Research article

ARTP mutagenesis of phospholipase D-producing strain *Streptomyces hiroshimensis* SK43.001, and its enzymatic properties

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ABSTRACT

Phospholipase D (PLD) is a group of enzymes that act on phospholipid molecules, which is widely used in the fields of food and medicine. PLD is extracted from animals and plants with low transesterification activity and high price. Therefore, it is benefit to screen an efficient PLD producing strain from microorganisms. A highly productive strain of PLD with transphosphatidylation activity, named *Streptomyces hiroshimensis* SK43.001, was screened from soil in our laboratory and mutated using atmospheric room temperature plasma (ARTP). A mutant strain SK43.001-11 with the highest enzyme activity and superior genetic stability was obtained, and its fermentation enzyme activity was 5.3 U/mL, which was 82% increased comparing with wild strain. The purification of PLD showed that the specific enzyme activity increased to 49.48 U/mg, which was 54.37-fold higher than that of the crude enzyme, with a recovery of 32.31%. In addition, enzymatic properties of PLD have revealed that the optimal pH and temperature were 7.0 and 60 °C, respectively. Metal ion Mg^{2+} and surfactant Triton X-100 made the enzymatic activity increased by 16% and 100%, respectively. The reaction kinetic parameters showed that the mutant PLD had higher affinity for the substrate of egg PC and better catalytic efficiency with K_m, V_{max} and K_{cat} of 30.20 mmol/L, 99.70 μmol/min and 76.33 s^{-1}, respectively. This study may provide important inspiration for obtaining high enzyme activity strains with PLD.

1. Introduction

Phospholipase D (PLD, EC 3.1.4.4), namely phosphatidylcholine phospholipid hydrolase, is a group of enzymes that act on the phosphodiester bond in phospholipid molecules. As shown in Figure 1, it catalyzes the hydrolysis of phospholipids to produce phosphatidic acid and hydroxyl compounds, as well as the combination of various hydroxyl-containing compounds with the bases of phospholipids to produce new phospholipids under certain conditions [1, 2].

PLD has a wide range of applications in food and medical fields. In food industry, the phospholipids through its transphosphatidylation activity of PLD can be used not only to convert and modify soy phospholipids to improve their nutritional value, but also to prepare some high-purity multifunctional phospholipids such as phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylserine (PS), etc [3, 4, 5]. In medicine, the transphosphatidylation of PLD is utilized to catalyze the binding of phospholipids to some nucleoside or polysaccharide drugs to prepare liposomes with specific medical benefits. Wang et al. [6] described an efficient enzymatic procedure for the

synthesis of phospholipid-inhibitor conjugates using PLD, such as aza-sugars, nucleosides, and peptides. Simultaneously, PLD is involved in the synthesis of anti-tumour drugs. Testetlamant et al. [7] found five 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (PAF-acether) analogues catalyzed from PAF-acether and cyclic primary alcohols by PLD, which opened up new directions for the enzymatic synthesis of antineoplastic drugs. Shuto et al. [8] and Koketsu et al. [9] synthesized phosphatidyl nucleoside analogues and phosphatidyl acetyl neuraminic acid with anti-tumour and anti-viral effects, respectively.

PLD is widely found in animals, plants and microorganisms. Animal-derived PLD mainly exists in tissues, such as brain and liver, and is mostly bound to cell membranes, making it difficult to be extracted [10]. In plants, PLD is mainly found in organs, such as roots, leaves and seeds, and has been extracted from carrots, cabbage, cottonseed and soybeans [11]. Among microorganisms, PLD is mainly produced by bacteria (*Escherichia coli*, *Bacillus pallidum*, *Bacillus cereus*, etc.) and actinomycetes [12, 13, 14] (*Streptomyces chromobium*, *Streptomyces cinnamon*, etc.) [15,16]. Compared with animal and plant-derived PLD, microbial-derived PLD has stronger substrate tolerance and broader substrate specificity [17].
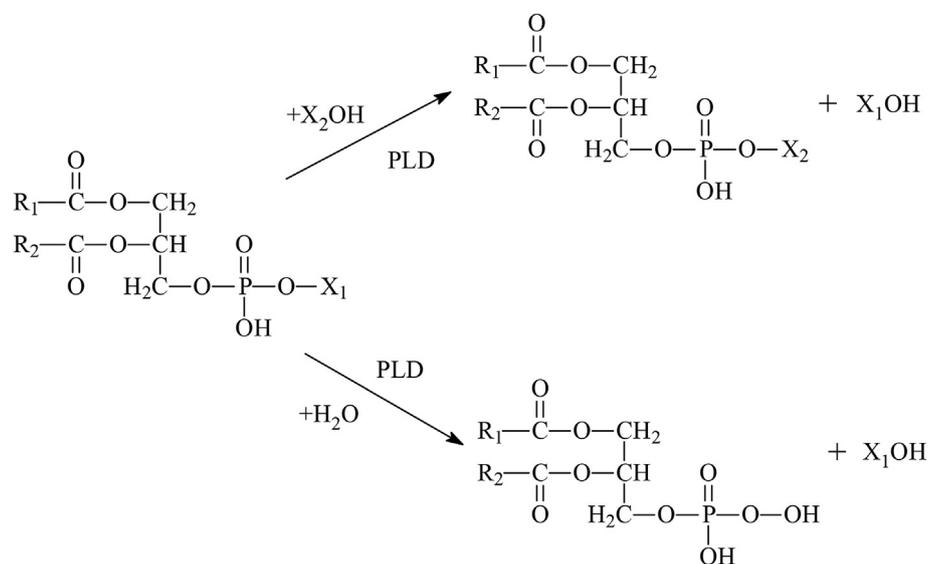
Figure 1. Catalytic reaction of PLD.

More microbial PLDs have been reported in recent years, ranging from viruses to prokaryotes and eukaryotes. PLDs from *Streptomyces* are known to exhibit high transphosphatidylation activity, broad substrate specificity for phospholipids and, more importantly, excellent stability to heat and organic solvents. Therefore, it is essential to screen strains with high PLD production capacity for industrial applications.

Atmospheric and room temperature plasma (ARTP) mutagenesis has been widely used to produce mutants of more than 40 microorganisms, including fungi, bacteria and microalgae [18]. Plasma from the ARTP has far-reaching effects on microorganisms, including heat, electromagnetic fields, ultraviolet radiation, charged particles and reactive oxygen species [19]. Compared to traditional physicochemical mutagenesis, ARTP mutagenesis is easier and more economical, and is free of hazards in terms of operator safety and environmental pollution [20]. The technique also offers outstanding features such as multiple damage to DNA, high mutation rates and good genetic stability [21].

In this study, the ARTP mutation system was used to mutate a strain of *Streptomyces hiroshimensis* SK43.001 from soil to screen the strain with higher PLD enzyme activity. The PLD in the fermentation broth was subsequently fractionally purified. Furthermore, the biochemical properties of the enzyme were evaluated in this study. The results of this study may provide important insights into strain mutation and the effective production of PLD.

2. Materials and methods

2.1. Strains and materials

The wild strain with PLD producing ability used in this study was *S. hiroshimensis* SK43.001, which was isolated from wetland soil in Wuxi, Jiangsu, China. The strain was identified from 16S rRNA and stored in our laboratory.

Choline oxidase and peroxidase were purchased from Sigma-Aldrich Trading Co., Ltd. (Shanghai, China). Other chemicals (analytical grade) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Culture conditions

The slanted agar medium contained the following components (g/L): glucose 15, maltose 10, yeast extract 2, and agar 20. The seed medium (pH 7.0) was as follows (g/L): glucose 10, yeast extract 20, peptone 5, $K_2HPO_4 \cdot 3H_2O$ 2 and $MgSO_4 \cdot 7H_2O$ 0.5. The fermentation medium (pH

7.0) consisted of glucose 10 g, beef extract 5 g, peptone 5 g, $K_2HPO_4 \cdot 3H_2O$ 2 g and $MgSO_4 \cdot 7H_2O$ 0.5 g per liter.

All medium used in this study were sterilized at 115 °C for 30 min (Glucose and maltose were added after separate preparation and sterilization.).

2.3. Mutation procedures for ARTP

The wild strain SK43.001 was inoculated on the slant medium and cultured at 28 °C for 7 d. The spore suspension was obtained with the aid of sterile water. The operational procedures for mutagenesis was carried out using ARTP-II (Wuxi Siqingyuan Biotechnology Co., Ltd., Wuxi, China) after appropriate dilution (Figure 2). The spore suspension was evenly coated onto the surface of sterilized metal plates and then irradiated for different times using a helium plasma jet. After processing the samples, wash the metal plate with 1 mL of sterile saline solution and shake for 1 min. Subsequently, the eluent was properly diluted, and the cell suspension was spread on plates to determine the mortality rate, which was calculated according to the following Eq. (1):

$$\text{Mortality rate } (\%) = \frac{\text{controlcolonies} - \text{survivalcolonies}}{\text{controlcolonies}} \times 100\% \qquad (1)$$

All colony numbers were obtained by the colony-forming units (CFU) method on agar plates.

2.4. Screening of the mutants

The mutants were screened after ARTP treatment. Select well-grown mutants from the plates and inoculate them into the slant medium. After 7 d of incubation at 28 °C, the organisms were inoculated into the seed medium and incubated for 1.5 d. The mutants showing high enzyme activity were selected by inoculating them in fermentation medium at 28 °C, 200 rpm for 7 d.

2.5. Genetic stability of the mutant strains

The genetic stability of mutants was determined by subculturing mutants for six generations. The selected mutants were cultured on plates, and then a single colony was taken from the aforementioned plates and transferred onto fresh plates once again. All plates were cultured at 28 °C for 7 d. The same step was repeated for six subcultures. The enzyme activity of each generation of mutants was measured by 250 mL shake flask fermentation experiments.
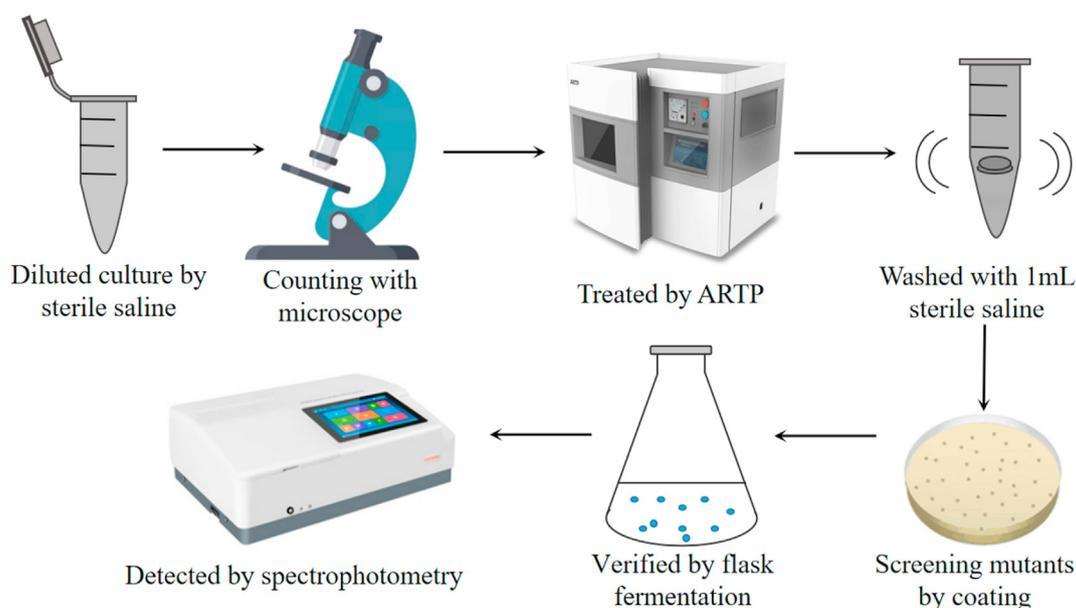
Figure 2. ARTP operational procedures for the mutation and selection of the mutants.

2.6. Enzyme activity assay

To determine the activity of PLD, the choline content released by phosphatidylcholine (PC) was measured according to the method of Imamura and Horiuti [22]. The reaction mixture (500 μL), which consisted of 100 μL of 4.5% egg PC emulsion (w/v, 0.5 g of egg PC dissolved in 1.0 mL of ether and shaken with 10.0 mL of deionized water to form emulsion), 100 μL of citrate buffer (10 mmol/L, pH 6.0), 150 μL of 7.5% Triton X-100 (v/v), 50 μL 0.1 mol/L $CaCl_2$ and 100 μL enzyme sample was incubated at 60 °C for 10 min, followed by the addition of 200 μL Tris-HCl buffer (0.1 mol/L pH 8.0, 50 mmol/L EDTA), and the reaction was terminated by boiling water bath for 5 min. After cooling at room temperature, 2 mL of choline identification solution consisting of 2 mg 4-aminoantipyrine, 1 mg phenol, 20 mg Triton X-100, 2 units of choline oxidase and 3 units of peroxidase was added and incubated at 37 °C for 20 min, followed by measurement of absorbance at 500 nm. The calibration curve was done with choline chloride. One unit was defined as the amount of enzyme required to generate 1 μmol of choline per minute from PC under the above conditions.

2.7. Purification of PLD

The fermentation broth was centrifuged at 4 °C for 20 min at 6000 rpm to obtain the crude enzyme solution. Ammonium sulfate was added to crude enzyme solution until the system saturation was 40%, and the precipitate was removed by centrifugation at 4 °C for 2 h. Then add ammonium sulfate to the supernatant until the system saturation reached 60%, centrifuge at 4 °C for 2 h to collect the precipitate, redissolve in citric acid-sodium citrate buffer (50 mmol/L, pH 5.0), and desalinate by dialysis at 4 °C. The dialysate was applied to a Hitrap SP HP ion exchange column 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 citric acid-sodium citrate buffer (50 mmol/L, pH 5.0) containing 0.5 mol/L NaCl. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to identify the protein molecular mass of the active fraction.

2.8. Enzymatic properties of PLD

2.8.1. Effects of temperature on PLD

To determine the optimal temperature, enzyme activity was assayed at different temperatures ranging from 30 to 80 °C. The highest enzyme activity was set at 100%. For thermal stability, the enzyme was incubated at 35–50 °C for 0–3 h and the residual enzyme activity was determined every 0.5 h intervals (the initial enzyme activity was set at 100%).

2.8.2. Effects of pH on PLD

To investigate the optimal pH of PLD, experiments were carried out under three buffer conditions: citric acid-sodium citrate buffer (50 mmol/L, pH 4.5–6.5), disodium hydrogen phosphate-sodium dihydrogen phosphate buffer (50 mmol/L, pH 6.5–8.0), and Tris-HCl buffer (50 mmol/L, pH 8.0–9.0). The relative activity was normalized to the maximum activity set at 100%. In order to study pH stability, the enzyme was pretreated at pH 3.0 to 9.0 for 24 h, respectively. The highest enzyme activity was set at 100%.

2.8.3. Effects of metal ion on PLD

The effect of metal ion was measured by assaying the activity at pH 7.0 and at 60 °C in the presence of 2 mmol/L of various metal ions. The tested metal ions included Ca^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, Ba^{2+}, Mg^{2+}, Co^{2+} and Al^{3+}, all supplied in chloride form. The metal ions and purified enzyme were placed at 4 °C for 6 h and a control group without metal ions was set up. The measured activity without metal ions was defined as 100% relative activity.

2.8.4. Effects of surfactant on PLD

The effect of surfactant was evaluated by assaying the activity at pH 7.0 and at 60 °C in the presence of different surfactants. The tested surfactants included Tween 20, Tween 60, Tween 80, Span 80, Triton X-100, deoxycholic acid, CHAPS, Brij-35 and SDS solution, all were at a mass fraction of 1.0%. The measured activity without surfactants was defined as 100% relative activity.

2.8.5. Kinetic parameters

The kinetic parameters of the purified PLD were measured at different concentrations of egg PC solutions (1–60 mmol/L) at 60 °C and pH 7.0. The K_m, V_{max} and K_{cat} values were calculated by fitting the nonlinear Hill function using OriginPro 9.1 (Origin Lab Inc., Northampton, MA, USA).

2.9. Statistical analysis

Three replicates were established for each group of experiments, with the data presented as the mean ± standard deviation. Analyses were performed using SPSS software (v.20.0; IBM Corp., Armonk, NY, USA),

with significant differences at $p < 0.05$. Maps were generated using OriginPro 9.1 (Origin Lab Inc., Northampton, MA, USA).

3. Results and discussion

3.1. ARTP exposure time for effective mutagenesis

The plasma irradiation dose affects microbial mortality and mutation efficiency [20]. In this study, to facilitate the selection of high-yielding mutants, the plasma exposure time was the main parameter for evaluating the irradiation dose. As shown in Figure 3A, there was a significant dose-effect relationship for lethality of ARTP against *S. hiroshimensis* SK43.001, i.e. lethality raised with increasing duration of mutagenic treatment. After irradiation for 40 s, the mortality rate was 95%. According to another study, a high lethality rate was helpful in increasing the probability of obtaining mutants with enhanced yield and high survivability [23]. In this study, an exposure time of 40 s was considered appropriate for mutagenesis.

3.2. Screening of high-yielding PLD mutants

From the enzyme activity assay curve (data not shown), it was observed that the enzyme activity of PLD correlated with cell growth and accumulation, i.e. the enzyme activity of PLD increased with the amount of cell growth. Therefore, mutant strains were screened for a stable period of cell growth. The results of screening of the mutagenically treated strains were shown in Figure 3B. Among the 15 screened mutants, 14 mutants had increased PLD enzyme activity. Mutant 11 was the optimal strain with the highest PLD enzyme activity of 5.3 U/mL, which was an increase of 82% compared to the wild strain. Apparently, ARTP mutagenesis had a significant effect on the increased enzymatic activity of PLD, which may be because it caused great DNA damage to living cells while maintaining their viability. The extent of DNA damage greatly influences the subsequent SOS repair process. SOS repair can enhance cell survival after DNA damage by either rigorous repair or by placing random deoxyribonucleotide triphosphates across the damaged site to ensure continuous replication, thereby generating mutations [21]. The above results suggest that ARTP is a potentially powerful mutagenic tool that can improve the characteristics of strains.

3.3. Genetic stability of mutants

If the genetic traits of the mutants are unstable, they are of no practical relevance for industrial production as they reflect potential future

mutations at the genetic level [24]. The genetic stability of *S. hiroshimensis* SK43.001-11 was assessed by culturing the mutant for several generations and measuring the enzymatic activity of PLD in each generation. As shown in Figure 3C, the strain maintained stable growth and PLD enzyme activity remained around 5.3 U/mL with no significant differences during the subsequent generations of culture. ARTP mutagenesis produced strains with high genetic stability due to the repairing effect of SOS. The active energy particles in the plasma cause damage to the genetic material of strains, thus inducing the SOS repair mechanism. SOS repair is a highly fault-tolerant process. During the repair process, various mismatch sites are created and stabilised to form genetic traits and produce mutants [21]. The results of this study show that mutant SK43.001-11 is genetically stable after mutation and has good potential for industrial application.

3.4. Purification of PLD

To determine the optimal dosage of ammonium sulfate, different quality of ammonium sulfate was added to the crude enzyme solution to achieve different saturation. As shown in Figure 4A, when the concentration of ammonium sulfate was increased from 0% to 40%, the residual enzyme activity decreased slowly and PLD remained mainly in the supernatant. When the concentration of ammonium sulfate was increased to 50%, the activity of PLD in the supernatant suddenly decreased from 90% to 35%, which indicated that PLD was precipitated from the supernatant in large quantities. The specific enzyme activity of the precipitated solution was highest when the ammonium sulfate saturation was increased to 60%, after which the specific enzyme activity decreased rather than increased with the continued addition of ammonium sulfate. This resulted in a determination of 40% for primary salting and 60% for secondary salting. The results of the AKTA avant protein purification system during ion exchange chromatography were shown in Figure 4B. Gradient elution was performed with buffer solution and three elution peaks appeared. It was found that only the first elution peak had enzyme activity of PLD. Afterwards, the enzyme activity of PLD in each elution peak was determined by gel chromatography, resulting in the first elution peak confirming the activity (Figure 4C).

The fractional purification of PLD were shown in Table 1. After the initial purification by ammonium sulphate, the specific enzyme activity increased to 2.58 U/mg, which was purified 2.84 times. Hitrap SP HP ion exchange chromatography removed a large amount of heteroproteins from the solution, achieving a specific enzyme activity of 40.67 U/mg and purifying PLD by 44.69-fold. The final purification by Superdex 200
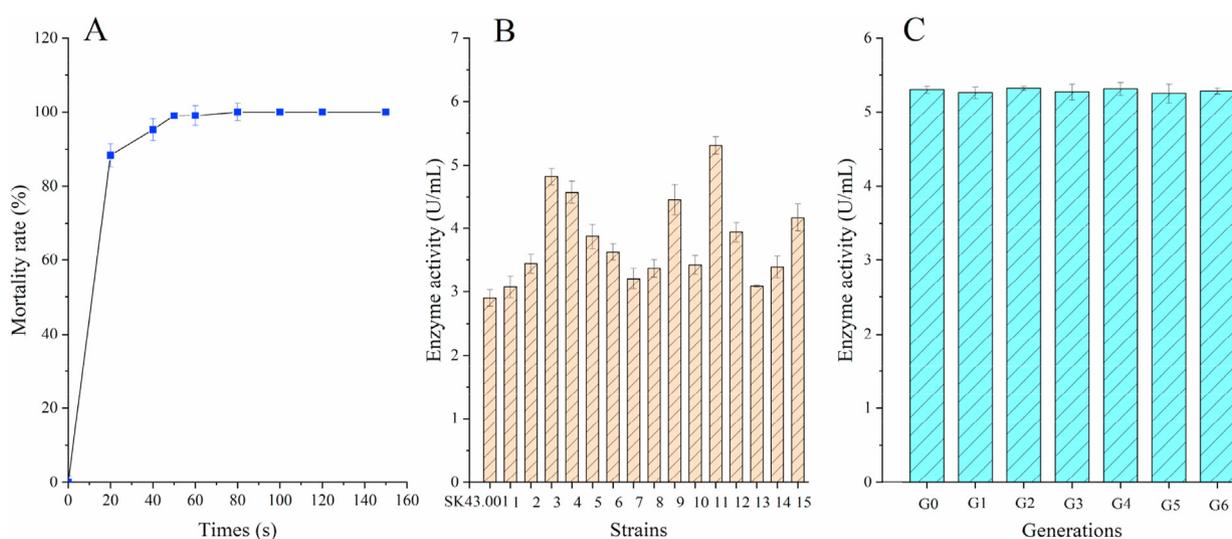
Figure 3. The conditions of ARTP mutagenesis and selection of the mutants. (**A**) The mortality rate at different exposure times. (**B**) The selection of mutants. (**C**) The validation of the genetic stability of mutants.
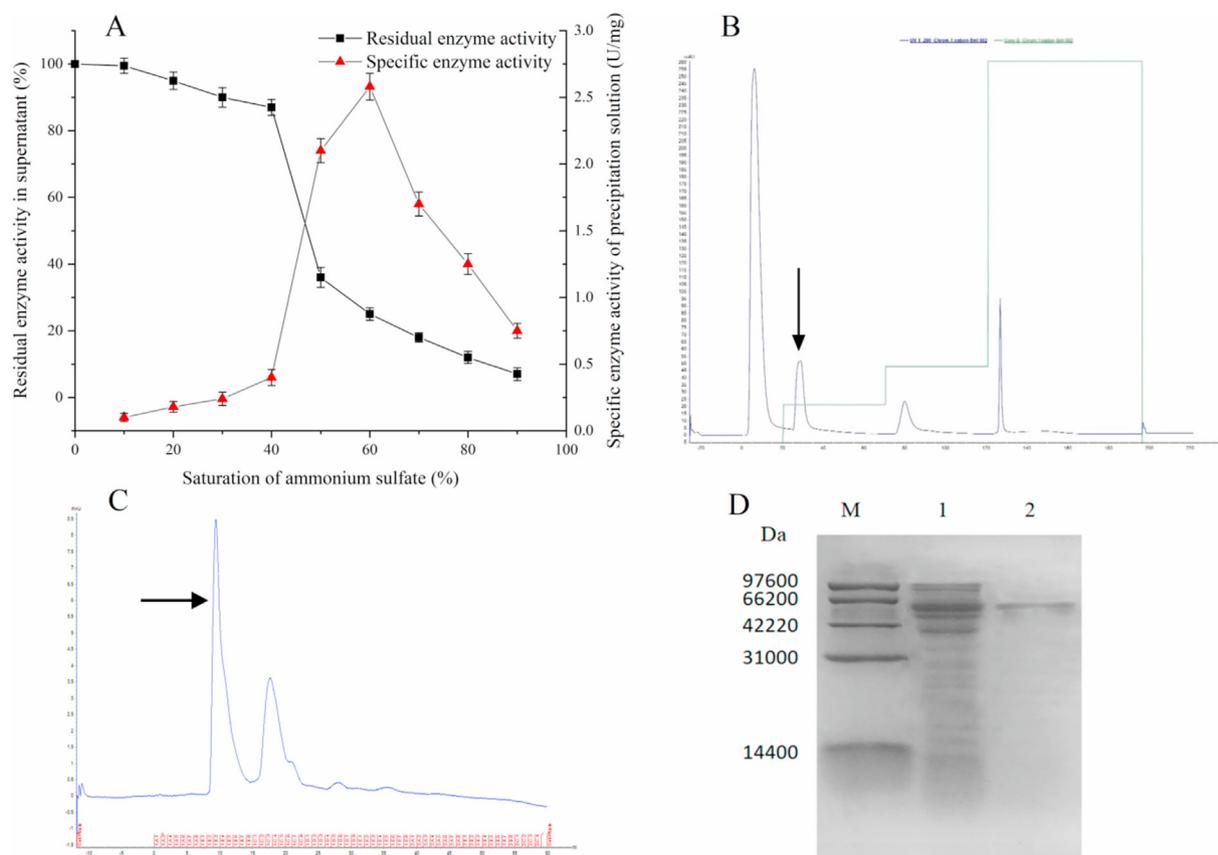
Figure 4. The purification of PLD. **(A)** Ammonium sulfate precipitation on PLD activity. **(B)** Hitrap SP HP ion exchange chromatography of PLD. (The black line represents the absorbance at 280 nm; the green line represents the eluent content; ↓ indicates the active peak.) **(C)** Superdex 200 10/300 GL gel chromatography of PLD. (The blue line represents the absorption value at 280 nm; → indicates the active peak.) **(D)** SDS-PAGE analysis of PLD. (M: middle relative molecular mass marker, 1: extract enzyme, 2: enzyme after Superdex 200 10/300 gel filtration).

10/300 GL gel chromatography gave a purification multiple of 54.37, a recovery of 32.31% and a specific enzyme activity of 49.48 U/mg of PLD. Active fractions were collected and analyzed by SDS-PAGE. Figure 4D showed that the enzyme solution was essentially electrophoretically pure by fractional purification, with a relative molecular mass of approximately 5.4×10^4.

3.5. Enzymatic properties of PLD

3.5.1. Effects of temperature on enzyme activity and stability

In industrial production, the value of enzyme applications is mainly influenced by temperature and thermal stability. The effects of temperature on PLD were evaluated at pH 7.0 over a range of temperatures, from 30 to 80 °C. As shown in Figure 5A, the activity was the highest at 60 °C and the enzyme activity decreased sharply at temperatures exceeding 60 °C. When the temperature was above 75 °C, the enzyme activity decreased by more than 90%. The optimal temperature largely depended on the microbial sources. For most PLD, such as *Streptomyces lydicus* D-121 [25], *Streptoverticillium cinnamoneum* [26], *Streptomyces antibioticus* [27], *Streptomyces septatus* TH-2 [28], *Streptomyces* sp. YU100 [29] and *Streptomyces*

cinnamoneum SK43.003 [30], the optimal temperature is typically consistent, ranging from 50 to 60 °C. However, its optimal temperature is more suitable for industrial production with low energy consumption compared to *Streptomyces olivochromogenes* [34] and *Streptoverticillium* sp. NA684 [35] (Table 2).

For the assessment of thermal stability, PLD was pretreated at 35, 37, 40, 45, and 50 °C. As shown in Figure 5B, about 80% residual activity was retained when the enzyme was incubated at 35 and 37 °C for 3 h. More than 60% activity was maintained at 45 °C for 0.5 h. However, there was only 20% and 15% residual enzyme activity after pretreating for 1 h and 1.5 h at 45 °C, respectively. The loss of enzyme activity was greater when the temperature was above 40 °C and decreased rapidly with increasing time. Residual enzyme activity was almost lost, after holding at 50 °C for 0.5 h. The thermostability of the PLD indicated that it was considerably stable below 40 °C, which was similar to those from *Streptomyces* sp. SC734 [14], *Streptomyces* sp. CS-57 [31], *Streptomyces* sp. CS684 [32], *Ochrobactrum* sp. ASAG-PL1 [33] and *Acinetobacter* [12] (Table 2). Compared with the reported literature, the thermal stability of this PLD needs to be improved, and attempts can be made by immobilization and molecular modification.

Table 1. Procedure for purification of PLD from *S. hiroshimensis*.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold	Yield (%)
Crude enzyme	574.03	524.02	0.91	1	100
Ammonium sulfate precipitate	167.90	432.91	2.58	2.84	82.61
Hitrap SP HP ion exchange chromatography	7.95	323.34	40.67	44.69	61.70
Superdex 200 10/300 GL gel chromatography	3.42	169.32	49.48	54.37	32.31
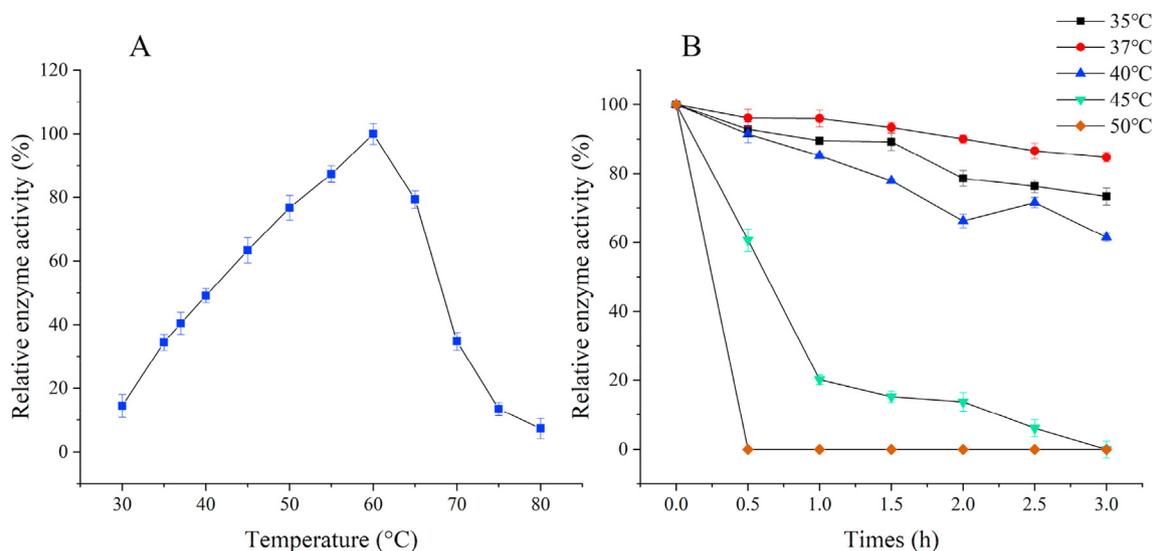
Figure 5. Effects of temperature on enzyme activity and stability of PLD. (**A**) Temperature-enzyme activity curve. (**B**) Temperature-enzyme activity stability curve.

3.5.2. Effects of pH on enzyme activity and stability

Changes in the pH of the reaction system can alter the dissociation state of the substrate from the enzyme, affecting the protein conformation and ultimately leading to changes in enzyme activity. As shown in Figure 6A, PLD exhibited the highest activity at pH 7.0, and 80% of the activity was retained at the pH of 6.0–8.0. Hence, the activity declined under acidic/alkaline conditions, suggesting that PLD is a neutral enzyme. Moreover, these data indicate that PLD, with an optimal pH of 7.0, which is similar with PLDs derived from *Ochrobactrum* sp. ASAG-PL1 [33], *Streptomyces* sp. SC734 [14] and *Streptomyces* sp. YU100 [29] that have an optimal pH ranging from 6.0 to 8.0 (Table 2). In terms of pH stability, more than 70% of the enzyme activity was retained after incubation at pH 5.0–8.0 for 24 h at 4 °C, respectively (Figure 6B). This result indicates that PLD is a relatively stable protein under slightly more acidic conditions. The pH stability of PLD indicates that it is superior to that of strains from *Streptomyces* sp. YU100 [29] and *Streptomyces olivochromogenes* [34]. The corrosion of equipment in industrial applications is much lower.

3.5.3. Effects of metal ions on enzyme activity

Metal ions affect the catalytic activity of enzymes by altering the ionic strength of reaction system, binding to amino acid residues at or near the active center of the enzyme and acting as cofactors for the enzyme to deliver chemical groups in enzyme-catalyzed reactions. To explore the effect of metal ions on PLD enzyme activity, nine metal ions were used, including Ca^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, Ba^{2+}, Mg^{2+}, Co^{2+} and Al^{3+}. As shown in Figure 7A, PLD was not strictly metal-dependent, and it exhibited strong catalytic activity without metal ions. The addition of Ni^{2+}, Mg^{2+} and Co^{2+} promoted the catalytic activity to different degrees, with Mg^{2+} showing the greatest enhancement, increasing the enzyme activity by 16%, while Cu^{2+}, Fe^{2+} and Al^{3+} significantly inhibited its catalytic activity. The addition of Ba^{2+} had no significant effect on the enzyme activity. Metal ions of Zn^{2+} and Ca^{2+} had a slight inhibitory effect on PLD. In contrast to the study by Simkhada et al. [31], Ca^{2+} did not have a strong promoting effect on the activity of PLD. This may be due to the fact that *Streptomyces*-derived PLDs, unlike plant-derived PLDs, lack the N-terminal domain and thus have no apparent dependence on Ca^{2+}. By

Table 2. Properties of PLD from partial microorganism sources.

Organisms	Subunit (kDa)	Optimum		Thermal stability[a]		pH stability[a]		References
		pH	T (°C)	T (°C)	Time (h)	pH	Time (h)	
Streptomyces lydicus D-121	56	5.5	60	60	0.5	4–7	2	[25]
Streptoverticillium cinnamoneum	54	6.0	55	NR	NR	NR	NR	[26]
Streptomyces antibioticus	64	5.5	60	50	0.5	4–8	2	[27]
Streptomyces septatus TH-2	55	5.0	55	60	1/6	4–9	16	[28]
Streptomyces sp. YU100	57	7.0	60	60	30	3–8	24	[29]
Streptomyces cinnamoneum SK43.003	54	6.0	60	50	5.0	4–7	24	[30]
Acinetobacter	60	6.0	45	45	2	7–9	40	[12]
Actinomadura sp. No. 362	58	5.5	50	30	2.0	4–8	2	[13]
Streptomyces sp. SC734	62	6–7	45	40	1.5	5.5–8	24	[14]
Streptomyces sp. CS-57	55	7.5	45	45	2.0	6–8	24	[31]
Streptomyces sp. CS684	29	6.0	45	45	2	7–9	40	[32]
Ochrobactrum sp. ASAG-PL1	37	7.0	40	NR	NR	7	24	[33]
Streptomyces olivochromogenes	60	8.0	75	75	1.5	7–13	63	[34]
Streptoverticillium sp. NA684	54	5.0	80	50	1.0	4–10	12	[35]
Streptomyces racemochromogenes	55	7.5	50	60	1/6	4–9	4	[36]
S. hiroshimensis SK43.001-11	54	7.0	60	40	0.5	6–8	24	This study

NR, not reported.

  [a] pH or temperature retaining at least 80% of the initial activity.
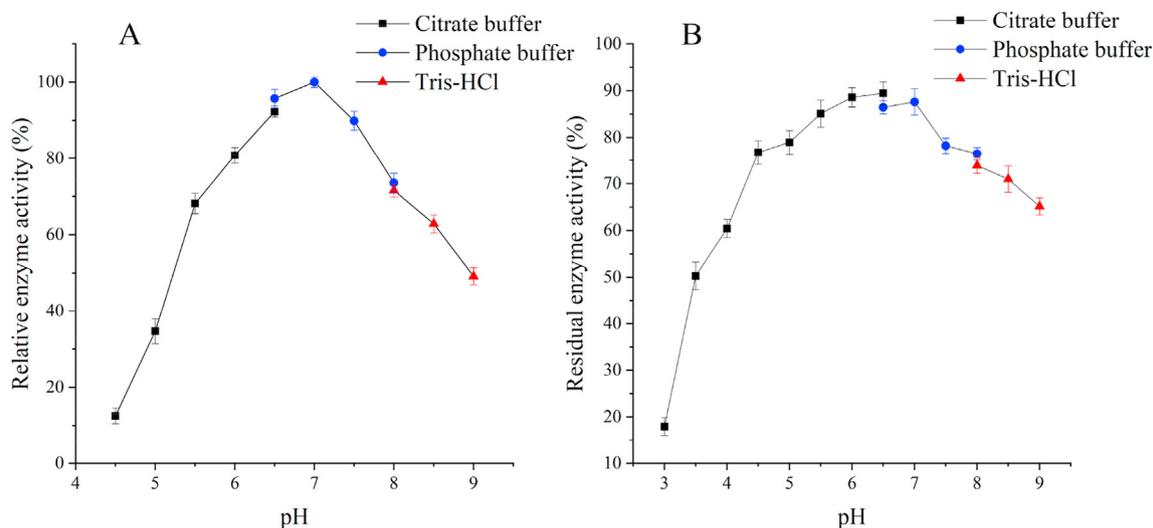
Figure 6. Effects of pH on enzyme activity and stability of PLD. (**A**) pH-enzyme activity curve. (**B**) pH-enzyme activity stability curve.

comparison, Mg^{2+} could significantly improve both the catalytic efficiency and stability of PLD. As this result, unless otherwise specified, the metal ions added in all the experiments of this article were Mg^{2+}.

3.5.4. Effects of surfactants on enzyme activity

The substrate PC is insoluble in water, thus the catalytic reaction of PLD usually occurs at the two-phase interface. The addition of surfactants is generally beneficial in improving the solubility of the substrate, changing the aggregation state of the substrate, increasing the contact opportunities between the substrate and the enzyme and enhancing the efficiency of the catalytic reaction [37]. To determine the effect of surfactants on PLD enzyme activity, nine surfactants were used, including Tween 20, Tween 60, Tween 80, Span 80, Triton X-100, deoxycholic acid, CHAPS, Brij-35 and SDS solution. As shown in Figure 7B, the addition of Triton X-100, Tween 60, Tween 20, Tween 80, Brij-35 and deoxycholic acid had different degrees of promoting effect on the catalytic reaction of the enzyme, among which Triton X-100 had the most obvious promoting effect on the enzyme activity, increasing the enzyme activity by 100%. However, CHAPS and SDS inhibited enzymatic activity, which may be due to their severe effect on the hydrophobicity of the protein, resulting in

denaturation of the enzyme and the loss of catalytic activity. In addition, Span 80 had an extremely slight effect on enzyme activity. As this result, the surfactants added in all the experiments were Triton X-100.

3.5.5. Kinetic parameters of PLD

Kinetic parameters provide insight into the affinity of the enzyme for the substrate and the effect of substrate concentration on the speed of the enzyme reaction. The kinetic parameters were obtained by fitting the Hill function under various substrate concentrations of egg PC (1–60 mmol/ L), with an optimal reaction temperature of 60 °C and an optimal pH of 7.0 (Figure 8). The K_m, V_{max} and K_{cat} values of PLD from mutant SK43.001-11 were 30.20 mmol/L, 99.70 μmol/min, and 76.33 s^{-1}, respectively. PLD from mutant SK43.001-11 showed a lower K_m value for egg PC as compared to PLD from SK43.001. It is speculated that the affinity of the substrate to the enzyme was changed after the mutant strain, so that PLD of the mutant strain showed better affinity to egg PC. The catalytic efficiency (K_{cat}/K_m) of the PLD from SK43.001-11 was 2.53 s^{-1} mM^{-1}, a 1.24-fold increase compared to wild strain, reflecting a higher substrate conversion rate (Table 3). Mutagenesis changed the catalytic activity of PLD on egg PC and improved the catalytic efficiency, and
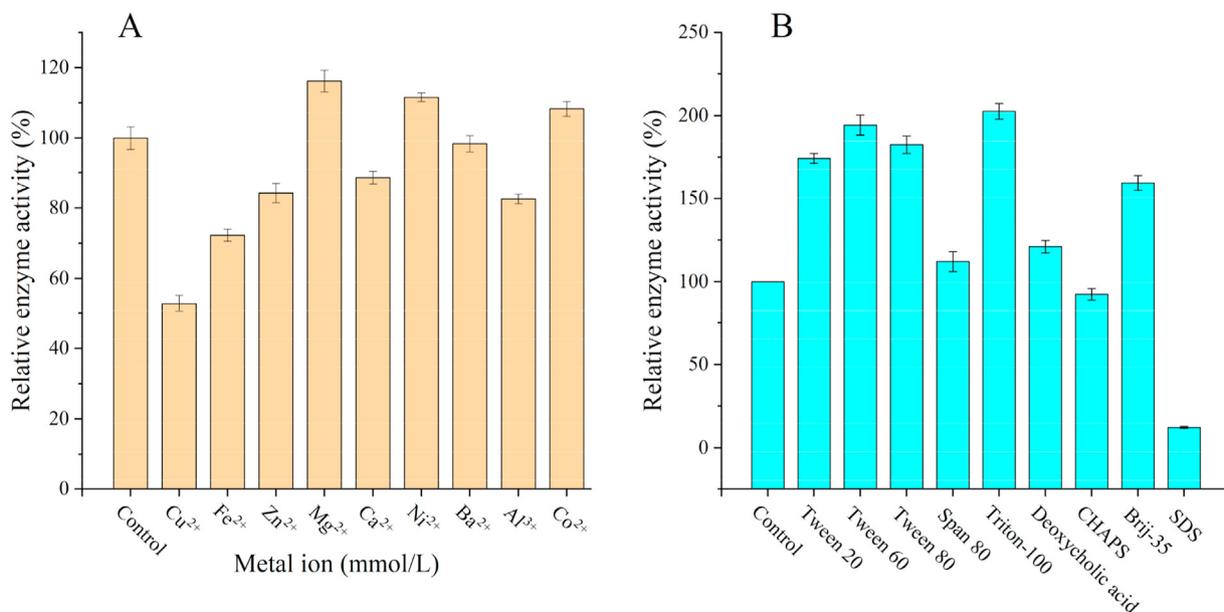
Figure 7. Effects of metal ions and surfactants on enzyme activity of PLD. (**A**) Metal ions-enzyme activity. (**B**) Surfactants-enzyme activity.
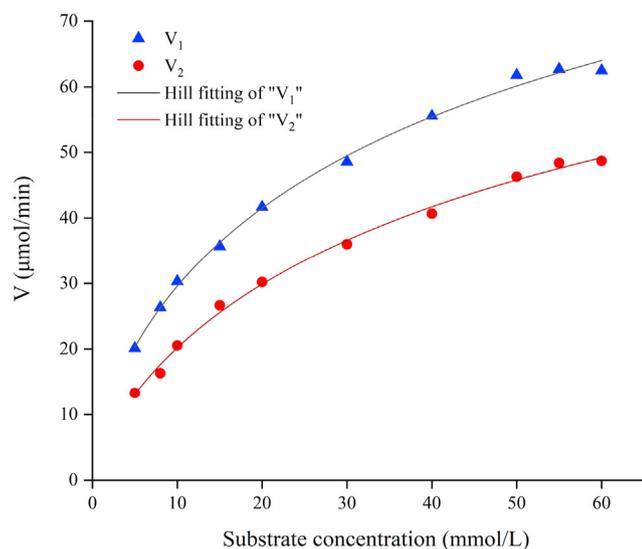
Figure 8. Hill function of PLD for egg PC. V_1 and V_2 represent the enzyme reaction rates of the mutant SK43.001-11 and wild strain SK43.001, respectively.

Table 3. Comparison of kinetic parameters of wild strain and mutant SK43.001-11.

Strains	K_m (mmol/L)	V_{max} (µmol/min)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} mM^{-1})
SK43.001	33.17 ± 0.47	97.46 ± 1.04	67.58 ± 0.58	2.04 ± 0.05
SK43.001-11	30.20 ± 0.67	99.70 ± 0.86	76.33 ± 0.62	2.53 ± 0.13

analysis of the mechanism from DNA and protein levels will be a future research direction.

4. Conclusion

In this work, the ARTP mutation system was successfully applied to *S. hiroshimensis* SK43.001, and a mutant strain SK43.001-11 with high PLD enzyme activity was obtained, which was 82% increased comparing to wild strain. Generation experiments showed that the strain has good genetic stability. After the extracellular crude enzyme solution was purified by grading, the specific enzyme activity of PLD was increased to 49.48 U/mg (54.37-fold that of the crude enzyme solution) and the recovery was 32.31%. Furthermore, enzymatic properties of purified PLD showed that the optimal pH and temperature were 7.0 and 60 °C, respectively. Both metal ion Mg^{2+} and surfactant Triton X-100 had the effect of increasing the enzymatic activity of PLD by 16% and 100%, respectively. The K_m, V_{max} and K_{cat} of PLD from SK43.001-11 were 30.20 mmol/L, 99.70 µmol/min and 76.33 s^{-1}, respectively, showing better affinity for the substrate egg PC and higher catalytic efficiency compared with the wild strain SK43.001. PLD can convert PC to PS, PG, etc. in a biphasic system, which makes this enzyme a promising candidate for the industrial production of rare and commercially important phospholipids. In the future, by studying the structure of PLD, the structure of the enzyme can be modified by methods such as random mutagenesis and site-directed mutagenesis to improve the catalytic activity and thermal stability of the enzyme, which can be used as a favorable direction.

Declarations

Author contribution statement

Chenchen Li, Tao Zhang: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yu Xia: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Mengli Li: Analyzed and interpreted the data.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no competing interests.

Additional information

No additional information is available for this paper.

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