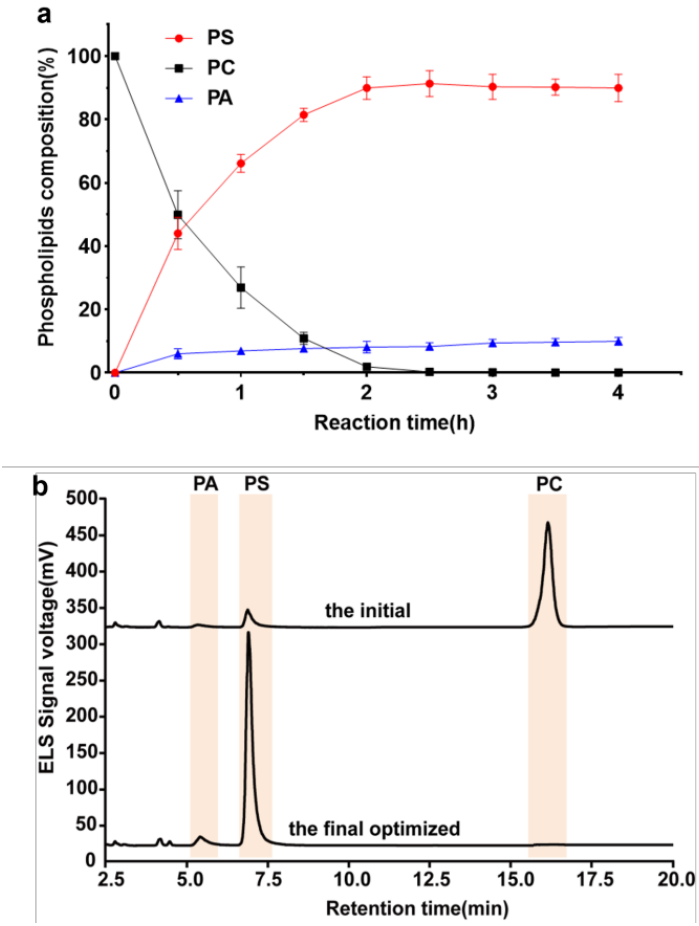

Figure 4. The recombinant PLD was used for the PC-to-PS conversion. (**a**) The transphosphatidylation reaction time course; and (**b**) the transphosphatidylation activity of the recombinant PLD towards PC.

4. Discussion

The recombinant PLD from *S. antibioticus* SK-3 was successfully expressed and purified, followed by its application for catalyzing the conversion of L-serine and lecithin to PS. The recombinant enzyme exhibited suitable hydrolytic activity on PC. Moreover, the enzymatic properties of this recombinant PLD were analyzed. The recombinant enzyme exhibits evident transphosphatidylation activity and could, therefore, catalyze the PS synthesis reaction. The recombinant PLD demonstrated a high conversion rate during the PS biosynthesis after optimizing the transphosphatidylation conditions. Under optimized conditions, PS production reached 91.35% within 2.5 h, with a PS productivity of 0.73 g/L/h. The findings of the present study establish a certain foundation for the application of this recombinant enzyme in industrial PS production, rendering the large-scale production of PS feasible. Importantly, the recombinant enzyme is revealed as a suitable potential candidate for use in the industrial production of uncommon phospholipids with commercial importance.

In the last 10 years, *Streptomyces* PLDs have been widely analyzed for their hydrolytic activities, while research on transphosphatidylation activity is scarce. The investigation of the transphosphatidylation properties of PLDs from *Streptomyces* is important for optimizing PS production conditions. As observed in the present study, PLD exhibited excellent thermostability in the transphosphatidylation reaction. Therefore, it was inferred that this enzyme could be applied at the industry level for the synthesis of PS. In addition, the resistance to certain metal ions was remarkably enhanced relative to the hydrolytic reaction. For instance, when the effects of Mg^{2+} and Ca^{2+} were compared, Mg^{2+} was observed to exert a higher effect compared to Ca^{2+}. This was inconsistent with the findings of most previous studies, which have reported Ca^{2+} to have a more favorable effect on the synthesis of PS [46,47]. Moreover, the hydrolysis activity was promoted upon the addition of Fe^{3+}, while the transphosphatidylation activity was almost completely inhibited upon the addition of this metal ion. On the one hand, this could be due to the different systems affecting the binding of the enzyme to the substrate. On the other hand, the different metal ions in the reaction systems could have affected the enzyme structure and stability and possibly the transcription of the gene encoding the protease, which could have ultimately affected the enzymatic activity of the extracellular protease. However, the mechanism underlying the effects of Mg^{2+} and Fe^{3+} remains unknown so far. Therefore, the effects of the different ions on the enzyme protein structure have to be explored further.

The PLD-mediated transphosphatidylation reaction for PS production is typically conducted in a biphasic solvent system, with a water-immiscible organic solvent phase because PS and PC are water-insoluble, while L-serine is water-soluble [12]. In this study, the optimal L-serine-to-PC mass ratio was found to be 80:1, which is higher than in most previous studies [30,38]. However, it appears that a large proportion of L-serine does not necessitate a higher water content (organic-to-aqueous phase volumetric ratio of 1:1) due to the diverse binding sites in PLD for both L-serine and water molecules [27,48]. Consequently, water content may not significantly impact PS production. Various organic solvents have been extensively evaluated for their effects on *Streptomyces* PLD-mediated transphosphatidylation for PS production, including ethyl acetate, diethyl ether, dichloromethane, and chloroform [26]. Although the diethyl ether system is favorable for PS production [14], its toxicity can lead to enzyme denaturation. In contrast, butyl acetate is less toxic and has a higher boiling point [12], making it advantageous for the large-scale industrial production of PS.

Previous studies on enzymatic PS production have primarily focused on using PLDs from *Streptomyces*. For instance, PS production with a yield of 88% was achieved using the PLD from *S. racemochromogenes* [12]. Duan and Hu evaluated five commercially available PLDs for PS production and reported that the PLD from *S. chromofuscus* resulted in a PS production of 90% after 12 h of bioconversion [35]. Table 2 presents the recombinant PLDs from *Streptomyces*, indicating that both the PS conversion rates and productivity values in the aqueous system and novel reaction medium are notably lower compared to the biphasic system. In our study, we obtained a PS productivity of 0.73 g/L/h with a PS yield of 91.35%

within 2.5 h of bioconversion, demonstrating that the recombinant PLD is a promising candidate for industrial-level PS production due to its higher efficiency and shorter reaction duration.

Table 2. Synthesis systems and the PS productivity achieved using these systems.

PLD Origin	System	Conversion Rate (%)	PS Productivity (g/L/h)	References
Streptomyces antibioticus	biphasic system/butyl acetate	91.35	0.73	this study
Streptomyces cinnamoneum	biphasic system/diethyl ether	92	0.61	[11]
Streptomyces racemochromogenes	biphasic system/butyl acetate	88	1.1	[12]
Streptomyces antibioticus	purely aqueous system	65	0.06	[31]
Streptomyces halstedii	purely aqueous system	53	0.07	[7]
Streptomyces chromofuscus	2-methyltetrahydrofuran	90	0.003	[35]
Streptomyces chromofuscus	biobased ionic liquids	86.5	5.8×10^{-4}	[33]

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