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Expression, Purification, and Characterization of Phosphatidylserine Synthase from *Escherichia coli* K_{12} in *Bacillus subtilis*

YE-NI ZHANG, FU-PING LU,* GUAN-QUN CHEN,† YU LI, AND JIAN-LING WANG

Tianjin Key Laboratory of Industrial Microbiology, College of Biotechnology, Tianjin University of Science and Technology, 300457 Tianjin, People's Republic of China

Although phosphatidylserine synthase (PSS) from *Escherichia coli* is an ideal enzyme for phospholipid production, its application in the food industry has been limited because of the low PSS yield. In this study, the *pss* gene was cloned from *E. coli* K_{12} and expressed in *Bacillus subtilis* DB104, and the recombinant PSS was characterized subsequently. PSS was purified to 39.59-fold, and the highest activity was detected as 13.62 U/mg. The enzyme was found to be stable in a pH range of 6.5−9.5, with optimal pH values of 8.0 for hydrolysis and 7.0 for transphosphatidylation, respectively. The optimal temperature for PSS activity was 35 °C. The enzyme activity could be detected after 1 h of heating at 65 °C. Among the detected detergents and metal ions, Triton X-100, Ca^{2+}, Mn^{2+}, and Co^{2+} could improve PSS activity. The transformation of phosphatidylcholine to phosphatidylserine under PSS catalyzation was carried out in a biphasic system, which confirmed the actual catalyzing ability of the recombinant protein.

KEYWORDS: *Bacillus subtilis*; phosphatidylserine synthase; characterization; transphosphatidylation

INTRODUCTION

Phospholipids, especially phosphatidylserine (PS), have many applications in functional food and pharmaceutical industries (*1*, *2*). Recent clinical studies have indicated that diets supplemented with PS play an important role in revitalizing the brain cell membranes and have important nutritional and biological functions, particularly in improving memory, increasing vigilance and attention, relieving depression, and decreasing stress (*3*−*5*). PS has been broadly identified in plants and animals, and the synthesis of PS by chemical reactions has been reported. However, the isolation and purification of high-quality PS from natural sources, as well as syntheses of PS by chemical reactions, are complicated, laborious, and expensive (*6*). To overcome this problem, investigators began to study the production of high-quality PS under the catalysis of microbial phosphatidylserine synthase (PSS; CDP-diacylglycerol:L-serine *o*-phosphatidyltransferase, EC 2.7.8.8).

PS biosynthesis in *Escherichia coli* has been well studied recently (*7*). The production of PS is catalyzed by PSS through a transphosphatidylation reaction. Experiments in vitro showed that PSS of *E. coli* could also effectively catalyze the biosynthesis of other scarce phospholipids, such as phosphatidylethanolamine, phosphatidylglycerol, and some novel artificial phospholipids. *E. coli* PSS is an ideal enzyme for industrial phospholipid production. As a typical membrane phospholipid synthesis enzyme, however, PSS presents in only small amounts in *E. coli*, which cannot satisfy the industrial demand.

Bacillus subtilis has the ability to efficiently express foreign genes with biological activity and secretes recombinant proteins directly into the culture medium (*8*). Furthermore, *B. subtilis* is not a human pathogen and is considered to be biologically safe. Therefore, the expression of the *E. coli pss* gene into *B. subtilis* might construct a desirable system to improve PSS productivity. The aim of this study, therefore, was to clone the *E. coli pss* gene and express it into *B. subtilis*. The purification and characterization of this recombinant enzyme were subsequently investigated. The results indicated that this reconstruction system had practical value for PSS production.

MATERIALS AND METHODS

Materials. L-Serine was purchased from Biodev (Shanghai, China). Choline oxidase, peroxidase, PS, and phosphatidylcholine (PC) were purchased from Sigma Chemical Co. All other chemicals used in this study were of analytical grade.

Strains and Plasmids. *B. subtilis* DB104, which was deficient in two proteases, was used as the expression host. *E. coli* DH5α was used in DNA manipulations. The vector pBES carrying the levansucrase gene (*sacB*) was used for gene expression in *B. subtilis*.

Cloning of the *pss* Gene. One pair of primers was designed on the basis of the corresponding sequence in the ExPASy database (primary accession no. P23830). The forward primer P1 was 5′-AAC TGC AGA ATT GTC AAA TTT AAG CGT AAT AAA C-3′, where the *Pst*I recognition sequence is underlined. The reverse primer P2 was 5′-CCC AAG CTT TTA CAG GAT GCG GCT AAT TAA T-3′, where the

* Author to whom correspondence should be addressed (telephone 86-22-60601667; fax 86-22-60602298; e-mail lfp@tust.edu.cn).

† Present address: Department of Biology, Hong Kong Baptist University, Kowloon Tong, Hong Kong.

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*Hin*dIII recognition sequence is underlined. The genomic DNA of *E. coli* K_{12} was used as a template. The PCR reaction was subjected to the following thermal cycles: one cycle at 94 °C for 5 min, 30 cycles at 94 °C for 1 min; 50 °C for 1 min 40 s; and 72 °C for 1 min, and a final extension at 72 °C for 10 min.

Construction of the Inducible Expression Vector and Transformation of *B. subtilis*. The cloned *pss* gene was digested by *Pst*I and *Hin*dIII restriction enzymes, and the resulting 1350 bp DNA fragment was purified and inserted into the same endonuclease sites of vector pBES. In this way, the plasmid pBES-*pss*, which harbored the *pss* gene under the control of the *sacB* gene promoter, was constructed.

Competence medium (SPI and SPII) and minimal medium were prepared as previously described (*9*). Cells were grown in SPI overnight at 37 °C. One hundred microliters of this primary culture was transferred to 5 mL of fresh SPI medium and incubated at 37 °C and 250 rpm to the early stationary phase (about 4 h). Subsequently, 0.2 mL of the early stationary phase culture was inoculated to 2 mL of SPII medium and cultured at 37 °C and 150 rpm for 1 h. DNA samples (1−3 μg) and competent cells (100 μL) were mixed and incubated at 37 °C and 100 rpm for 30 min and then at 250 rpm for 90 min. The cells were plated on a Luria−Bertani (LB)/agar plate containing kanamycin (20 μg/mL) and incubated at 37 °C for 12 h. The recombinant vector was transformed into the strains of *B. subtilis* DB104 and used as control.

Induction and Expression of the Recombinant Protein PSS. The recombinant plasmids were transformed into *B. subtilis* DB104 to construct the recombinant strain DB104/pBES-*pss*. The recombinant strain was inoculated to the LB medium containing 20 μg/mL kanamycin and shaken at 37 °C and 200 rpm for 2 h. Subsequently, sucrose (2%, w/v) was added to induce the expression of the *pss* gene. A negative control without sucrose induction was carried out simultaneously. After 28 h of cultivation, the culture was centrifuged at 8000 rpm and 4 °C for 20 min. The supernatant was harvested for enzyme activity analysis and purification. The expression of the *pss* gene was examined by SDS-PAGE (12%, w/v) and visualized by Coomassie Brilliant Blue R-250.

Purification of Recombinant Protein. The supernatant was precipitated by ammonium sulfate (75%, w/v). The precipitate was redissolved in 100 mL of 10 mmol/L Tris-HCl buffer (pH 7.0) and dialyzed at 4 °C for 12 h. The solution obtained after dialysis was subsequently purified on a SP-Sepharose HP column. The fractions containing PSS were pooled and purified again on a Sephadex G-75 gel column. The protein was eluted with 10 mmol/L Tris-HCl buffer in 1.0% NaCl at a rate of 0.5 mL/min. The fractions containing PSS were used for the analysis of enzyme activity.

Assay of PSS Activity. The PSS activity assay is based on the hydrolysis of PC to choline (*10*). PC solution was prepared by mixing 200 mg of L-α-phosphatidylcholine with 4 mL of distilled water and 0.4 mL of diethyl ether. The reaction mixture, which was composed of PC (0.16%), Triton X-100 (0.375%), $CaCl_2$ (50 mM), Tris-HCl (30 mM, pH 8.0), and enzyme (0.04 mL), was incubated at 35 °C for 10 min. A solution containing Tris-HCl (375 mM, pH 9.0) and EDTA (18.75 mM) was added to terminate the reaction. The reacted samples were then boiled for 10 min, followed by chilling on ice for 20 min. Subsequently, 0.15 mL of solution composed of 1.7 U of peroxidase, 7.98 mM 4-aminoantipyrine, 4.3 mM phenol, and 0.14 U of choline oxidase was added to the samples and incubated at 37 °C for 2.5 h. The amount of quinoneimine dye formed during the reaction was detected by a spectrometer at a wavelength of 500 nm. One unit of enzyme was defined as the amount of enzyme required to produce 1 μmol of choline from PC.

The assay of PSS transphosphatidylation activity was based on the biosynthesis of PS from water immiscible PC and water miscible L-serine. The reaction was performed in water−organic solvent emulsions. Five milliliters of sodium acetate buffer (0.1 M, pH 7.0) containing 50 mM $CaCl_2$, 0.375% Triton X-100, 3.4 M L-serine, and 0.2 mL of recombinant PSS enzyme solution was added to 5 mL of ethyl acetate containing 17 mM PC. The mixture was stirred vigorously to obtain a homogeneous emulsion and reacted at 35 °C. The reaction was terminated by adding 100 mM citric acid (pH 6.0). The content of PS was analyzed by normal phase high-performance liquid chromatography (NP-HPLC) (*11, 12*).

Table 1. Active Motif Assay of the *pss* Gene from *E. coli* K_{12}

site	length	description	sequence
133−159	27	phosphodiesterase 1	ALGVL**H**F **K**GFII **D**DSVLYSG-ASLNDVY
352−379	28	phosphodiesterase 2	DDNTY**H**L **K**GMWV-**D**DKWMLITGNNLNPRA
119−154	36	hydrophobic	VDVPVYGVPINTREALGVL**HFKGFIID**-DSVLYSGAS
239−284	46	hydrophobic	SVTPLVGLGKSSLLNKTIFHLMPCAE-QKLTICTPYFNLPAILVRNI

Influence of Temperature and pH on PSS Activity and Stability. For identifying the optimal temperature and pH, PSS activity was tested from 20 to 70 °C and from pH 3.0 to 12.0, respectively. For PSS thermostability determination, the purified enzyme was maintained at 25, 35, 45, 55, and 65 °C in a water bath for 15, 30, 45, 60, 75, 90, 105, and 120 min, respectively, before activity analysis. For testing pH stability, the purified enzyme was adjusted to different pH values and kept at 4 °C for 24 h before activity assay. All measurements were taken in triplicate, and the variations were determined to be <5%.

Effect of Metal Ions and Detergents on PSS Activity. The standard reaction mixture described above was amended to detect the effects of metal ions and detergents on the hydrolysis and transphosphatidylation activities of PSS. For the determination of the influence of metal ions and EDTA, 20 mM Mg^{2+}, Fe^{3+}, Zn^{2+}, K^{+}, Ca^{2+}, Mn^{2+}, Co^{2+}, and EDTA were added to the standard reaction mixture, respectively. To detect the effect of detergents, Triton X-100, Tween 20, Tween 80, deoxycholic acid, and SDS were added to the reaction mixture at the concentration of 0.45%, respectively.

RESULTS

Sequence Analysis. Compared to the sequence in ExPASy database (primary accession no. P23830), the *pss* gene cloned in this study had a 99.33% similarity with only three different encoding positions: Asp^{202} was replaced by Asn, His^{258} by Arg, and Asp^{383} by Gly. The predicted active motif of the recombinant protein is shown in **Table 1**. Two phosphodiesterase active motifs were identified in the sequence of the recombinant PSS. The mutation sites mentioned above did not locate in these motifs.

Expression of PSS in *B. subtilis* DB104. As shown in **Figure 1**, the recombinant plasmid pBES-*pss* was transformed into *B. subtilis* DB104. The expression of the inserted *pss* gene in the recombinant strain was induced by the addition of sucrose. The molecular mass of the expressed protein was about 53 kDa, which was in good agreement with that calculated (52802 Da) from the amino acid sequence (**Figure 1**). The hydrolysis activity of the recombinant PSS in the supernatant of the culture medium was 1.68 U/mL. The results revealed that PSS was secreted from *B. subtilis* successfully.

Purification of Recombinant Protein. The PSS purification results are presented in **Table 2**. After purification with ammonium sulfate fractionation, anion exchange, and gel filtration, the crude supernatant of PSS was purified 39.59-fold with a specific hydrolysis activity of 13.62 U/mg. A single band was identified in the SDS-PAGE gel, and its molecular mass was found to be 53 kDa (lane 3, **Figure 1**).

Influence of pH and Temperature on PSS Activity and Stability. The optimum temperature for both hydrolysis and transphosphatidylation activities was found to be 35 °C (**Figure 2**). The enzyme was found to be stable for up to 1 h at 65 °C (**Figure 3**), which indicated that the recombinant PSS was neither highly thermostable nor highly heat sensitive. The

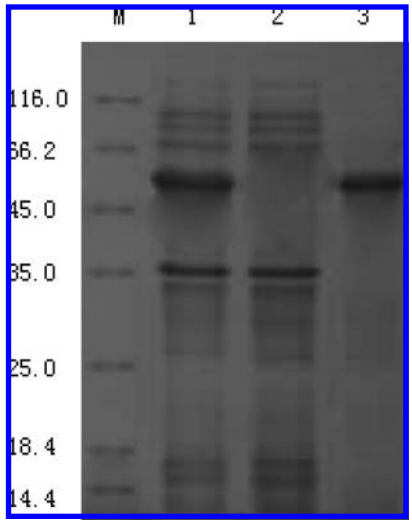

Figure 1. SDS-PAGE analysis of proteins secreted by *B. subtilis*: lane M, molecular mass markers (kDa); lane 1, concentrated culture supernatant of *B. subtilis* harboring pBES-pss; lane 2, concentrated culture supernatant of *B. subtilis* harboring pBES; lane 3, purified recombinant phosphatidylserine synthase.

Table 2. Purification of Phosphatidylserine Synthase

treatment	total protein (mg)	total activity (U)	specific activity (U/mg)	recovery (%)	purification fold
extract supernatant	4412	1518	0.34	100	1
ammonium sulfate	533.4	1396	2.62	91.96	7.61
SP-Sepharose HP	151.1	1187	7.86	78.19	22.84
Sephadex G-75	67.3	917	13.62	60.38	39.59

optimum pH was observed to be 8.0 for the hydrolysis reaction and 7.0 for the transphosphatidylation reaction, respectively (**Figure 4**). In addition, the enzyme was found to be stable within a tight pH range of 6.5−9.5, but its activity was lost under highly acidic and basic conditions (**Figure 5**).

Effect of Metal Ions and Detergents on PSS Activity and Stability. As shown in **Table 3**, Mn^{2+} and Ca^{2+} enhanced the hydrolysis activity of PSS by 16 and 15%, respectively. Ca^{2+} can effectively improve the transphosphatidylation activity of PSS by 21%. The stimulation effect of Ca^{2+} on the transphosphatidylation activity was higher than that on the hydrolysis activity, which suggested that Ca^{2+} might play important roles in the action of the OH^- ion and in the recognition of L-serine as an acceptor of the phosphatidyl group. The enzyme was also found to be active in the presence of EDTA. The effect of various nonionic and anionic detergents on PSS activity was detected, and the results are listed in **Table 4**. Only Triton X-100 could enhance PSS activity.

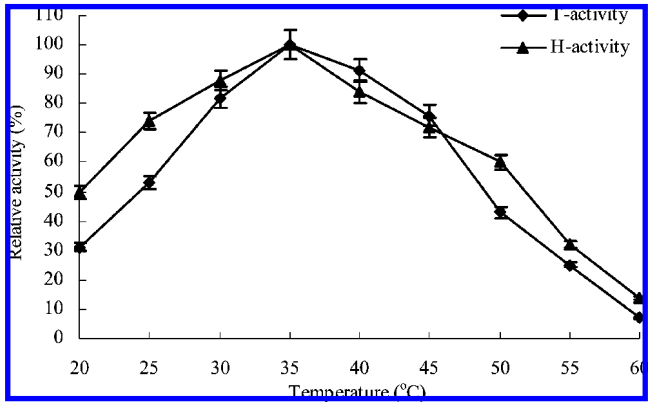

Figure 2. Effects of temperature on the hydrolysis and transphosphatidylation activities of phosphatidylserine synthase.

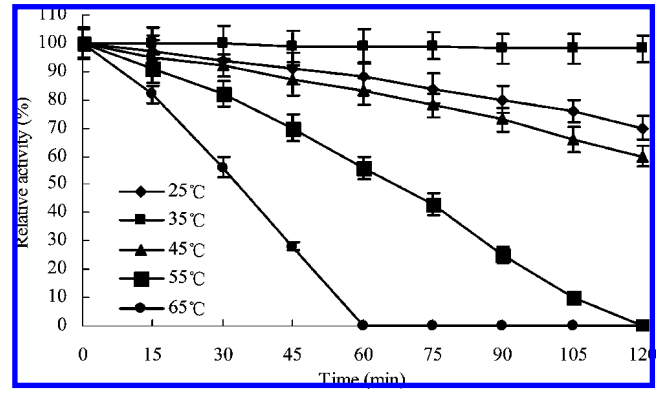

Figure 3. Effects of temperature on the stability of phosphatidylserine synthase.

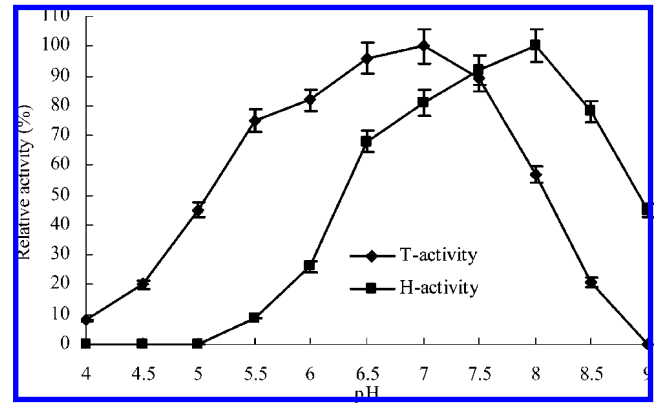

Figure 4. Effects of pH on the hydrolysis and transphosphatidylation activities of phosphatidylserine synthase.

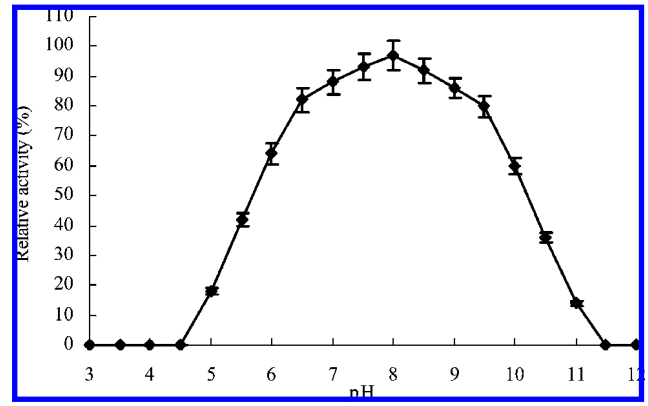

Figure 5. Effects of pH on the stability of phosphatidylserine synthase.

Synthesis of Phosphatidylserine by Phosphatidylserine Synthase. Recombinant PSS exhibited prominent transphosphatidylation activity in the presence of PC and L-serine (**Figure 6**). The rate of PC degradation and the rate of PS formation were found to be almost identical. The time course of transphosphatidylation reaction indicated that the reaction was nearly complete at 240 min with a conversion rate of 86% (**Figure 6**).

DISCUSSION

With a few exceptions, members of the phospholipase D (PLD) superfamily including PSS from *E. coli* shared two copies

Table 3. Effects of Various Reagents on Phosphatidylserine Synthase Activity

reagent	concn (mM)	H activity[a] (%)	T activity[b] (%)
none		100	100
$CaCl_2$	20	115	121
$MgSO_4$	20	93	95
$MnSO_4$	20	116	106
$FeCl_3$	20	84	87
$ZnSO_4$	20	87	85
$CoSO_4$	20	106	103
KCl	20	79	81
EDTA	20	104	97

[a] Hydrolysis activity. [b] Transphosphatidylation activity.

Table 4. Effects of Various Detergents on Phosphatidylserine Synthase Activity

detergent (0.45%)	type of ion	H activity[a] (%)	T activity[b] (%)
none		100	100
Triton X-100	non ionic	120	123
Tween 20	non ionic	30	37
Tween 80	non ionic	50	45
deoxycholic acid	anionic	27	24
SDS	anionic	12	22

[a] Hydrolysis activity. [b] Transphosphatidylation activity.

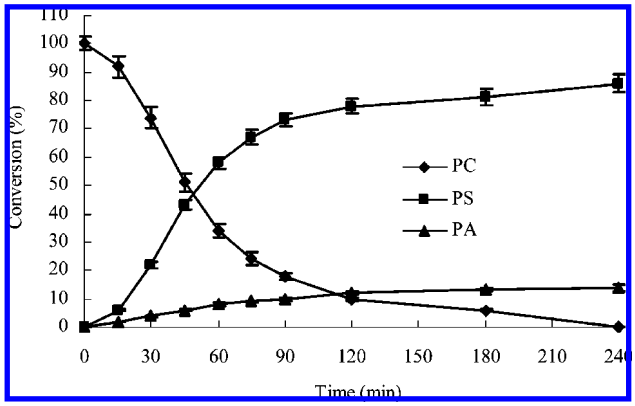

Figure 6. Time course of transphosphatidylation reaction between phosphatidylcholine and L-serine: (◆) phosphatidylcholine (PC); (■) phosphatidylserine (PS); (▲) phosphatidic acid (PA).

of the conserved sequence motif $HXK(X)_4D$ (in single-letter amino acid code and where X might be any amino acid) and denoted the HKD motif (*13*−*15*). The ExPASy database was used to predict the active motif of the recombinant protein. The result showed that the two phosphodiesterase active motifs existed in the sequence of recombinant PSS, which were identified with the $HXK(X)_4D$ motifs. In addition, two hydrophobic sites were also identified in the recombinant PSS sequence. One of the $HXK(X)_4D$ motifs was located in the hydrophobic site. Our results matched the fact that the transphosphatidylation reaction usually occurred in biphasic systems consisting of water-insoluble organic solvents (*12*, *16*, *17*).

In this study, the *pss* gene was successfully cloned and expressed in *B. subtilis* DB104. Under the control of the *B. subtilis sacB* promoter and signal peptide, the *pss* gene was expressed and the activity of the corresponding enzyme in the culture supernatant was 1.68 U/mL. The PSS activity was much higher than that of the original strain, where the content of PSS was only <0.1% of the total protein. To the best of our knowledge, this might be the first paper describing a high-efficiency and inducible expression system of the *pss* gene under the induction of the *sacB* gene. It was reported that the expression of the *sacB* gene was not only induced by sucrose but also stimulated by pleiotropic regulatory genes such as degQ, degU, and degS. Therefore, the inducible expression of the *pss* gene could be further improved in this system if a recombinant vector linking to the pleiotropic regulatory genes was constructed (*18*).

Enzymatic conversion of PC to PS with PLDs from different resources has been reported previously (*19*−*21*). Most PLD- and *E.coli* PSS-catalyzed transphosphatidylation reactions take place through the formation of a phosphatidyl-enzyme intermediate, which is either transphosphatidylated with a suitable nucleophile to form a new phospholipid or hydrolyzed to phosphatidic acid. PLDs are concluded to be of two types according to the results of transphosphatidylation experiments with PLDs from different strains. One type of PLD clearly had transphosphatidylation over hydrolysis, whereas it is vice vesa for the other type (*22*). The transphosphatidylation reaction with the recombinant PSS as enzyme was investigated in this study. To overcome the obstacle caused by the poor solubility of phospholipids in water and the favoring of the hydrolytic side reaction under conditions of excess water, the transphosphatidylation reaction from PC and L-serine to PS was carried out in a biphasic system. The time course of the conversion of PC to PS indicated that transphosphatidylation was much more pronounced than hydrolysis.

Although the biphasic system works well in our study, the exclusion of inflammable and toxic organic solvents would be advantages for environmental protection and cost reduction (*23*). Moreover, the application of phospholipids in nutritional and medical industries requires the exclusion of organic solvents. Therefore, the transphosphatidylation of PC in aqueous systems might be attractive, despite the poor solubility of PC in water. The possibility of transphosphatidylation reaction in purely aqueous media with immobilized recombinant PSS is being explored in our laboratory.

ABBREVIATIONS USED

NP-HPLC, normal phase high-performance liquid chromatography; PA, phosphatidic acid; PC, phosphatidylcholine; PLD, phospholipase D; PS, phosphatidylserine; PSS, phosphatidylserine synthase.

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