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Master's Thesis

Production, purification and characterization of phospholipase D from *Streptomyces* sp. VN-3

Graduate school of Chosun University

College of Pharmacy

Vu Thi Ngoc Bich

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Advisor: Prof. Jin-Cheol Yoo

Thesis submitted for the degree of Master of Science

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Abbreviations

DAG..... Diacylglycerol

PC..... Phosphatidylcholine

PA ..... Phosphatidic acid

Sn ..... Stereo chemical numbering

PLA .....Phospholipase A

PLC .....Phospholipase C

IP_2 ..... Phosphatidylinositol 4,5-bisphosphate

IP_3 .....Inositol 1,4,5-triphosphate

PKC .....Protein kinase C

PLD .....Phospholipase D

$CaCO_3$ .....Calcium carbonate

SDS-PAGE..... Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

EDTA .....Ethylene deamine tetra acetic acid

ABSTRACT

Production, Purification and Characterization of phospholipase D from *Streptomyces* sp. VN-3

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VN-3 strain, isolated from Vietnam sea showed a close phylogeny to *Streptomyces cheonanensis* (99.43%) and *Streptomyces spinoverrucosus* (97.12%). The 16S rDNA data suggested that this species might be *Streptomyces cheonanensis*. This study is an attempt to produce, purify and characterize phospholipase D from *Streptomyces* sp. VN3. The optimum culture medium was determined as glycerol 1.0%, soybean 1.0%, $CaCO_3$ 0.1% at 28°C. The phospholipase D produced in the culture broth exhibited hydrolytic activity as well as transphophatidylation activity on phosphotidylcholine. In particular, the culture broth showed 1.97-units/mg of hydrolytic activity. The phospholipase D was purified through ammonium sulfate fractionation (5-45%), ultra membrane (YM10) filtration, Sepharose

CL-6B column chromatography and Poros 20 HQ strong anion exchange column chromatography, which produced a major band of 58 kDa on 10% SDS-PAGE. The enzyme showed an optimum pH of 8.0 and temperature of 45°C for hydrolytic activity. The enzyme was relatively stable at high temperature and alkaline pH. The detergent 1.5% Triton X-100 and the metal ion Mn^{2+} (2 mM) were found to be enhanced the enzyme activity.

초록

Streptomyces sp.VN-3 균주로부터 Phospholipase D의 생산, 정제 및 특성분석

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베트남 해안에서 분리된 VN-3는 *Streptomyces cheonanensis* (99.43%)과 *Streptomyces spinoverrucosus* (97.12%)에 가까운 계통발생을 보여졌다. 이에 16S rDNA 데이터를 통해 이 종은 *Streptomyces sp. (Streptomyces cheonanensis)*라 생각된다. 본 연구는 *Streptomyces* sp. VN-3로부터 Phospholipase D의 생산,정제 및 특성분석이 이루어졌다. 최적 배양 배지는 28°C 하에서 Glycerol 1.0%, Soybean 1.0%, $CaCO_3$ 0.1%라 보여진다. 배양액으로부터 생산된 Phospholipase D는 phosphotidylcholine의 hydrolytic activity뿐만 아니라 phosphatidycholine에 대해 transphosphotidlation activity까지 지니고 있다. 그 중에서도, 배양액의 hydrolytic activity는 1.97 units/mg의 활성이 나타났다. Phospholipase D 는 5-45%의 ammonium sulfate fractionation, ultra membrane (YM10) filtration, Sepharose CL-6B column chromatography와 Poros 20 HQ strong anion exchange column chromatography를 통해 정제 되었

고 10% SDS-PAGE에서 58kDa의 밴드를 나타냈다. Hydrolytic activity의 경우 이 효소는 pH 8.0, 45 ºC에서 최적의 반응을 나타내었다. 이 효소는 비교적 높은 온도와 알칼리 pH에서 안정함을 보였다. 효소의 활성을 증가시키기 위해 유화제와 금속이온은 각각 1.5% Triton X-100 과 Mn^{2+} (2 mM)을 사용하였다.

I. Introduction

Phospholipase constitutes an important class of lipolytic enzymes that catalyzes the hydrolysis of the ester or phosphodiester bonds of phospholipid. [1,2,3] These enzymes clearly play important "housekeeping" roles in phospholipid catabolism and turnover associated with membrane synthesis, degradation, and organelle biogenesis, [4,28, 47, 48] Phospholipase can also generate molecules with important biological activities by degradation of various phospholipid substrates. [4, 28, 29, 30] For example, hydrolysis of arachidonic acid containing phospholipids by phospholipase A_2 released arachidonic acid for synthesis of protanoids, while phospholipase C catalyzed hydrolysis of inositol head group containing phopholipids, generating inositol phosphate and diglyceride, second messengers that control intracellular Ca^{2+} levels and protein kinase C activity, respectively.[27, 31, 32, 33] In mammalian cells, phosphotidylcholine (PC) specific phospholipase D (PLD) are the central effectors enzymes in a widespread receptor regulated signaling pathway. Hydrolysis of PC by these enzymes generates phosphatidic acid (PA) and choline. PA can regulate the activity of a number of important proteins and enzymes *in vitro*. [43, 47] Dephosphorylation of PLD generated PS by phosphatidate phosphohydrolase (PAP) is a significant route for the generation of diacylyglycerol in stimulated cells. Further metabolism of PA can produce the cell surface receptor active compound *lyso*PA, and PA may also be an important source of arachidonic acid for synthesis of prostaglandin's and leukotrienes. [50, 51,

[52] The hydrolysis of phosphotidylcholine by phospholipase is shown in Fig 1: [34, 35]

Phospholipase D belongs to a class of lypolitic enzymes which functions in heterogeneous systems, hydrolyzing water insoluble substrate. [1, 2, 3] PLD activity has been identified in mammals, plants and bacteria. PLD hydrolyzes phosphotidylcholine to phosphatidic acid and choline by breaking its phosphodiester bond. [2, 5, 7, 9] PLD also acts on other phosphatidyl esters and catalyzes a transphosphatidylation reaction when alcohol is present as a nucleophile donor such as the transformation of phosphatidylcholine (PC) and serine to phosphatidylserine (PS) and choline. This transphosphatidylation is important process for the synthesis of scarce phospholipid such as phosphotidylethanolamine, phosphotidylserine or phosphotidylglycerol and novel artificial phospholipids. [7, 9] These phospholipids as an abundant lipid component in nature possess glycerol structure with a polar head moiety and two fatty acid chains. This amphiphilic property facilitates the application of phospholipid in various industrial fields such as pharmaceutical, cosmetics and foods. For example, phosphatidylserine (PS) is used as a liposome for drug delivery systems (DDS), a surfactant, an emulsifier for cosmetics, and dietary supplement for foods. PS is also known to improve memory performance in patients suffering from age-associated memory impairment or Alzheimer's disease. [3, 4, 12] In addition, the PLD in mammalian cells plays a role in membrane vesicular trafficking, regulation of mitosis and signal transduction. In the brain, the PLD

activities of synaptic vesicle were reported to have the highest specific activity (Waite, 1985). [18, 20, 32] It was suggested by Hattori and Kanferi that this reaction could serve as a mechanism by which choline can be provided in the brain for acetylcholine synthesis (1984). PLD-catalyzed hydrolysis and transphosphatidyllation reaction is shown in Fig 2. [4, 25 27] PLDs have been isolated from bacteria such as *Streptomyces* species and *Corynebacterium* species. Unlike the plant, yeast and mammalian enzymes, these bacterial PLDs can hydrolyze *lyso*PC and sphingomyelin. [11, 12, 13] These enzymes have an alkaline pH optimum (pH 8-9), while enzymes from other sources are generally most active at neutral pH. Enriched preparations of the *Streptomycine antibioticus* PLD are commercially available.

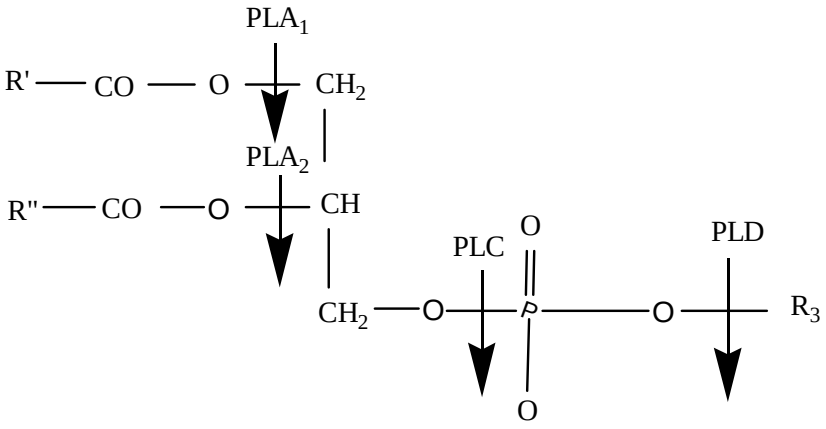

Fig.1 Hydrolytic reaction of phospholipid by phospholipase

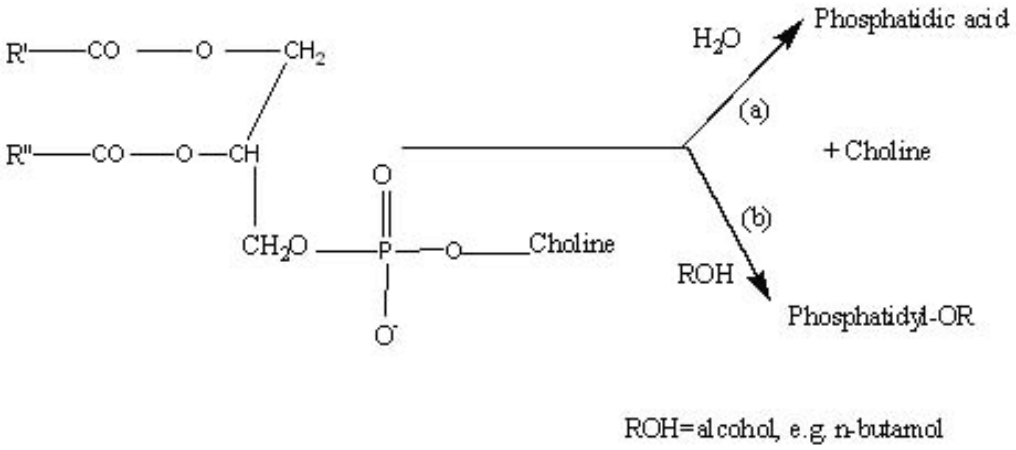

Fig.2 Reaction scheme of hydrolysis (route a) and transphosphatidylation (route b) of phosphatidylcholine by PLD

II. Material and methods

II-1. Materials

II-1-1. Chemicals and instruments

Choline oxidase from *Alcaligenes* sp., 4.05 IU mg^{-1} and peroxidase 120 purpurogallin units mg^{-1} were from the Sigma.

L-α-phophotidylcholine (from dried egg yolk), phenol, 4-aminoantipyrine, Triton X-100, as well as other chemicals of analytical grade were purchased from Sigma. Similarly, Sepharose CL-6B and Poros 20- HQ were from Pharmacia.

II-1-2. Bacterial strain

Streptomyces sp. VN-3 strain was isolated from Darang beach of Phu Yen province (Central area of Viet Nam). The seawater was taken offshore from 4.7 m depth. Salinity of the water was 3%.

II-2. Methods

II-2-1. Growth conditions

VN -3 strains streaked on agar medium. When colonies formed 2mm in diameter and violet in color, they was seeded in OSYM medium containing 2% oatmeal, 1% dried yeast, 1% soybean, 1% mannital at 28℃, 140 rpm in 250 ml baffled flasks. After 48 hours seed culture, VN-3 was transferred to GSC medium (1% glycerol, 1% soy bean, 0.1% $CaCO_3$) and shook at 28℃, 140 rpm for 3 days.

II-2-2. Ammonium sulfate precipitation

VN-3 cells were grown in GSC medium for 3 days. Cells were harvested and centrifuged (6,000 x g for 30 minutes at 4℃). Ammonium sulfate was poured slowly into the supernatant over a period of ten minutes allowing the salt to slowly dissolve. The supernatant was continually stirred on ice for an additional 1 hours. Precipitates were recovered by centrifugation (6000-x g for 1 hour at 4℃, and dissolved in 10 mM Tris-HCl (pH 8.0). [6, 8, 15, 19]

II-2-3. Sepharose CL-6B column chromatography

The dialyzed ammonium sulfate fractions were concentrated using an ultra filtration (Amicon, Danvers, MA) through a membrane pore size > 50,000 MW (Diaflo, Danvers, MA). The resulting concentrated samples (5 - 20 ml) were applied to a column (116 cm length, 2.2 cm in diameter) of Sepharose CL-6B. Sample eluted by Tris-HCl buffer (10 mM, pH8.0), and colleted in tubes (4ml/20min).

Fractions that have PLD activity were colleted and concentrated using filtration through a membrane pore size 50,000 MW. [53]

II-2-4. Poros 20 HQ strong anion-exchange chromatography (AEC)

AEC was carried out on a Poros 20 HQ column (10 x 1 cm; Perseptive Biosystem) at room temperature with the following mobile phases: buffer A, 10 mM Tris-HCl pH8.0 and buffer B, 10 mM Tris-HCl pH8.0 containing 1 M KCl. The flow rate was 0.5 ml/min. Sample (1ml) was filtered and injected. 30 minutes after of injection, a gradient from 0% to 100% of buffer B over 60 min

was applied, followed by 10 min of buffer A. The total run time was 220 minutes. Fractions (1ml) were collected and PLD assay was preformed.

II-2-5. Enzyme assay (PLD assay)

The assay for the determination of the hydrolytic activity of PLD was an adaptation of Imamura and Horiuti (1978), which is based on the enzymatic determination pf the release by the hydrolysis of phosphodylcholine (PC) by PLD []. The substrate solution PC was prepared by adding 200 mg of L-α-phophotidylcholine to 4 ml water and 0.4 ml Diethyl ether, son iced on ice in 1 hour. In a typical experiment, to make PC emulsion 200㎕ PC was added 100㎕ 1M Tris-HCl buffer, pH 8.0 and 66㎕ $MnCl_2$ 0.1 M. The reaction was started by the addition 40㎕ of the enzyme sample, and 60㎕ PC emulsion, followed by incubation at 55℃ for 20 minutes and arrested by the addition of 60 ㎕ of 1M Tris-HCl buffer, pH 8.0 containing 50 mM EDTA. The samples were boiling 10 minutes then chilled 20 minutes in ice. Immediately to the above standard reaction was added 150 ㎕ of following mixture; peroxidase reagent (containing 65 ㎍ peroxidase, 1.5 mM 4-aminoantipyrine and 2.1 mM phenol) and choline oxidase (1.7 mg) in 10 mM Tris-HCl buffer, pH 8.0, followed by incubation at 37℃ for 1 hour. The sample was diluted with 1% Triton X-100 to remove turbidity. The qiuoneimine dye formed was measured at 500 nm. One per minute under the assay conditions defines one unit of the enzyme as the amount that changes the O.D at 500 nm. [3, 4, 26,)]

II-2-6. Optimization of temperature and thermal stability

PLD activity was determined at different temperatures (22-70℃) by incubating the PLD catalyzed standard reaction mixture at different temperatures for 10 minutes.

The sample of enzyme was taken at different time intervals of 10 mins and included in the reaction mixture. After that, it was incubated at 37℃ and 60℃ and stopped reaction at 5, 10, 15, 20, 30 minutes.

II-2-7. Optimization of pH and pH stability

PLD activity was determined at different pH values (3-10) by taking 10 μl buffer of different pH (sodium bicarbonate buffer, pH 3.0, 4.0, 5.0, 6.0. 7.0, Tris-HCl, pH 7.5, 8.0, 8.5, 9.0, 10.0) in the PLD catalyzed standard reaction mixture as described above.

II-2-8. Effect of some metal ions on PLD activity

The effect of different ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, Cr_2O_3, Cu^{2+}, Ni^{2+}, EDTA) on the activity of PLD was studied, using the above standard reaction mixture.

II-2-9. Effect of various detergents on PLD activity

The effect of various detergent (Triton X-100, Tween 20, Tween 80, Polyethylene-4-Lauryether, Deoxycholic-acid, N-Lauryl-Sarcosine, CHAPS, SDS) on the activity of PLD was studied, using the above standard reaction mixture.

II-2-10. SDS -Polyacrylamide Electrophoresis (SDS-PAGE)

SDS-PAGE of *Streptomyces* sp. VN-3 strain in growth medium, filtered

culture supernatant, ammonium sulfate, after GPC and Poros 20 HQ fractions containing PLD activity were carried out as described by Laemmli (1970) using 5% stacking gel and 12% polyacrylamide resolving gels (1.5 mm thickness). Low molecular weight standards (Fermentos) samples of protein preparations and molecular weight standards were dissolved in a solution containing 60 mM Tris-HCl (pH 6.8), 14.4 mM β-mercaptoethanol, 2% SDS, 25% (vol/vol) glycerol, and 0.01% (wt/sol) Bromophenol Blue and immediately heated at 100℃ for 10 minutes.

Electrophoresis was carried out at a constant current of 100V/40mA per gel until the tracking dye (bromophenol blue) reached at the bottom of the gel (approximately 1 hour total running time). SDS-PAGE gels were stained by using a Coomassie Brilliant Blue Stain (Bollag et al., 1991).

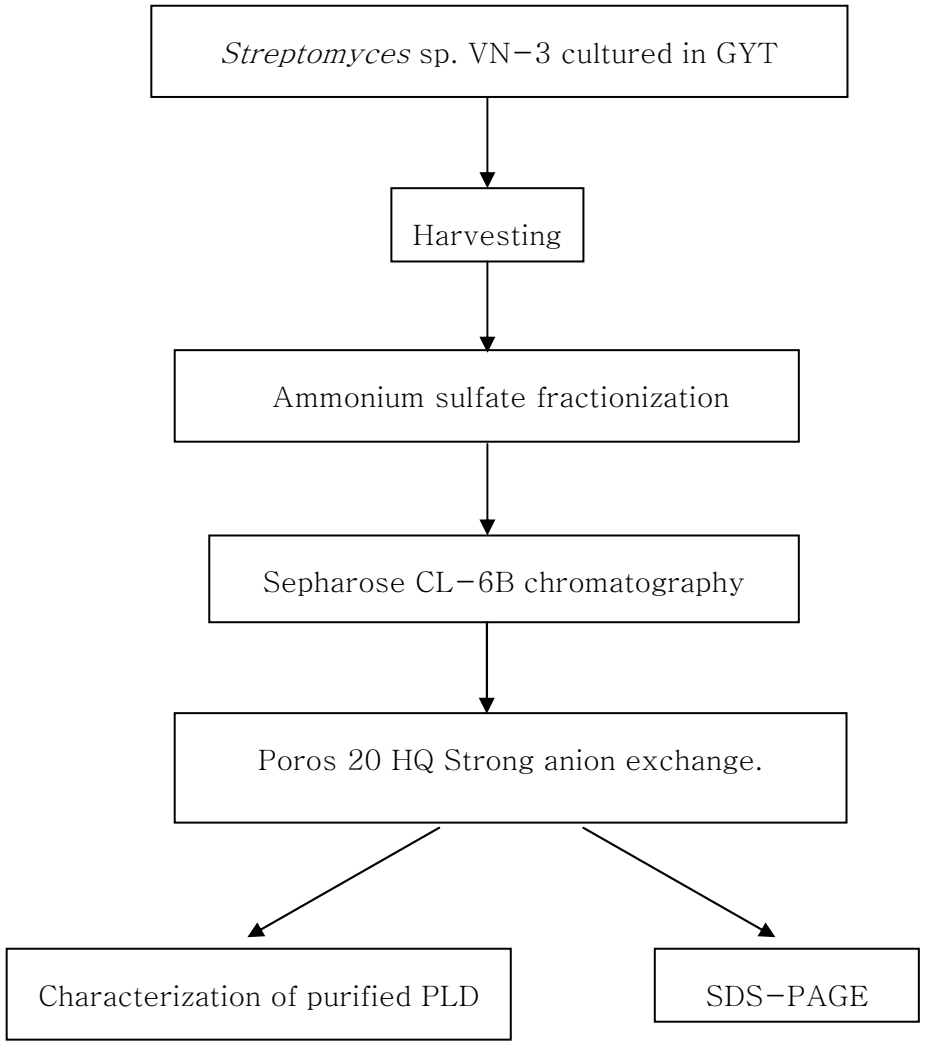

Fig. 3 Purification scheme of PLD from *Streptomyces* sp. VN-3

III. Results and discussion

III- 1. *Streptomyces* sp. VN-3 morphology

Colony morphology of *Streptomyces* sp. VN-3 was growth on marine media.

After 3 days incubated at 28℃, Colonies of *Streptomyces* sp. VN-3 was 0.3 mm in diameter, violet color. (Fig. 4)

III-2. Production of PLD from *Streptomyces* sp. VN-3

Streptomyces sp. VN-3 began to show its exponential growth approximately in between 24 to 36 hours of culture, and entered into stationary phase approximately up to 100 hours. PLD activity was appeared around 50 hours and continuously increased up to 125 hours of the culture period. For the maximum production and better enzyme isolation, the culture was harvested at 72 hour. (Fig. 5)

III-3 Purification of PLD

III-3-1. Ammonium sulfate precipitation

Ammonium sulfate precipitation was performed as the fist step pf PLD purification. Ammonium sulfate fractions collected at levels of saturation of 10 % were collected and assayed for specific PLD activity after dialysis. Fractions collected at 0-20% and 20-40% ammonium sulfate contained the highest percent of specific PLD activity, with a total of 64.3% of the total protein concentration (Table 1). More precisely to define the ammonium sulfate fraction containing the highest amounts of specific PLD activity, smaller fractions were

collected. It was determined that the 5-45% ammonium sulfate fraction contained 75.6% of the PLD activity whereas 0-5% and 45-70% fractions contained 12,7%of the PLD activity. [7, 8, 54] An ammonium sulfate precipitation between 5%-45% was used for purification of the PLD.

III-3-2. Partial purification of phospholipase D of *Streptomyces* sp. VN-3

The broth of VN-3 was concentrated by ammonium sulfate precipitation at 5-45% saturation at 4℃. The pellet, collected by centrifugation at 10,000xg for 1 hour at 4℃, was dissolved in 10 mM Tris-HCl pH8.0 buffer. After filtering by YM 10 (pore 50,000 MW), the supernatant was applied to Sepharose CL-6B column (2.2x116 cm). Sample was eluted by 10 mM Tris-HCl, pH 8.0. Fig.6 shows the graph of fractions that collected from Sepharose CL-6B chromatography column. [54]

Sample collected from Sepharose CL-6B chromatography, was applied on Poros-20-HQ Strong anion exchange column. Elution was carried out with a gradient of 1M KCl at a flow rate of 0.5 ml/min (Fig. 7).

Increase of the VN-3 PLD activity using Ammonium sulfate (2.23-fold increase) is consistent with published literature. Subsequent increases in PLD activity (3.04-fold increase) using Sepharose CL-6B column chromatography were also consistent. By using Poros 20 HQ, PLD activity was increased 3.73 fold.

The enzyme was purified to homogeneity on SDS-PAGE by the Poros 20 HQ

procedure (Fig.8), resulting in a 3.73-fold purification of PLD with a yield of 2%.

The band was seen in lane 5 that can be concluded that the PLD is approximately 58 kDa. [50]

III-4 Optimization of temperature and thermal stability

Using the partially purified phospholipase D from VN-3, its reaction characteristics were examined.

Similar to phospholipase D from other actinomycetes, PLD from VN-3 was strongly active from 40℃ to 60℃. The optimum reaction temperature was 45℃ for the hydrolysis of PC (Fig. 9). [26, 38]

III-5 Optimization of pH and pH stability

The effect of pH on the activity of PLD is shown in Fig. 11. The enzyme showed good activity in the pH ranges 7-9. The optimum pH was observed to be 8.0.

When investigating the enzyme stability at various pHs, VN-3 phospholipase D exhibited high stability within a range from neutral to base (7.0-10.0), and lost its activity above acidic pH (2.0-6.0). VN-3 PLD is more stable within a base pH range (Fig. 12). [1, 3]

III-6 Effect of various detergents on activity of phospholipase D

To identify the effect of the detergent on PLD activity, I used Non-ionic, anionic, and zwitter-ionic detergents in hydrolytic reaction of PLD.

As shown in Table 3, supplementation of 1.5 % Triton X-100 in the reaction

medium, the hydrolytic activity of the enzyme was increased by as much as 1.67 times compared to the case without Triton X-100. No other detergents produced any significant increment in the enzyme activity, except N-laurylsarcosine and SDS, which inhibited the hydrolytic activity of the enzyme.

III-7 Effect of metal ions on the hydrolytic activity of phospholipase D of *Streptomyces* sp. VN-3

Metal ions such as Mn^{2+}, Ca^{2+}, Mg^{2+}, Co^{2+}, and Fe^{2+} increased the activity of VN-3 PLD, whereas Zn^{2+}, Ni^{2+}, Cu^{2+}, as well as EDTA as chelating agent, no significant effects were observed in the PLD from VN-3 (data shown in Table 4). (51)

Fig. 4 Photographs showing the colony morphology of *Streptomyces* sp. VN-3

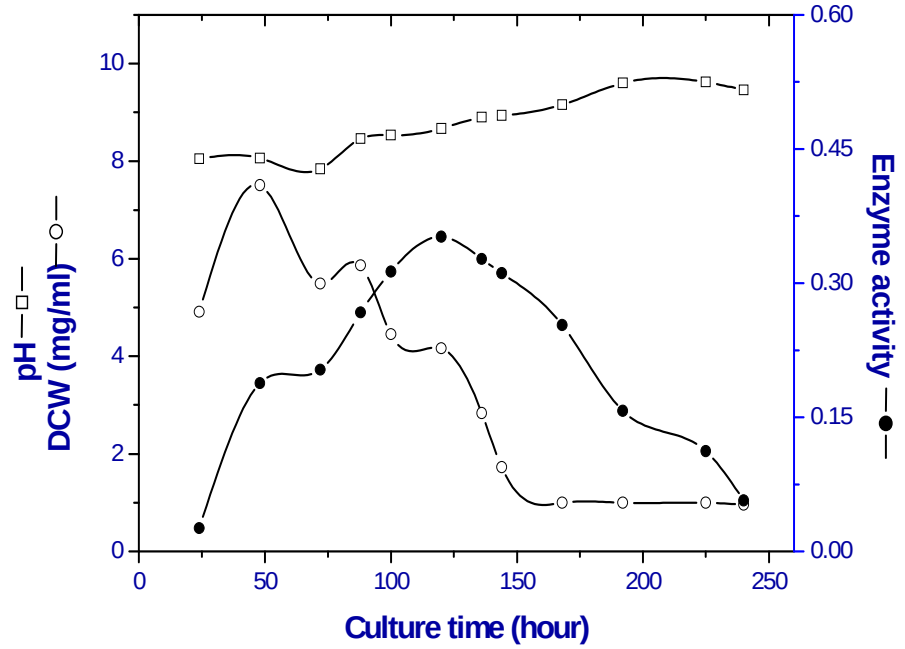

Fig. 5. Fermentation profiles for the production of phospholipase D from *Streptomyces* sp. VN-3

Table. 1. Pattern of Ammonium sulfate precipitation

Salt added	% Of enzyme specific activity	% Of Total protein concentration
0-20%	53.0	46.4
20-40%	36.3	17.9
40-60%	6.6	14.8
60-80%	3.9	6.3
Sup	0	13.9

Salt added	% Of enzyme specific activity	% Of Total protein concentration
0-5%	3.9	33.7
5-45%	**75.6**	**41.6**
45-70%	8.8	12.2
Sup	2.0	6.0

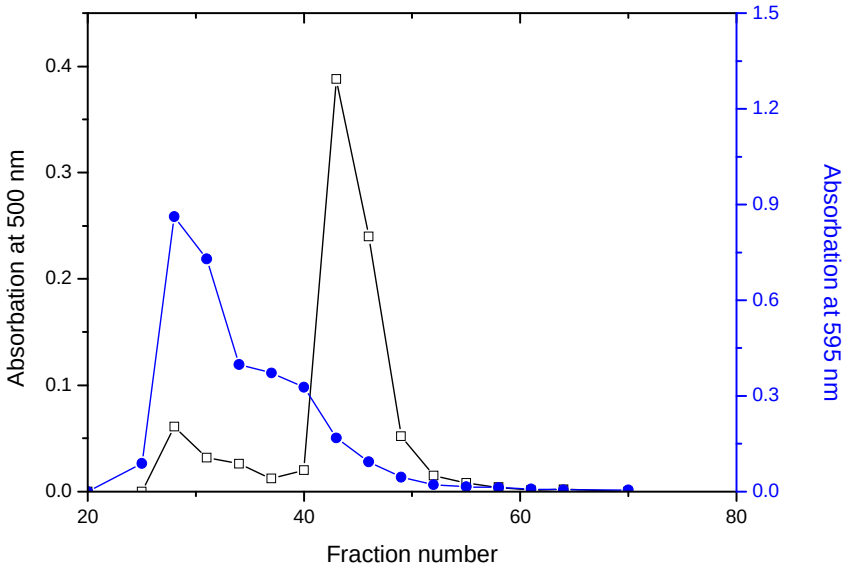

Fig.6. Elution profile of phospholipase D from Sepharose CL-6B column chromatography

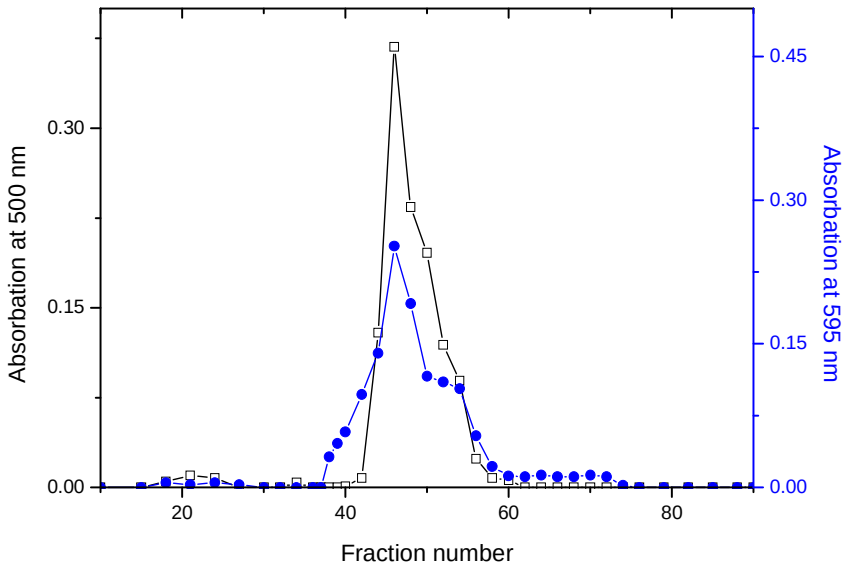

Fig. 7. Profiles of PLD from Poros-20 HQ strong anion exchange column

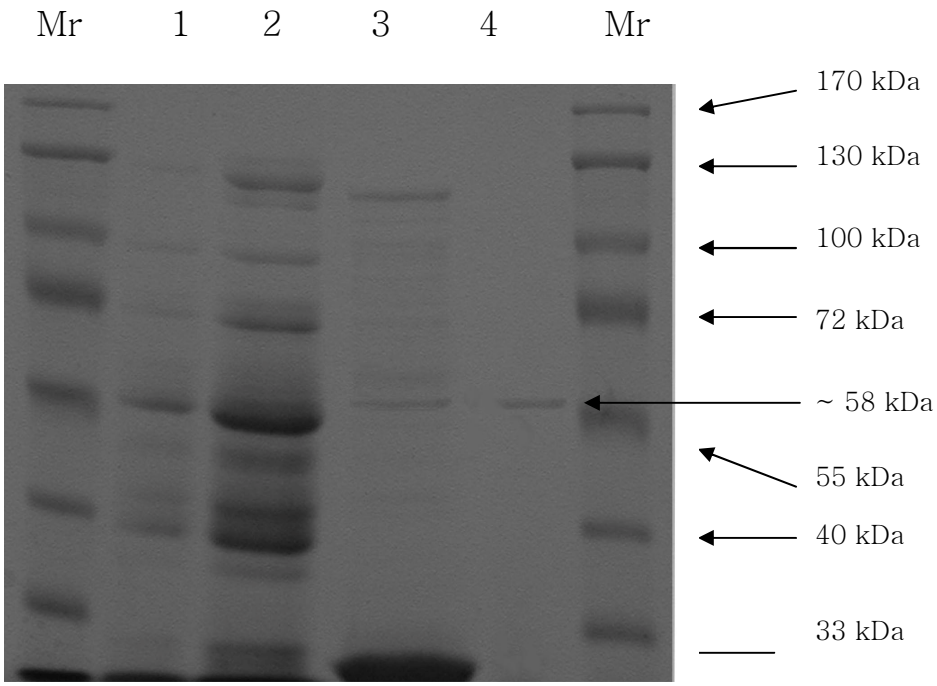

Fig. 8. Molecular weight determination of phospholipase D from *Streptomyces* sp. VN-3 by SDS-PAGE

Mr: Size marker

Lane1: Crude extract of VN-3

Lane 2: Ammonium sulfate (5-45%) fraction

Lane 3: Sepharose CL-6B

Lane 4: Poros 20 HQ strong anion exchange

Table. 2. Purification of phospholipase D from *Streptomyces* sp. VN-3

Step	Total protein (μg)	Total activity (unit)	Specific activity (Unit/μg)	Fold	Yield (%)
Crude extract	6682	13205	1.97	1	100
Ammonium. Sulfate	2780	9983	4.38	2.23	75.6
Sepharos CL-6B column chromatography	694	4170	6.00	3.04	31.5
Poros-20 HQ column chromatography	359	2638	7.35	3.73	2

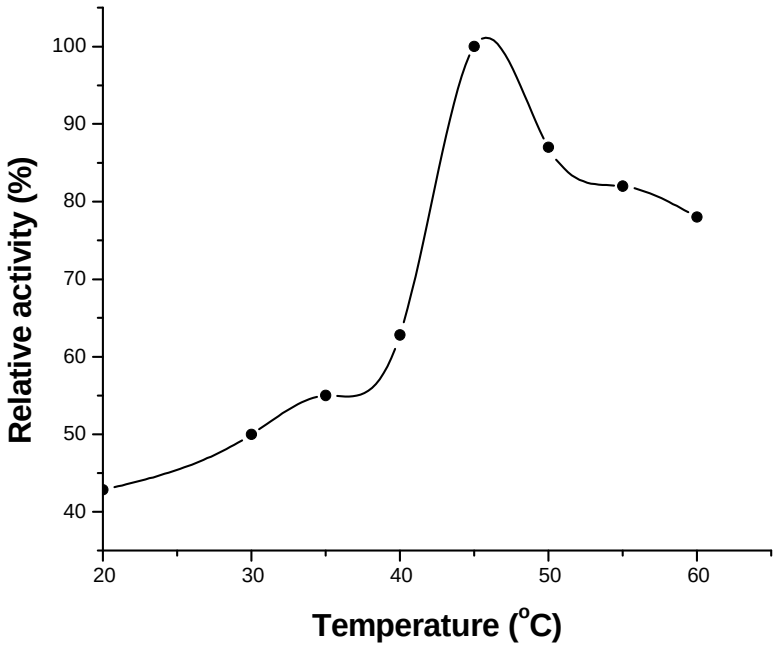

Fig. 9. Effect of temperature on activity of phospholipase D of *Streptomyces* sp. VN-3

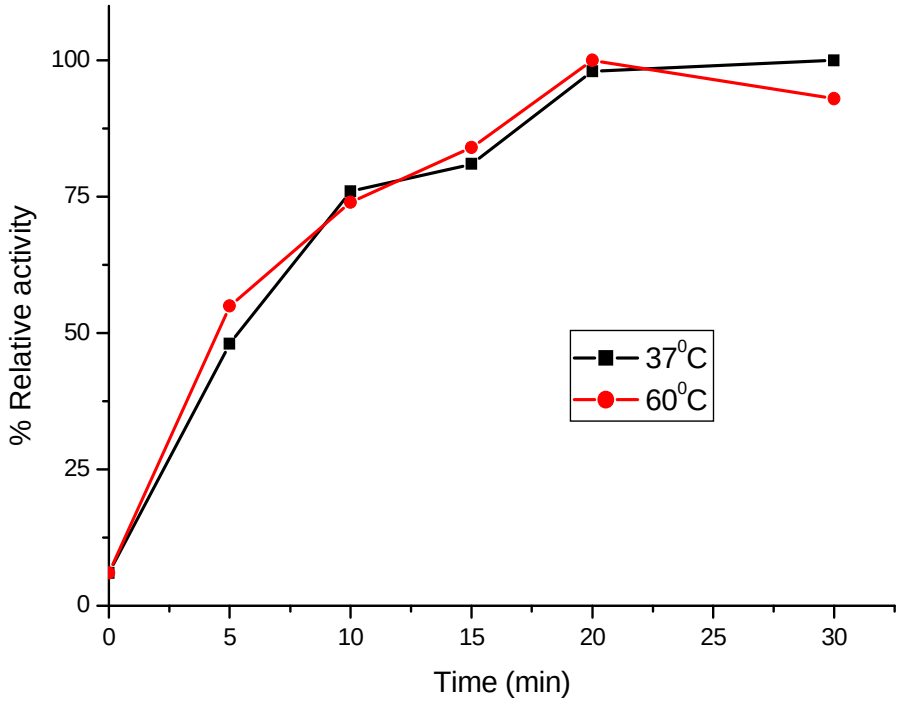

Fig. 10. Thermal stability of phospholipase D from *Streptomyces* sp. VN-3

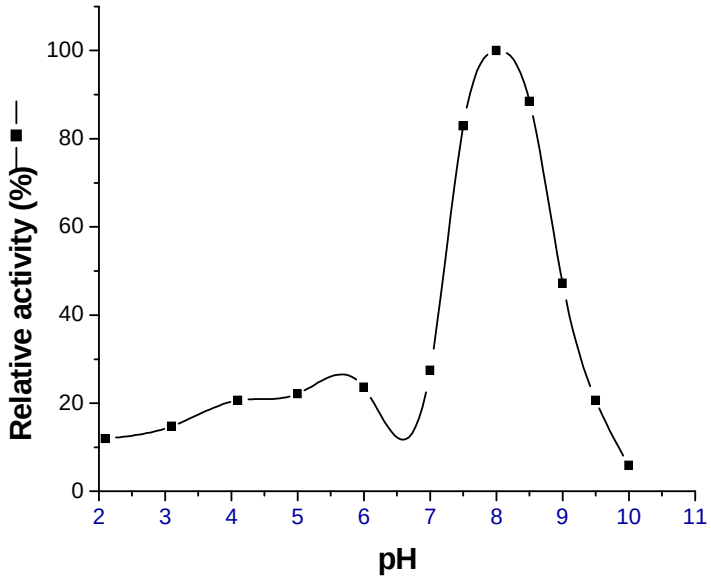

Fig 11. Effect of pH on activity of phospholipase D of *Streptomyces* sp. VN-3

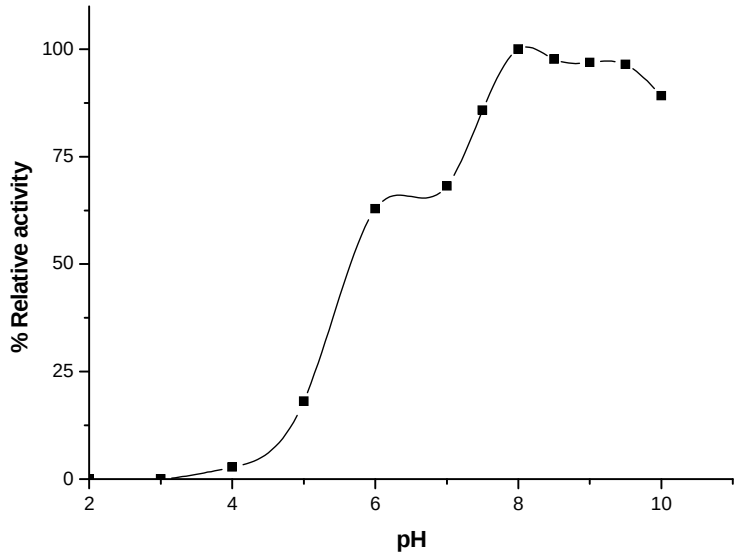

Fig. 12. pH stability of PLD enzyme during treatment on pH 2-10 for 20 min at 45℃

Table. 3. Effect of various detergents on the hydrolytic activity of phospholipase D from *Streptomyces* sp. VN-3

Biological Detergents	Type of ion	Relative Activity(%)
None		**100**
Triton X-100	Non-Ionic	**167**
Tween 20	Non-Ionic	**95.2**
Tween 80	Non-Ionic	**17.1**
Polyoxyethylene -4-laurylehter	Non-Ionic	**12.8**
N-laurylsarcosine	Anionic	**0**
CHAPS	Zwitter-Ionic	**66.6**
SDS	Anionic	**0**

Table. 4. Effect of metal ions on the hydrolytic activity of phospholipase D from *Streptomyces* sp. VN-3

Metal ions	Relative activity (%)
None	100
Ca^{2+}	225
Mg^{2+}	306
Mn^{2+}	437
Zn^{2+}	0
Co^{2+}	393
EDTA	0
Fe^{2+}	300
Ni^{2+}	56
Cu^{2+}	1.87
CrO_3	143

IV. Conclusions

Streptomyces sp. VN-3 was grown in GSC medium containing 1% glycerol, 1% soybean and 0,1% $CaCO_3$ at 28℃, 180 rpm/min. PLD enzyme was purified from culture broth after 72 hours.

An ammonium sulfate precipitation between 5-45%, Sepharose CL-6B and Poros 20 HQ column chromatography were used for purifying the PLD, resulting in a 3.73-fold purification of PLD with a yield of 2%.

Phospholipase D of *Streptomyces* sp. VN-3 was purified with specific activity 7.35-unit/μg protein at pH 8.0 and 45℃ in the hydrolytic reaction. Its molecular weight was determined as 58 kDa

Like most convention bacterial PLDs, triton X-100 and divalent cation such as Mn^{2+}, Mg^{2+}, and Ca^{2+} were required for enzyme activity. N-laurylsarcosine and SDS were strong inhibitor of PLD from *Streptomyces* sp. VN-3

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논문제목	**한글** : *Streptomyces* sp.VN-3 균주로부터 Phospholipase D의 생산, 정제 및 특성분석				
	영문 : Production, Purification and Characterization of phospholipase D from *Streptomyces* sp. VN-3				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함.
   다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의( ) 반대( )

2006 년 12 월 6 일

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