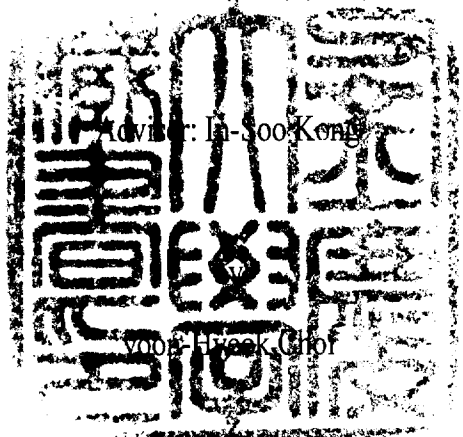
Purification and Characterization of Recombinant Phospholipase A_2 from *Vibrio mimicus*

Vibrio mimicus 유래 재조합 인지질가수분해효소의 정제 및 특성 연구



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Purification and Characterization of Recombinant Phospholipase A_2 from *Vibrio mimicus*

A Dissertation

By

Yoon-Hyeok Choi

Approved as to style and content by :

Chairman

Member

Member

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주 심 이 학 박사 홍 용 기 (인)

부 심 공 학 박사 김 성 구 (인)

부 심 공 학 박사 이 형 호 (인)

Table of Content

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Abstract

We have previously isolated the phospholipase gene from *Vibrio mimicus* ATCC 33653. Due to investigate the biochemical chracteristics, the recombinant phospholipase from *V. mimicus* was overexpressed in E. coli BL21(DE3) with 1mM IPTG and purified homogeneous protein which has a molecular weight of 53 kDa with Ni-NTA affinity chromatogram. In addition, we identified that this purified enzyme was specific to sn-2 position ester likage of phosphatidylcholine. So, we determinated the purified enzyme is phospholipase A_2 (PLA_2). The recombinant PLA_2 ($rPLA_2$) was found to be a typical neutral activity and calcium ion dependency. And we can suggest that the phospholipase A_2 from *V. mimicus* can cause massive tissue distruction of fish cell as primary source of infection

Introduction

Many microbial virulence factors including hemolysin, cytotoxin, protease, hemagglutinin, cell adhesion factors, phospholipase and lipase are suggested to play an important role in host infection and intestinal disease(1). In terms of pathogenesis, microbial phospholipases play an important role in the initiation of infection because phospholipids are the basic components of natural membranes and cell walls(2). And also, phospholipases are known to be considerable virulence factors for bacterial species that cause massive tissue destruction. Phospholipid turnover by phospholipases action lead to the signal transduction in mammalian cells and plants(3,4).

Phospholipase A_2 (PLA_2, phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4) is one of them. It hydrolyses L-α -phosphatidylcholine, to L- α - lysophosphatidylcholine and fatty acid. The enzyme for biocatalytic applications is found in invertebrates (bee), in the venom of snakes (*Crotalus adamanteus, Naja naja, Crotalus atrox*), in mammal (bovine pancreas,

porcine pancreas) (5, 6) and in microorganisms (*Streptomyces violaceoruber, S cinnamonoeus, S. griceus*) (7, 8). Use of this enzyme allows the removal/replacement of the acyl chain at position sn2 either via hydrolysis and subsequent chemical reestrification or through direct interesterification with an acyl donor. In our lab., the phospholipase gene (1.4kb) from *Vibrio mimicus* was indentified and constructed with pET 22b, and this recombinant plasmid (pPHL13) was transformated in *E. coli* BL21(DE3), which was expressed by *lac* promoter, previously (9).

In this study, the recombinant enzyme, Phl13 was determined the type of enzyme to PLA_2 by TLC method and done optimum experimental condition with respect to pH and temperature and metallic cation as cofactor. And, we examined the cytotoxic effect against cultured fish cell as primary source of *Vibrio* sp. infection.

Materials and methods

Bacterial strain, plasmid and culture condition

Strain was used *E. coli* BL21, and pPHL13 is previously constructed plasmid consisting of the phospholipase open reading frame on a *Bam*HI *Eco*RI from *V. mimicus* cloned into the same site of pET22b (9).

Bacteria were grown in Luria-Bertani(LB) broth containing ampicillin (100mg/L) at 37℃. Due to express *lac* promotor, when the absorbance was 0.6 at $O.D_{600}$, IPTG was added into culture with final concentration 1mM, and then the culture were incubated same temperature for 5hrs.

Enzyme purification

Induced cells were harvested by centrifugation at 7000 × g. To obtain intracellular inclusion body, Centrifugated cells were resuspended in 100 ml of 20 mM Tris-HCl (pH 8.0) buffer and then using sonicator (Sonifier 250, Branson) and centrifuger, they were homogenized and gathered 2 times. The

precipitates which were accumulated finally were the bulk of inclusion bodies, those were inactive form enzymes. Due to convert in active form, the precipitates were resuspended in solubilization buffer (2M Tris 2M Urea, pH 12) and stirred at 4℃ until dissolved completely (10). Then soluble fraction was taken by centrifugation at 12000 × g and filtration through 0.22 ㎛ membrane filter. The soluble protein was refolded and separated at the same time by gel filtration. Sephadex G-100 and 20mM Tris-HCl (pH 8.0) were used as gel filtration matrix and eluent.

The eluted solution was applied directly to Ni-NTA resin (Qiagen Co.) equilibrated with 50mM $NiSO_4$. The proteins were eluted by 20mM imidazole buffer. The purified fractions were dialyzed against 20mM Tris-HCl buffer (pH 8.0) containing 10% glycerol(V/V) at 4℃ for 12 hrs. The protein concentrations in the course of enzyme purification were determined by the dye reagent method (11). All purification procedures were carried out at 4℃. The purity of the enzyme was analyzed by SDS-PAGE.

Thin-Layer Chromatography

To determine the action mode of site-specific deacylation against phospholipid, L-α -phosphatidylcholine(L-α -PC), L- α -lysophosphatidyl-choline(L- α -LPC), 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine and 1-O-Hexadecyl-2-[(cis)-9-octadecenoyl]-rac-glycero-3-phosphocholine were employed as substrates. The reaction was initiated by the addition 10 $\mu\ell$ of 10 $\mu g/\mu\ell$ substrate to 100 $\mu\ell$ enzyme reaction buffer which was composed 20 mM Tris-HCl(pH 8.0), 5.0 mM Na-deoxycholate, 2.7 mM $CaCl_2$ and 1mg of dried enzyme. The reaction mixture was incubated at 37℃ for overnight and terminated by the addition of 650 $\mu\ell$ of chloroform : methanol (2 : 1 vol.). The mixture was voltexed and centrifuged, The bottom layer was collected and dried under vacuum. The dried sample was redissolved in 20 $\mu\ell$ of chloroform and then spotted on a silica gel plate. The plate was developed in hexane : diethyl ether : acetic acid (80 : 20 : 1 vol.) until the solvent front was 1cm from the top of the plate. The free fatty

acid was visualized using a 50% sulfuric acid as detection solution and drying at 115℃ for 30mins.

Measurement of enzyme activity

PLA_2 activity assay of crude or purified fraction was routinely assayed using the following method. Three milliliter of reaction buffer (10mM Tris-HCl, 10mM $CaCl_2$, 100mM NaCl ; pH 8.0) was combined with 50 $\mu\ell$ of substrate stock solution [4-Nitro-3-(octanoyloxy)benzoic acid, 3.1mM in acetonitrile] was then added. Each tube was voltexed and placed in a water-bath at 37℃ for 1hr. To stop the reaction, tubes were held on ice for 5 mins and spectrophotometically measured absorbance at OD_{410} every 30mins.

The enzyme was assayed at different pH (from 3 to 11) and temperatures (from 4℃ to 50℃) and the enzyme stability against pH was measured after incubation of enzyme at various pH (from 3 to 11) and its stability against temperature was measured after 120 mins incubation at various temperature

(from 4℃ to 70℃)

Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electerophoresis (SDS-PAGE) analysis of fractions collected during the various steps of purification were performed as follows; the sample was boiled for 5 mins in the presence of an equal volume sample buffer. The sample was then loaded onto 0.75 mm-thick slab gels. Electrophoresis was run at a constant voltage of 100 volts. The presence of protein bands was detected by Coomasie blue staining.

Effect of metal ion on PLA_2 activity

In order to investigate the effect of metal ion on $rPLA_2$ activity, various metal ions were added to a metal free enzyme solution at 10mM concentration. Metal free enzyme solution was made by chelating the metal ion using 10mM EDTA, then, the metal chelating enzyme solution was

dialyzed against distilled water for 12hrs to remove EDTA. The enzyme activity was determinated as previously.

Pathogenicity of V. mimicus rPLA$_2$ on CHSE cell line and LDH determination

Chinook salmon embryo cells, CHSE-214 cell line was innoculated MEM-medium containing penicillin G and streptomicin to 48-well plate 1ml each and cultivated at 17℃ for 24hrs. Then, 100 $\mu\ell$ of enzyme was added and incubate in same temperature.

The CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega) was used to measure the amount of lactate dehydrogenase (LDH) released in the cell culture medium.

From each well of the cell culture plate, 50 $\mu\ell$ of cell culture medium was transferred to a new microtiter plate and then 50 $\mu\ell$ of substrate mixture was added to each well. The plate was incubated at room temperature in the dark

for 15 mins and added 50 $\mu\ell$ reaction stop buffer to each well. Finally, the absorbance of the red reaction product was measured at 490nm with a microplate reader.

Results

Purification of the enzyme

In order to investigate the biochemical properties of *V. mimicus* phospholipase A_2, we overexpressed its gene in *E.coli* BL21(DE3). After the *phl*13 gene expression was induced with 1mM IPTG, we could detect a distinct overexpressed band which was not present in *E.coli* cells harbouring only the expression vector pET22b(+). The expressed Phl13 encodes a 6x His-tag for convenient purification using a Ni-NTA affinity column. The His tagged protein was purified as described in materials and methods. The purification scheme of the overexpressed Phl13 is summarized in Table 1.

Table 1. Purification steps of phospholipase A_2 from recombinant *E. coli*

Purification step	Total protein (mg)	Activity*	Total activity	Yield (%)	Purification Fold
Cell homogenate	78.33	2.21	172.89	100	1.00
Refolded Inclusion body	8.33	15.73	132.01	76	7.13
Ni-NTA affinity chromatography	4.44	21.75	96.57	56	9.86

*nmoles product/min/mg protein

As shown in Fig. 1, the molecular weight of expressed protein was estimated to be 53 kDa. This value is coincident with the molecular weight calculated from the predicted amino acid sequences.

To investigate the efficiency of temperature on the induction of the phospholipase, the recombinant was expressed by 1mM IPTG as inducer at 25℃ and 37℃. The expression level of phospholipase incubated at 25℃ was higher than 37℃. (data was not shown)

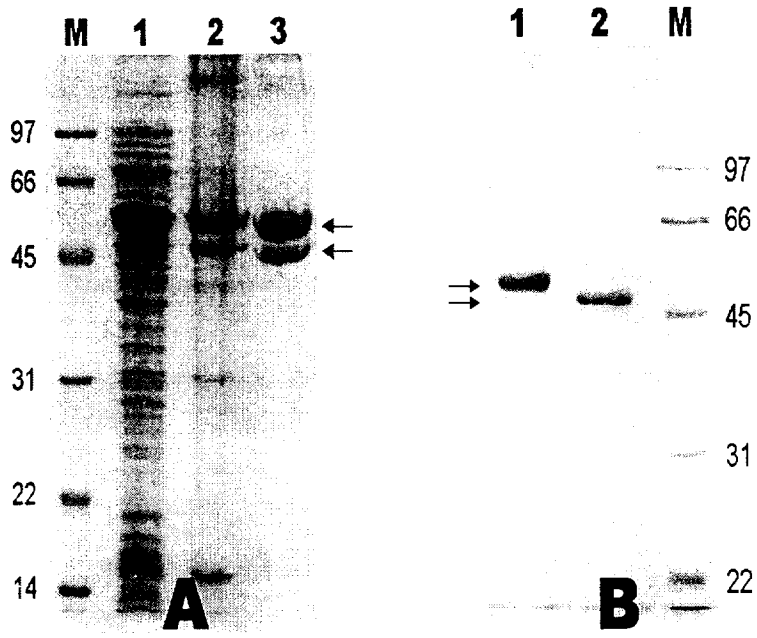

Figure 1. SDS-PAGE analysis of *V. mimicus* rPLA$_2$ during purification steps,

(A) Lane 1 : The homogenate of pPHL13 cell induced with 1mM IPTG for 4hrs at 25℃, lane 2: isolated and refolded inclusion body by Sephadex G-100 gel chromatography, lane 3: Ni-NTA affinity column elute fraction. (B) Using membrane which MWCO is 50,000Da, The rPLA$_2$ was isolated. Lane 1: The designated pPHL13 protein 53kDa, lane 2: the lower band of pPHL13 protein which size is 47Kda, M: low range molecular marker

Type determination of enzyme

Various phospholipids were tested as substrates. Both egg yolk lecithin and L- α -PC were hydrolyzed by this protein, but ρ -nitrophenylphosphorylcholine (NPPC) was not hydrolyzed by the enzyme compare with *C. perfringens* phospholipase C activity. For specificity towards phosphatidylcholine, The enzyme reaction products were analyzed by thin-layer chromatography.

As shown Fig. 2., the releasing of LPC from L- - PC could be found. Rf values of LPC and PC were 0.2 and 0.57, respectively. When L- -LPC was adapted as a substrate, the FFA spot was not on the plate. Also, 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine and 1-O-hexadecyl-2-[(cis)-9-octadecenoyl]-rac-glycero-3-phosphocholine were used as substrates, FFA spot were detectable.

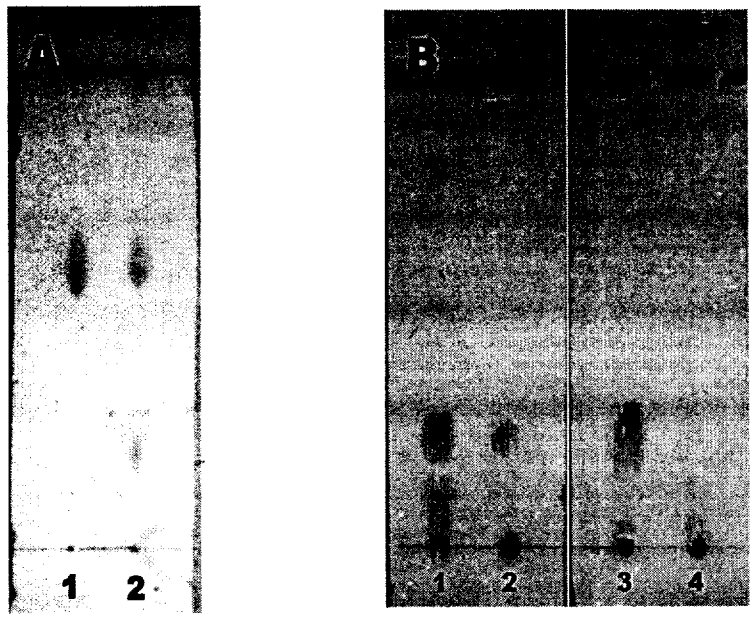

Figure 2. Thin-layer chromatogram detection of reaction product

(A) lane 1 : only PC, lane 2 : enzyme reaction solution between $rPLA_2$ and PC after 10hr incubation, the RF values of PC and LPC were 0.57 and 0.2, respectively (B) lane 1 : enzyme - 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine reaction solution, lane 2 : enzyme - 1-O-hexadecyl-2-[(cis)- 9-octadecenoyl]-rac-glycero-3-phosphocholine reaction solution, lane 3 : enzyme - PC reaction solution, lane 4 : enzyme - L-α -LPC reaction solution. Every sample was incubated for 10 hrs. The spot of free fatty acid (FFA) were detectable on lane 1, 2, 3 but , the any spot was not detected on lane 4.

Considering of substrates specificity and TLC analysis, it seems that the purified enzyme acts as phospholipase A_2.

Effect of pH and temperature against $rPLA_2$

As shown in fig. 3-A, $rPLA_2$ showed maximal enzyme activity at pH 8.0. To evaluate the stability of the enzyme of pH, the PLA_2 was pre-incubated in various pH of buffer at 37℃ for 2 hrs. The remaining activity was measured pH 8.0 at 37℃. The enzyme was stable at a pH range between 7.5 ~ 9.0 (fig. 3-B)

Due to determination of optimal pH, various pH of buffer was used enzyme reaction as reaction buffer ; pH 3.0 ~ 6.0 (0.1N citric acid, sodium citrate buffer), pH 7.0 ~ 9.0 (0.1N Tris buffer), pH 10.0 ~ 11.0 (0.1N sodium carbonate buffer).

The effect of temperature on enzyme activity and stability was investigated at different temperature. The maximal activity of the enzyme under the condition employed was observed between 35 ~ 40℃.

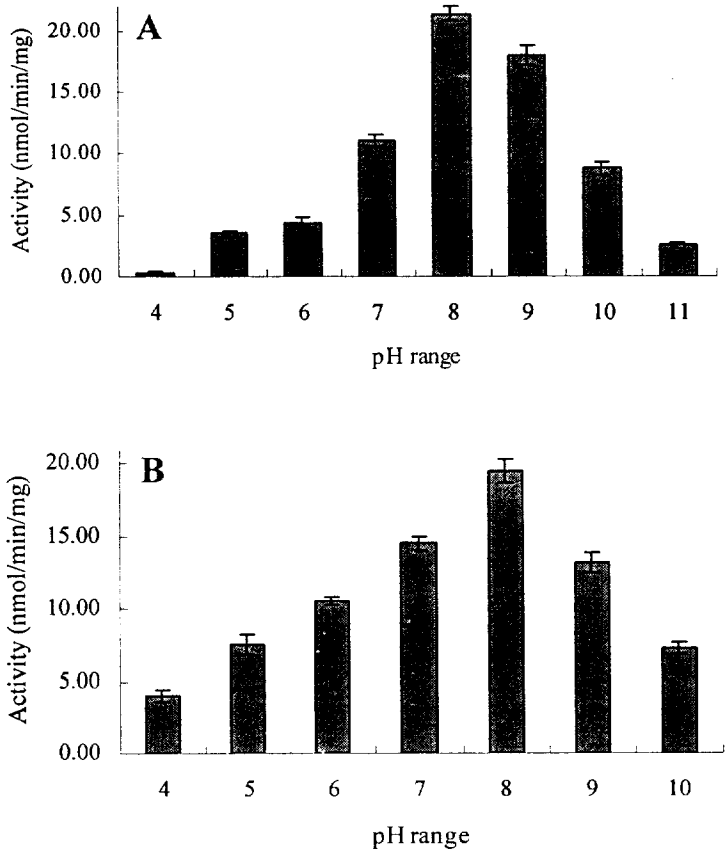

Figure 3. The pH-dependency and pH stability of *V. mimicus* rPLA$_2$ activity

(A) The pH-dependency of PLA$_2$ activity was examined using buffered solutions of 0.1N citric acid, sodium citrate (pH 3.0~6.0), 0.1N Tris (pH 7.0~9.0) and 0.1N sodium carbonate (pH10.0~11.0). (B) To examine the pH stability of Phl13-PLA$_2$, the enzyme was incubated in the above buffered solutions at 4℃ for 24hrs and then the enzyme activity was measured under the standard assay condition

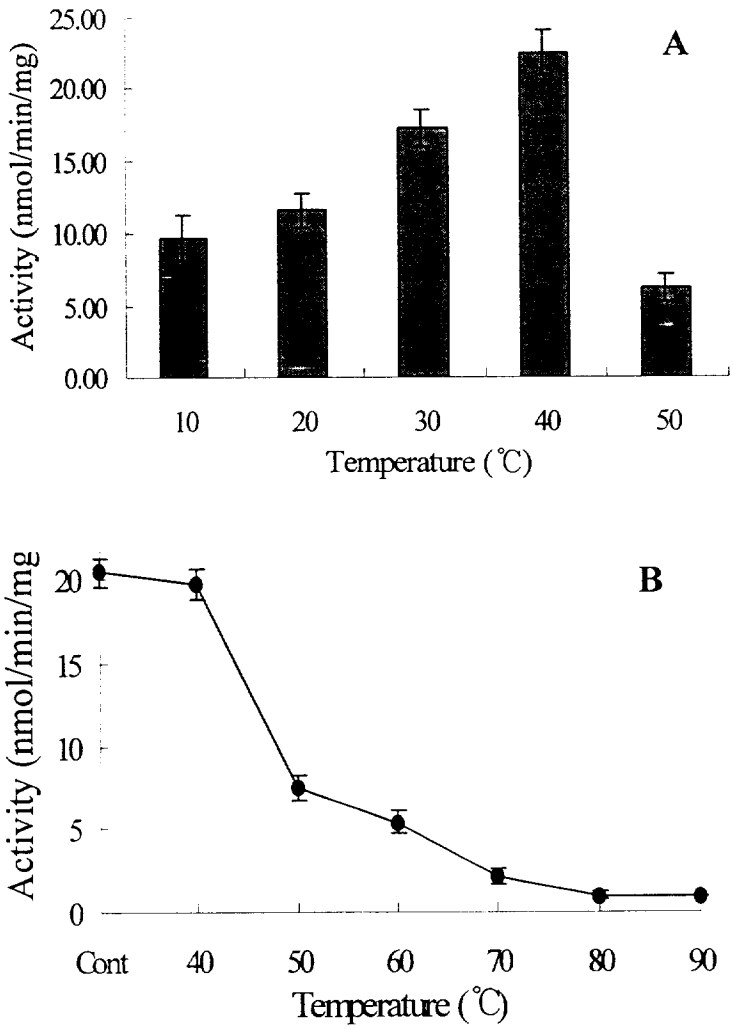

Figure 4. The Optimal reaction temperature and temperature stability of *V. mimicus* rPLA$_2$ activity

(A) The Optimal temperature of rPLA$_2$ activity was examined under various temperature for 1hr. (B) To investigate the temperature of the enzyme, each enzyme was preheated in various temperature for 2hrs. Finally, the enzyme activity was measured under the standard assay condition

To investigate thermal stability the enzyme, the enzyme was incubated at several temperature for 1hr at pH 8.0 and residual enzyme activity was determined. The results showed that showed that this enzyme was not stable up to 50℃, at 66% of initial enzyme activity was inactivated after 2 hrs of incubation (fig.4-B).

Effect of metallic cations

Phospholipases from various sources were reported to be activated by certain metal ion (e.g. Ca^{2+} and Mg^{2+}). The effect of metal ions on the activity of the Phl13 were investigated by adding various metal salts (20mM each of $CaCl_2$, $MgCl_2$, $MnCl_2$, Zn Cl_2, $CuCl_2$, $NiSO_4$ and KCl). As a control, EDTA was added same concentration to chelate metal ions from the reaction mixture. The mixture without metal ion was measured a very weak enzyme activity. In contrast, many of metal ions examined stimulated the PLA2 activity. (Table 2)

Table 2. Effects of metalic cation against V. mimicus rPLA2 activity.

Metal ion (10mM)	Activity (nmole/min/mg)
EDTA	0.00
None	1.56
$CaCl_2$	16.88
$CuCl_2$	ND
$NiSO_4$	8.75
$CoCl_2$	15.31
$ZnCl_2$	ND
$MgCl_2$	5.31
KCl	1.88

Above all, Ca^{2+} ion was enhanced the activity most significantly

The activation of PLA_2 vs the increase of calcium ion concentration followed a saturation curve (fig. 5).

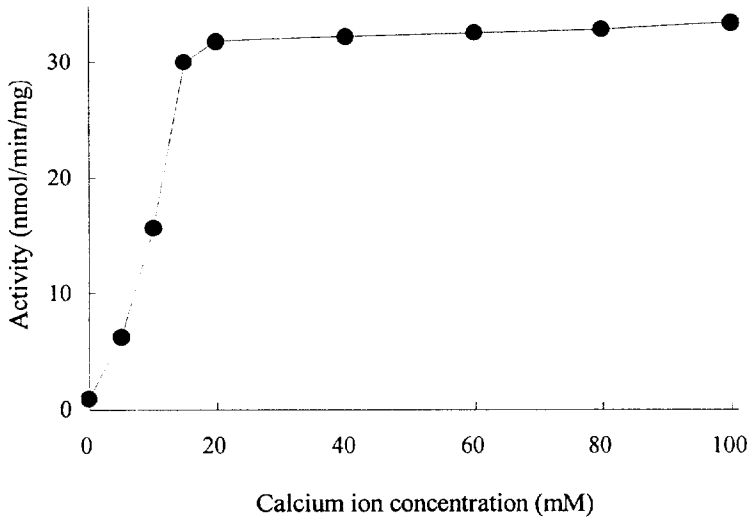

Figure 5. *V. mimicus* rPLA$_2$ activity as function of the calcium concentration.

The enzyme solution was incubated with various concentration of calcium ion for 30 mins. The activity was determinated under standard assay condition

Discussion

Phospholipase are known to be one of the important virulence factors in the pathogenicity of a number of microorganisms. Few phospholipases from *Vibrio* sp. have been characterized in detail with an exception which is the bifunctional hemolysin-phospholipase C of *V.cholerae* O139 (12). Accordingly, the purified phospholipase-hemolysin from *V.cholerae* O139 differed from *V.cholerae* El Tor hemolysin in pI and its thermostability. *V.cholerae* El Tor hemolysin has been shown to be enterotoxic but its phospholipase C activity was not identified. Recently, DNA sequence of phospholipase (or lecithinase) gene was revealed in *V.cholerae* El Tor and *V. mimicus*. Immediately upstream of the hemolysin lies phospholipase gene with opposite translational direction. *V.mimicus* was classified as atypical strain of *V.cholerae*. The high similarity of two genes hemolysin and phospholipase, between *V.cholerae* and *V.mimicus* was shown in our previous reports, but we could not any hemolitic activity from purified

enzyme. Although phospholipase (or lecithinase) genes of *V.cholerae* and *V.mimicus* were cloned earlier, the biochemical function of this enzyme has not been investigated.

In this report, we overexpressed *V. mimicus* phospholipase from reconbinant *E. coli* and purified the enzyme by Ni-NTA affinity chromatogram. Through the thin-layer chromatogram analysis, we could say the action mode of this enzyme to phospholipase A_2.

So, We adapted to use 4-Nitro-3-(octanoyloxy)benzoic acid (PLA2 chromogenic substrate) as substrate for liquid assay. This enzyme was shown its activity under existance of calcium ion. Some of metalic cations stimulated the PLA_2 activities. But the anzyme activity was shown maximum when calcium ion was used as additional metallic cation. This tendency could find many reported paper about phospholipase (13, 14, 15, 16)

The purified enzyme was found to be a typical neutral activity, displaying its

activity predominantly in the neutal region (pH 7.5 ~ 8.5) (fig. 3-A).

When the Phl13 was incubated in various buffers over a broad pH range (pH 4~11) for 1hrat 37℃, the enzyme showed maximal stability and optimal reaction condition at pH 8.0, respectatively. The enzyme activity decreased about 80 % and 19 % of optimal activity being retained at pH 6.0 and 9.0.

The purified enzyme exibited maximum activity at 35 ~ 40 ℃. At lower temperature, the activity was gradually decreased below 59 % of its maximal activity. The enzyme activity was highly stable at 40℃, whereas a rapid loss of enzyme activity was exibited at temperature above 50℃. Based on these results, it could be presumed that the optimal conditions on the Phl13 activity might be related with the habitable condition for aquatic environment.

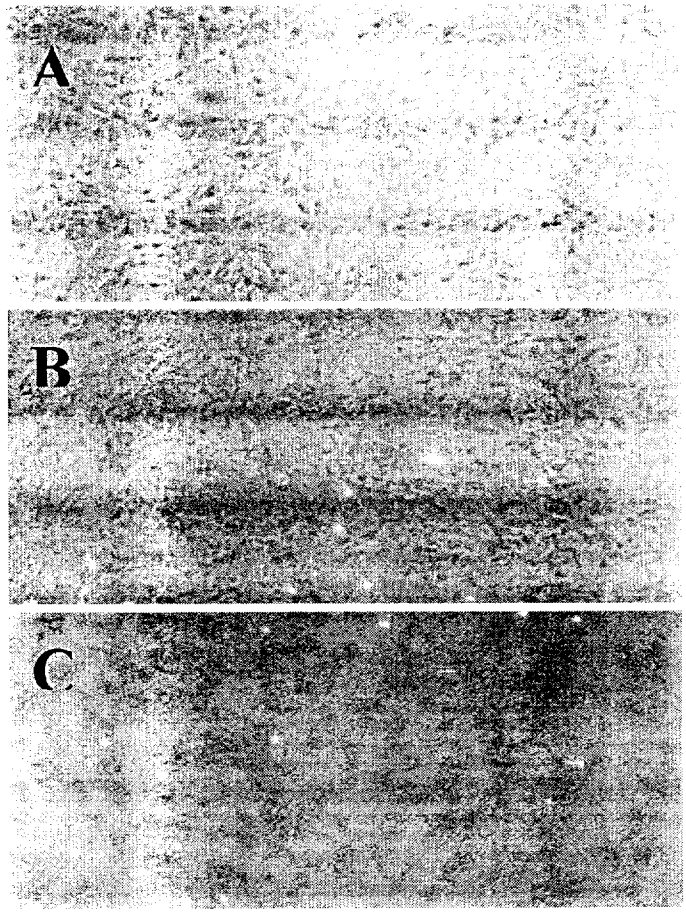

Figure 6. Effect of $rPLA_2$ activity against cultivated CHSE-214.

To observe the effect of $rPLA_2$ on cultivated fish cell, CHSE-214 cell line was grown in MEM medium on 48-well plate at 17℃ for 24hrs. Then 100 ㎕ of enzyme which was reconstituted in phosphate buffered saline (pH 7.5) was added to each well and cultivated for a day. A; No enzyme, B; 30 ㎍ of $rPLA_2$ was added, C; 50 ㎍ of $rPLA_2$ was added

There is evidence that phospholipases A2 (PLA2) play an important pathophysiological role in various inframmatory diseases, such as septic shock (17), adult respiratory distress syndrome (18), arthritis (19) and acute pancreatitis.

So far, the action of phospholipase from bacterial cell against cultured fish cell has not been published. For better understanding of the role of the PLA_2 of bacterial infection, we examined the the cytotoxic effect on CHSE-214 cell line.

As shown in Fig. 6 CHSE-214 cell line was distrupted by the enzyme and compeletely bursting of cell was taken a day with 50 μg of enzyme (fig. 6-C).

Cytotoxicity was evaluated by the dectection of released LDH from CHSE-214 cells after addtion to the purified enzyme vs. various concentration of enzyme or various reaction time.

Fifty microgram of Phl13 caused the release 88 % of the total LDH from

CHSE-214 cells, while 10 μg of Phl13 caused of only 30 % of the LDH (fig. 7-B).

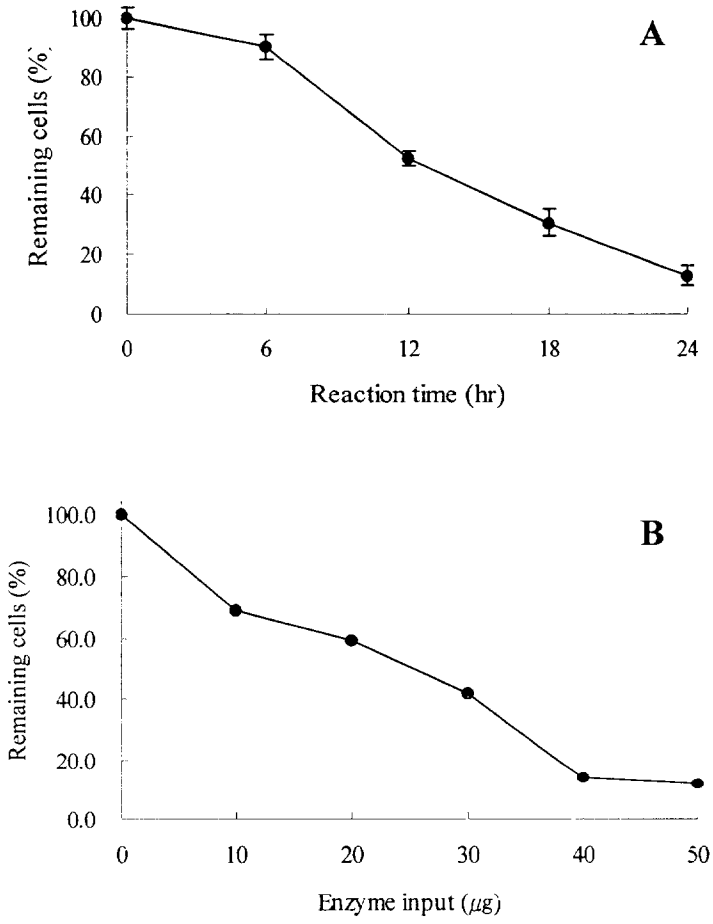

Figure 7. Cytotoxicity of rPLA$_2$ against CHSE-214 cell line

To investigate the cytotoxicity of rPLA$_2$, the released LDH was measure using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit.

When 50 μg of enzyme was added, 50 % LDH was released after 12 hr. In case of a day, the LDH was released nearly completely.

In these results, we can suggest that the phospholipase A_2 from *V. mimicus* can cause massive tissue distruction of fish cell as primary source of infection and the enzyme has important role of bacterial infection.

Summary

1. The purified enzyme was determinated the phospholipase A2 by Thin-layer chromatogram.
2. The molecular weight of purified enzyme is approximately 53kDa
3. the purified enzzme was shown typical neutral activity. Optimal temparature and pH were 35 ~ 40℃ and pH 8.0.
4. The enzyme was founded calcium ion dependency.
5. The enzyme could cause massive tissue distruction of cCHSE-214 cell line as primary source of infection

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그동안의 실험실 생활에서 많은 도움을 주신 윤수철, 김구택, 하정철, 김영옥, 박기재, 김대경, 김현국, 최선영, 이상봉, 신승렬, 진철호 선배님께도 감사드리고 특히 대학원 생활의 모든부분을 지도해주셨던 이종희 선배님께 진심으로 머리숙여 감사드립니다. 그리고 항상 힘이 되어주었던 동기들 남현, 정현 그리고 일형이와 뒤바라지 해주느라 고생많았던 후배들인 민래, 선희, 선회, 은미, 윤희, 승하, 나리와 막둥이 나영이에게도 고마운 마음을 전합니다.

언제나 큰 그늘이 되어주셨던 아버지, 어머니, 누나, 자형, 형님, 우혁, 태현과 차마 지면에 옮기지 못한 많은 고마운 분들께 큰 감사를 드립니다.

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