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Cloning and expression of phospholipase D gene *pld* from *Streptomyces chromofuscus*

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Abstract - The phospholipase D (PLD) gene pld encoding the mature PLD enzyme from *Streptomyces chromofuscus* was cloned and sequenced. The recombinant protein has been expressed in *Escherichia coli* and purified. The yield of aim protein was up to 27.5 mg/l culture medium. Because it was targeted with a 6 x his-tag, the recombinant protein could be much more easily purified by using Ni-NTA His•Bind Purification Kits. After concentration, the concentration of rPLD was 1.0 mg/ml buffer. With transphosphatidylation activity of rPLD, phosphatidylserine (PS) could be produced from phosphatidylcholine (PC). The optimum organic phase was chloroform, optimum pH was 7.5, optimum temperature and time of transphosphatidylation reaction that catalysed by rPSS were 30 °C and 6 h, respectively. The highest transphosphatidylation rate of rPSS was up to 31% and the specific enzyme activity was up to 39 U/mg.

Key words: phospholipase D (PLD), phosphatidylserine (PS), transphosphatidylation, *Streptomyces chromofuscus*, HPLC assay.

INTRODUCTION

The importance of large scale production of phosphatidylserine is increasing because of their functions in industrial processes, health and nutrition applications (Lekh *et al.*, 1992; Masashi *et al.,* 2000). For example, in clinical trials conducted in the United States and Europe, it indicated that phosphatidylserine (PS) supplemented in the diet plays important roles in the support of mental functions in the aging brain. So the benefits of PS have received a great deal of attention (Masashi *et al.,* 2000).

The isolation of PS in pure and homogeneous forms from natural sources and also PS synthesis by chemical reactions are complicated, laborious and expensive. Although PS has been prepared by a variety of methods, most of them have limitations at the industrial-scale (Lekh *et al.,* 1992).

Enzymatic conversion of phosphatidylcholine (PC) to PS using phospholipase D (PLD, EC 3.1.4.4) from different resources, including from plants and microorganisms, has been examined by many researchers (Yang *et al.,* 1967; Comfurius and Zwaal, 1987; Shuto *et al.,* 1987; Masashi *et al.,* 2000; Tairo *et al.,* 2000; Shnigir and Kisel, 2004). PLD was firstly discovered in plants (Hanahan and Chaikoff, 1947) and later identified in microorganisms (Soucek *et al.,* 1967) and mammals (Satio *et al.,* 1974). PLD hydrolyzes phosphatidylcholine to phosphatidic acid and choline by breaking its phosphodiester bond (Ponting and Kerr, 1996). PLD also acts on other phosphatidylesters and catalyses a transphosphatidylation reaction when alcohol is present as a nucleophilic donor (Uhm, 2005). In particular, Actinomycetes (especially *Streptomyces*) PLDs showed high transphosphatidylation activities than plant and mammalian PLDs (Tadashi *et al.*, 2002). Although the biosynthesis has many advantages in phosphatidylserine production, there are also some limitations for industrial scale: the yield of PLD from *Streptomyces* was low and cost nearly one week to culture (Tadashi *et al.*, 2002, Ogino *et al.*, 2004).

To over-express the enzyme rapidly, we cloned PLD gene from *Streptomyces chromofuscus* into *Escherichia coli* BL21 (DE3) with pET-22b (+) vector. And the recombinant protein was targeted with a 6 x his-tag, it could be much more easily purified by using Ni-NTA His•Bind Purification Kits. With its transphosphatidylation activity, the rPLD could generate PS by using PC and serine as a substrate. This research also investigated the optimum organic phase, pH, temperature and time of the transphos-

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phatidylation reaction by the rPLD. It has found a foundation for biosynthesis PS by rPLD from *E. coli*.

MATERIALS AND METHODS

Materials. L-Serine was purchased from Biodev (Shanghai, China). PS and PC (from soybean) were purchased from Sigma Chemical Co. LA Taq with GC buffer was purchased from TaKaRa (Japan). Ni-NTA His•Bind Purification Kits was purchased from Novagen.

Strains and plasmid. The gene encoding PLD was isolated from *Streptomyces chromofuscus* AS 4.331. *Escherichia coli* JM109 was used for cloning and *E. coli* BL21 (DE3) was used to express PLD protein. The vector pET-22b (+) was used for expression in *E. coli* BL21.

Culture medium. *Streptomyces chromofuscus* was grown on YME medium (4 g yeast extract/100 ml, 10 g malt extract/100 ml, 4 g glucose/100 ml, pH 7.3~7.5). *Escherichia coli* K_{12} and BL21 (DE3) were grown on LB (Luria Bertani) medium. The recombinant *E. coli* was selected on LB medium containing 50 Ìg/ml ampicillin.

Plasmid construction. Two oligonucleotide primers were designed based on the *S. chromofuscus* PLD DNA sequence recently reported (Yugo *et al.,* 1999; Yang and Roberts, 2002). 5′-cgGGATCCggccgaccaggcccccgcctccct-3′ (containing a *Bam*HIsite as indicated by the capitals) and 5′-cccAAGCTTctactcggggtcgtaggtgcgc-3′ (containing a *Hind*III site as indicated by the capitals) were used to amplify the 1530 bp PLD gene with LA Taq. The 30-cycle PCR products were digested by *Bam*HI and *Hind*III?then ligated to *Bam*HI-*Hind*III linearised pET-22b (+) vector. The purified recombinant plasmid, pET-S, was transformed into *E. coli* JM109. The recombinants were selected on the ampicillin resistant plate. Sequencing, PCR and double restriction enzyme digestion were carried out to confirm the positive transformants. Then, the recombinant plasmid was transformed into BL21 (DE3) competent cells for *pld* gene expression.

Expression and purification of recombinant PLD cloned in pET-22b (+). A single colony of BL21 (DE3) containing pET-S plasmid was grown in 10 ml of LB medium containing ampicillin until OD_{600} reached 1.0. This culture was used to inoculate into 1 l fresh LB medium. Culture were grown with rapid shaking (200 rpm) at 37 °C to OD_{600} = 0.8.

Overexpression of PLD was induced by the addition of IPTG to a final concentration of 0.8 mmol/l. After induction, cultures were incubated at 25 °C overnight. Cells were harvested by centrifugation and stored at -80 °C until needed.

Frozen pellets or fresh cells were thawed at room temperature and resuspended in 30 ml PBS (pH 7.0). The suspensions were sonicated for 2 x 4 s (total 5 min) on ice, and the supernatant was separated from cell debris by centrifugation (3600 rpm for 20 min). The recombinant protein was purified by Ni-NTA His•Bind Purification Kits. The supernatant was slowly (1 ml/min) loaded onto an affinity column of the nickel gelose gel (10 ml). After loading, the column was washed with 50 ml of 50 mmol/l PBS pH 7.4. rPLD was eluted from the resin with 50 mmol/l PBS pH 7.4 containing 50 mmol/l and 400 mmol/l imidazole, respectively.

Total proteins, supernatant or the soluble protein fraction, and precipitate or the insoluble protein fraction after sonication were subjected to SDS-PAGE (12% resolving gel). Proteins were stained with Coomassie Brilliant Blue. Protein concentration was estimated by the method of Bradford (1976).

Assay of PLD activity. Transphosphatidylation reaction from PC and L-serine to PS was carried out in a biphasic system because water miscible serine and immiscible PC were used as substrate. In this study, the activity of the enzyme was determined as follows: 30 ml of 0.1 mol/l sodium acetate buffer pH 7.0 (containing 5 mmol/l $CaCl_2$, 0.15 mol of serine) and 1 ml purified rPLD enzyme solution were added with 15 ml acetonitrile containing 0.75 mmol (0.56 g) phosphatidylcholine (Lekh *et al.,* 1989; Masashi *et al.,* 2000). The mixture was stirred vigorously (200 rpm) at 30 °C to obtain a homogeneous emulsion. After 3 h reaction, the reaction mixture was stopped to demix dual-liquid phase and 1 ml organic phase was extracted. After volatilisation, the remainder was washed by chloroform twice and resolved by 50 Ìl chloroform. Samples (15 Ìl) were analysed by HPLC operated at 206 nm. The eluting solvent was acetonitrile/methane/85% phosphoric acid (100:10:0.8, v/v). The flow rate was 1 ml/min. Each run took 30 min.

In particular, one unit of rPLD was defined as the amount of enzyme trasphosphatidylation 1 Ìmol of pure PC per hour at optimum temperature and optimum pH in the biphasic reaction time and the specific activity is defined as U/mg of protein.

The optimum organic phase (dichloromethane, chloroform, ethyl acetate and hexane), pH (ranging from 5 to 8.5), temperature (ranging from 20 to 40 °C), and reaction time (ranging from 1 to 8 h) of transphosphatidylation reaction were tested at the conditions described above.

RESULTS AND DISCUSSION

Construction of recombinant plasmid pET-S

The amplified PLD gene (1530bp) was inserted into

*Bam*HI and *Hind*III restriction sites of the pET-22b (+) vector to construct an expression vector pET-S, which harbours PLD gene under the control of T7 promoter.

Sequence analysis

The sequence of the *pld* gene cloned from *S. chromofuscus* AS 4.331 had 4 differences from the counterpart of *S. chromofuscus* from ATCC (#23616) (Yang and Roberts, 2002). The identity is 99.22%. Gly^{69} was replaced by Glu, Gly^{198} by Ala, Val^{206} by Ile, and Ser^{285} by Ala.

Several PLDs from *Streptomyces* have been sequenced and shown significant sequence similarity. In particular, the duplicated $HxK(x)_4D$ (or HKD) motifs were identified (Yugo *et al.,* 1999; Leiros *et al.,* 2000; Uhm *et al.,* 2005). But PLD that isolated from *S. chromofuscus*, has very little sequence homology with other *Streptomyces* PLD enzymes. The primary amino acid sequence of *S. chromofuscus* PLD exhibits no $HxK(x)_4D$ motif. However, $^{187}HxK(x)_3D^{193}$ and $^{200}HxK(x)_7D^{210}$ in the same region of the protein as the $HxK(x)_4D$ motifs in the other *Streptomyces* PLDs might be variations of that catalytic motif. And some experiments showed that the mutant of the single Cys123 in *S. chromofuscus* to Ala or Ser generated well-folded protein with greatly reduced activity (Yang and Roberts, 2002). Actually, the amino acidic sequence variation deduced for the *pld* gene of *Streptomyces chromofuscus* AS 4.331 are not located in region that should affect the enzymatic activity of the phospholipase.

Expression and Purification of PLD

The recombinant PLD protein had a calculated molecular size of 55~58 kDa similar with the report published previously (Tadashi *et al*., 2002). In contrast, no band was detectable in the uninduced control culture. With the method of Lowry *et al*. (1951), the total protein concentration of the crude lysed cell supernatant is 125 mg/l culture. And the expression of the *S. chromofuscus* PLD gene in BL21 (DE3) is about 20% (27.5 mg/l), based on the SDS-PAGE of the crude lysed cell supernatant. Because of short culture time (12 h) and high expression of the rPLD in *E. coli*, the rPLD could be fully restored, easily. As to the initial strain, highest yield of PLD is 30 mg/l culture medium (Hatanaka *et al.,* 2002). But the production periods was very long, for 5 days. Its relative yield was no more than 3 mg/12 h in one litre medium. The relative yield was higher than the results reported previously (Yang and Roberts, 2002; Ogino *et al.,* 2004).

The recombinant protein was expressed with a six-histidine tag at the C-terminus, allowing purification by Nickel-chelate affinity chromatography. After 400 mM imidazole washed from the affinity column, the PLD protein was more than 90% pure (Fig. 1). After concentration, the concentration of rPLD was up to 1.0 mg/ml buffer.

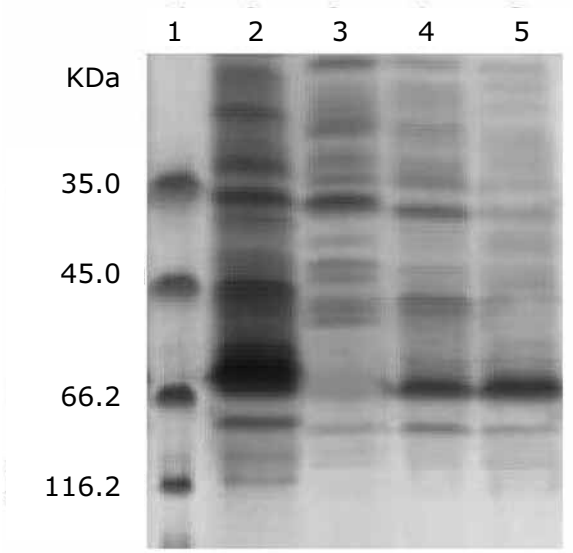

FIG. 1 - SDS-PAGE showing purification of PLD from pET expression. Lane 1: molecular masses for the standard proteins, lane 2: protein content of the crude cell extract, lane 3: protein content of wash from the purification column, lane 4: protein eluted after incubation of column with 50 mM imidazole, lane 5: protein eluted after incubation of column with 400 mM imidazole.

Standard curve for transphosphatidylserine

Phosphatidylserine, the product of transphosphatidylation reaction, was determined to calculate the transphosphatidylation rate of rPLD. When the standard PS solution was subjected to the present transphosphatidylation-activity assay, a linear standard curve exhibiting a correlation coefficient of 0.9965 (R^2) was obtained (Fig. 2).

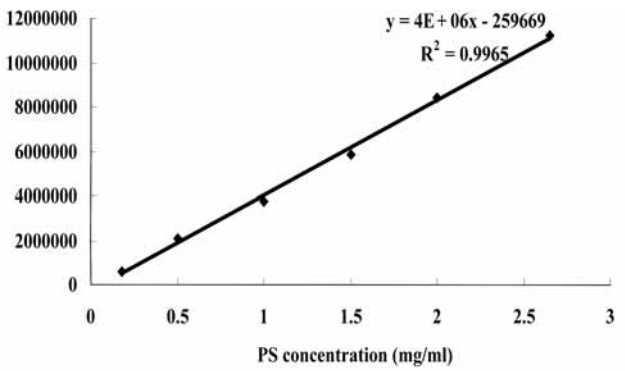

FIG. 2 - Phosphatidylserine concentration standard curve by HPLC.

Assay of PLD activity

Four organic reagents were chose to investigate the effect of organic phase to the transphosphatidylation reaction. And the polarity of the reagents mentioned above increased as the same sequence.

Figure 3 showed that chloroform and ethyl acetate were suitable for organic phase medium. It might seem that the polarity of phospholipid compounds was similar to the polarity of the two reagents. So it could dissolve in chloroform and ethyl acetate. Chloroform was chose as the organic phase of the transphosphatidylation reaction for the reason that phosphatidylcholine emulsified better in chloroform than in ethyl acetate, so the PC could disperse homogeneously in chloroform.

Figure 4 showed transformation curve for pH 5~8.5 Transphosphatidylation rare was slightly increased by increasing the pH from 5 to 7.5. At the higher pH, the rate of transphosphatidylation decreased prominently. The result proved that recombinant PLD has had well transphosphatidylation ability at pH 7~8. And the optimum growth pH of *S. chromofuscus* is pH 7.3~7.5. The alkaline condition was suitable for enzyme stability. Actually, at low pH, the PLD would occur to hydrolyzation more easily than transphosphatidylation (Gottlin, 1998). So the optimum pH of transphosphatidylation carried with rPLD is about 7.5.

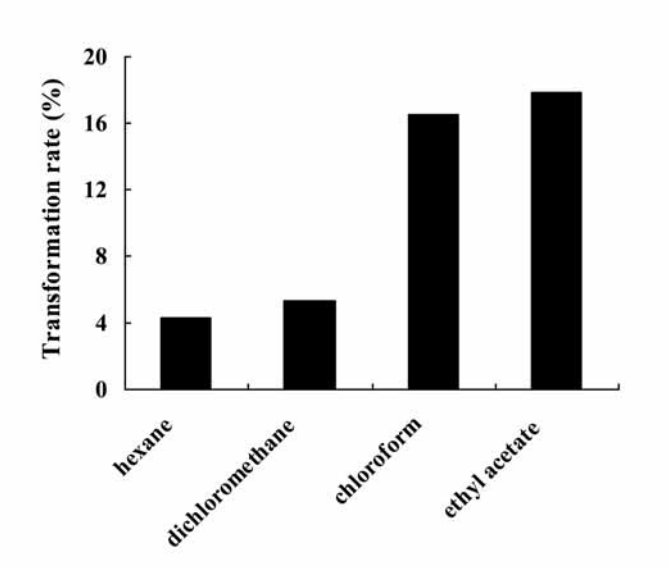

FIG. 3 - The effect of organic phase to transphosphatidylation reaction.

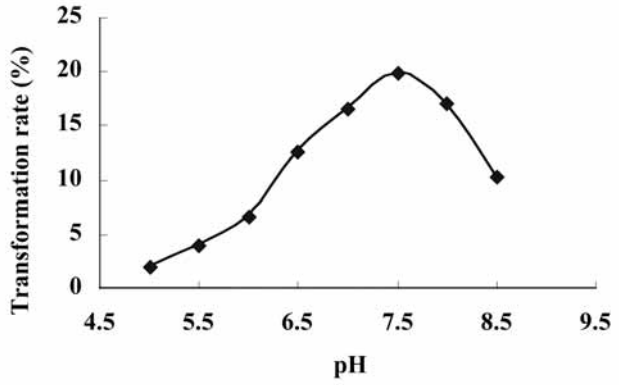

FIG. 4 - The effect of pH to transphosphatidylation reaction.

Transphosphatidylation rare was slightly increased by increasing the temperature from 20 to 30 °C. At the higher temperature, the rate of transphosphatidylation decreased prominently. The result proved that recombinant PLD has had well transphosphatidylation ability at 28~32 °C (Fig. 5).

Through the transformation curve (Fig. 6), the reaction time could be determined: after 6 h the reaction stabilised, and the transphosphatidylation rate was up to 31%. It meant that 233 Ìmol PC was transformed to PS in 6 h. The enzyme activity was 39 U/mg proteins.

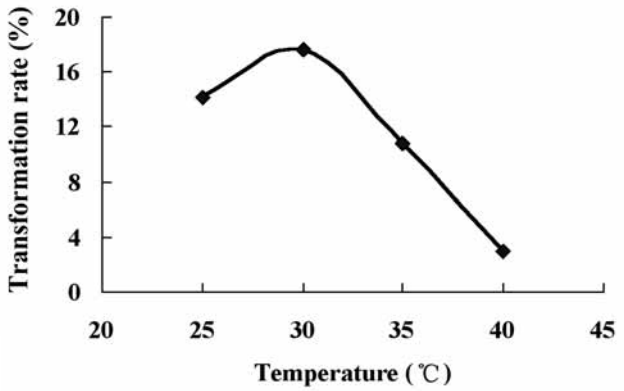

FIG. 5 - The effect of temperature to transphosphatidylation reaction.

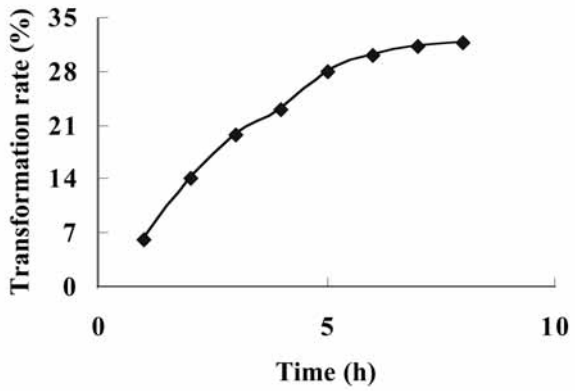

FIG. 6 - The effect of reaction time to transformation rate.

CONCLUSION

In this research, the PLD gene encoding the mature PLD was cloned from the genomic DNA of *S. chromofuscus* and its gene sequence was analysed. There were four mutants appeared, $Gly^{69}\rightarrow Glu$, $Gly^{198}\rightarrow Ala$, $Val^{206}\rightarrow Ile$, and $Ser^{285}\rightarrow Ala$. With the method described previously, the transphosphatidylation rate of PC to PS by rPLD could be calculated. And the optimum organic phase, pH, temperature and time of rPLD transphosphatidylation reaction are: chloroform, 7.5, 30 °C and 5~6 h, respectively. The highest transphosphatidylation rate is up to 31%. In other word, the IU of the rPLD is up to 39

U/mg. And because of short culture time of expression host and high expression of the rPLD in *E. coli*, the rPLD could be fully restored, easily. This research has found a foundation for industrial-scale biosynthesis PS by rPLD.

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