Full Length Research Paper

Overexpression and characterization of a thermophilic and hemolytic phospholipase of *Vibrio vulnificus* cloned in *Escherichia coli* (phospholipase of *V. vulnificus*)

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A phospholipase (PLase) gene of *Vibrio vulnificus* was cloned in *Escherichia coli* and the properties of the gene product were investigated. The PLase structural gene was composed of 1,251 bp, encoding 417 amino acids for a protein with a predicted molecular mass of 47,187 Da including a putative signal sequence. The predicted protein sequence was 87 and 82% identical to those of hemolysins from *Vibrio* spp. and that of lecithinase from *V. cholerae*, respectively. A lipid binding motif, GDSL, conserved among various PLases and lipases was also observed. Over-expression of PLase caused inclusion body formation in *E. coli*, but not that of the PLase subclone without the signal sequence (45 kDa). Purified PLase exhibited hemolytic activity on red blood cells and hydrolyzed phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and soya-lecithin mainly to fatty acid and 1,2-diacylglycerol, indicating that it was a PLase with unique catalytic activity. PLase from *V. vulnificus* had temperature and pH optimum at 45°C and 7.0 in 50 mM Tris-HCl buffer, respectively, but was quite active at temperatures up to 55°C and in a broad range of pH 5 to 10. The activity of the enzyme was enhanced by divalent cations such as Ca^{2+}, Co^{2+}, Mg^{2+}, and Mn^{2+}, but not by ethylenediaminetetraacetic acid (EDTA).

Key words: Phospholipase, *Vibrio vulnificus*, hemolytic, *VplA*.

INTRODUCTION

Vibrio vulnificus is a Gram negative marine bacterium that is pathogenic to both human and marine animals. The bacterium has become a great concern during summer time in Korea due to the increase of serious wound infections and fatal septicemia with high mortalities (>50%; Blake et al., 1979). The life cycle of *V. vulnificus* between marine environment and human hosts implies that the pathogen has delicate regulatory systems sensing and responding to environmental changes and that there is a close inter-relationship among the systems that affect the survival of the pathogen. Virulence of the bacterium is likely to be controlled by such complicate and delicate networks of various regulatory systems.

V. vulnificus produces various virulent factors such as polysaccharide capsules (Simpson et al., 1987), cytolytic hemolysins (Chang et al., 1997; Lee et al., 2004), elastolytic protease (Kothary and Kreger, 1987), siderophore (Simpson and Oliver, 1983), collagenase (Smith and Merkel, 1982), and phospholipase (PLase; Testa et al., 1984) etc. Among them, PLases are known to be associated with virulence in many pathogens (Titball, 1993; Mengaud et al., 1991; Portnoy et al., 1981; Gustafson and Tagesson, 1990). Due to their catalytic capability, PLases may also be involved in scavenging phosphate when it became a factor limiting bacterial growth. PLase C of *Pseudomonas aeruginosa* has well been characterized for its role in phosphate scavenging and pathogenesis (Gray et al., 1982). Many members of the family Vibrionaceae produce and secrete PLases,

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some of which have been shown to be hemolysins (Testa et al., 1984; McCarter and Silverman, 1987; Pal et al., 1997; Shinoda et al., 1991) and others glycerophospholipid-cholesterol acyltransferases (Shaw et al., 1994; Thornton et al., 1988). PLases from other family also have been reported to be involved in the induction of apoptosis (Kirschnek and Gulbins, 2006) or in the regulation of flagella (Schmiel et al., 2000), indicating that their expression might be under the control of multiple regulatory systems.

In previous studies, the PLase activity in the lysate of *V. vulnificus* was shown to be dependent on phosphate concentration, temperature, pH, osmotic pressure, and also subjected to catabolite repression by glucose (Oh et al., 2007). Therefore, PLase that is controlled by multiple environment factors would make a good system to investigate inter-relationship among various regulatory systems. As the first step toward such research goal, a gene encoding PLase of *V. vulnificus* was cloned based on its capability of forming hemolytic zone on a blood agar plate and over-expressed in *E. coli*. The physico-chemical properties of the purified enzyme were also characterized in this study.

MATERIALS AND METHODS

Bacteria, plasmids and media

V. vulnificus ATCC29307 was cultured either in Luria-Bertani broth (LB; 0.5% yeast extract, 1% bacto tryptone , Difco, USA) containing 2.5% NaCl or heart infusion broth (beef heart infusion 1.0%, tryptose 1.0%, Difco, USA) containing 2.5% NaCl at 37°C with shaking at 200 rpm. *E. coli* MC1061 [*hsdR, merB, araD*139Δ(*araABC- leu*)7679, Δ*lacX*79, *galU, galK, rpsL, thi*] and pUC119 was used for cloning the PLase gene; *E. coli* BL21 (DE) (*cIts*857, *ind*1, *sam*7, *nin*5, *lac*UV5-T7 gene1) and pET22b(+) for over-expression of the clone. The *E. coli* strains were cultured in LB broth and ampicillin (100 µg/ml) was added when it was necessary.

Cloning of the PLase gene

The PLase gene of *V. vulnificus* ATCC29307 was cloned in *E. coli* MC1061 by shotgun cloning. The *V. vulnificus* genomic DNA was digested partially with either *Sau*3AI or *Hind*III and the DNA fragments in the range of 2.0 to 9.4 kb were isolated from agarose gel by electro-blotting onto DEAE membrane. Isolated DNA fragments were ligated to pUC119 at the *Bam*H1 or *Hind*III site to construct a sub-genomic DNA library and the resulting library was transformed into *E. coli* MC1061. Putative PLase clones were isolated based on their hemolytic activity on sheep blood agar plates. Nucleotide sequence analysis of the insert on the positive clone (pHPLC3) was determined by the dideoxy chain termination sequencing method using an AB1377 PRISM DNA sequencer (Perkin-Elmer, USA). Nucleotide sequence was analyzed using Lasergene (DNASTAR Inc., USA) and putative signal sequence was predicted using SignalP 3.0 Server (Technical University of Denmark).

Over-expression of the PLase gene

The PLase structural gene (*VplA*) cloned on pHPLC3 was subcloned onto pET22b(+) for over-expression in *E. coli*. The structural gene was amplified by polymerase chain reaction (PCR) using two primers, VPLF2 (5′-GAAGATAGT<u>CATATG</u>AAGAAGATA-3′) and VPLR1 (5′-GTTGAC<u>CTCGAG</u>ATTAAAGCGTTG-3′), carrying a *Nde*I and a *Xho*I site (underlined), respectively. In order to remove the signal sequence from the gene another primer set, VPLF3 (5′-AAA<u>CATATG</u>GATGAGCCAGCTCTCTCTC C-3′) and VPL4 (5′-AAA<u>GTCGAC</u>GCTAAAAATTA AAGCGTTGC-3′), carrying an *Nde*I and a *Sal*I site (underlined), respectively, was used. The 1.25 kb long PCR product was digested with *Nde*I and *Xho*I (or *Sal*I) and ligated to the corresponding sites on pET22b(+) to place the *VplA* gene under the control of the T7 promoter and to tag the 3′ end of the gene with the sequence encoding 6xHis. Putative subclones were screened for their capability of forming clear zone around the colony by producing hemolysin and/or PLase activities upon induction with 0.5 mM Isopropyl-ß-D-thiogalactopyranoside (IPTG) on a blood agar plate and a lecithin agar plate containing 1% lecithin, respectively. The subclone of PLase with or without the signal sequence was designated as pVPL10 and pVPL11, respectively.

Purification of recombinant PLase

PLase was over-expressed in *E. coli* BL21 (DE3) carrying one of the subclones, pVPL10 or pVPL11 from the T7 promoter by inducing with 0.5 mM IPTG during early exponential growth phase in LB broth containing 100 µg/ml of ampicillin. However, PLase with the signal sequence from pVPL10 was found in the insoluble fraction as inclusion body and the enzyme was refolded and purified according to Wingfield et al. (1995). The cell extract in the His-Tag binding buffer containing 8 M urea was subjected to chromatography using a Ni-NTA column (Qiagen, Germany) after sonication in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF) and filtration of the supernatant through 3M paper (Whatman, USA). The enzyme bound to the resin was renaturated by sequential wash and incubation with renaturation buffers [100 mM NaCl, 20% glycerol, 20 mM Tris-HCl (pH 7.4)] containing 6, 4, 2, 1, and 0 M urea. Finally, the enzyme was washed with 20 mM Tris-HCl (pH 8.0) and eluted with 20 mM Tris-HCl (pH 8.0) containing 100 mM imidazole. PLase without the signal sequence from pVPL11 was purified from the supernatant of the cell lysate using regular buffers for Ni-NTA chromatography. Protein concentration was determined by the Bradford method (Bradford, 1976) and the purified enzyme was analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gel.

Enzyme assay

PLase activity was determined using an artificial substrate, *p*-nitrophenylphosphorylcholine (*p*NPPC; Sigma Co., USA; Kurioka and Matsuda, 1976). Enzyme solution (200 ng/100 µL) was mixed with pre-warmed 50 mM *p*NPPC (60 µL) dissolved in dH$_2$O and 2 M Tris-HCl (pH 7.0) containing 1 mM ZnCl$_2$ (140 µL). The reaction mixture was incubated at 37°C for 10 min or until yellow color was developed. The reaction was stopped by boiling for 3 min and mixing with 800 µL of ethanol and 200 µL of 2 M Tris-HCl (pH 7.0). Optical density of the supernatant was measured at 420 nm. One PLase unit was defined as the amount of enzyme that released 1 mmole of *p*-nitrophenol at 37°C for 1 h (Kuroshima and Hayano, 1982). Moreover, hemolytic activity of PLase was analyzed using sheep red blood cells according to Lee et al. (2002a) or on sheep blood agar plates for the capability of the clone forming clear zone. Lecithinase activity of PLase was examined using agar plates containing 1% lecithin based on the formation of clear zone around colonies (Sibinovic et al., 1971).
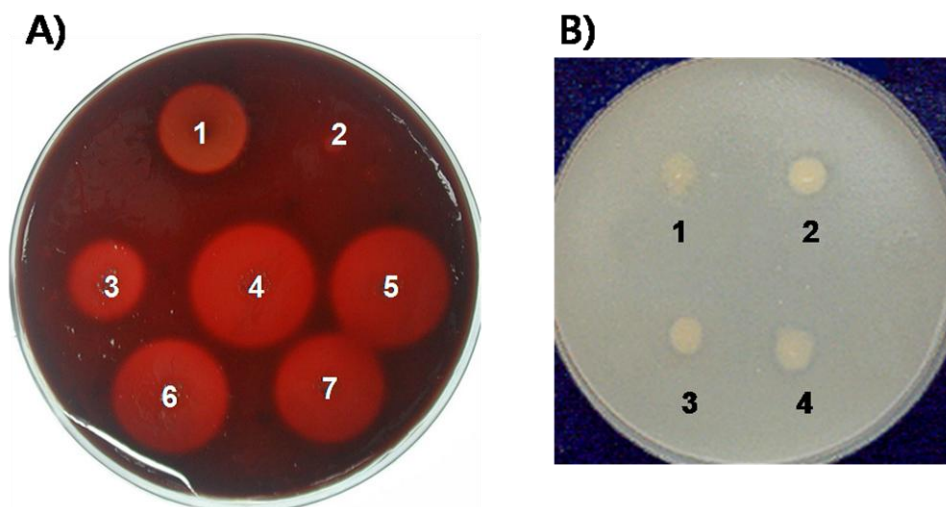
Figure 1. Agar plate assay of hemolytic (A) and lecithinase (B) activities of the *VplA* clones. Panel A represents a blood agar plate inoculated with *V. vulnificus* 29307 (1), *E. coli* BL21 (2), *E. coli* MC1061 harboring pHPLC3 (3), and *E. coli* BL21 harboring pVPL10 (4 to 7). Panel B represents a lecithin (egg yolk) agar plate inoculated with *E. coli* BL21 (1) and *E. coli* BL21 harboring pVPL10 (2 to 4).

Thin layer chromatography (TLC)

In other to assay phospholipid hydrolyzing activity of the cloned PLase, TLC was carried out as follows. Purified PLase was mixed with various phospholipids (0.5%; Sigma Co., USA.) homogenized in 50 mM Tris-maleic acid-NaOH buffer (pH 7.0) and incubated at 37°C for 1 h. The reaction was terminated by adding a mixture of methanol and chloroform (1: 2). The reaction mixture was spotted on a silica gel plate (Whatman, England) and separated using a mixture of n-hexane: diethyl ether: acetic acid (80: 20: 4). The plate was developed by dipping into the mixture of 5% H_2SO_4 and methanol.

RESULTS AND DISCUSSION

Screening of the sub-genomic DNA libraries for the putative PLase gene

From the two sub-genomic DNA libraries of *V. vulnificus*, several putative clones were isolated based on their capability of hemolyzing red blood cells. Five hemolytic clones from the *Sau*3AI sub-genomic DNA library (pSPLC1-5) had inserts of different lengths but with a common open reading frame (ORF) for the zinc metalloprotease gene of *V. vulnificus* (Chuang et al., 1997). The hemolytic zone of the clones on the blood agar plate was hazy and small. Three hemolytic clones from the *Hind*III sub-genomic DNA library (pHPLC1-3) carried an identical insert of 3.5 kb long (Supplementary data). *E. coli* MC1061 carrying pHPLC3 formed a clearer hemolytic zone than *V. vulnificus* (Figure 1a) and the metalloprotease clones (data not shown) on blood agar plates. They also showed small clear zone around the colonies on a lecithin agar plate (Figure 1b). The coding

region for the hemolytic and *p*NPPC hydrolyzing activities was defined to the 2.5 kb *Hind*III fragment by deletion mapping (data not shown). Therefore, nucleotide sequence analysis of the DNA fragment was carried out to determine the primary structure of the PLase gene.

Primary structure of the PLase gene

Nucleotide sequence analysis of the insert revealed an open reading frame (ORF) of 417 amino acid residues for a protein with a predicted molecular mass of 47,187 Da (Figure 2). The ORF was preceded by a putative ribosome binding site and potential promoter sequences at the 5' upstream region and followed by an inverted repeat for transcriptional termination at the 3' downstream. The gene was designated as *VplA*. The first 19 amino acids of the ORF with a stretch of hydrophobic residues were typical of a signal sequence for secretion, correlating well with the secreted nature of PLase in *V. vulnificus*.

A homology search of the GenBank/EMBL database revealed that the ORF shared homology with various PLases and hemolysins. The ORF was 99% identical to the PLase encoded by a gene (Gene ID 1181413, VV2_1483) on chromosome II of *V. vulnificus* CMCP6 strain. It also shared 87% identity with hemolysin of *Vibrio harveyi* (Zhang et al., 2001), 83% with thermolabile hemolysin of *Vibrio parahaemolyticus* (Taniguchi et al., 1986), 82% with lecithinase of *V. cholerae* (Fiore et al., 1997), and 69% with hemolysin of *Vibrio campbellii* (Luis and Hedreyda, 2006), and 64% with PLase A of *Vibrio mimicus* (Kang et al., 1998; Lee et al., 2002b) at the amino
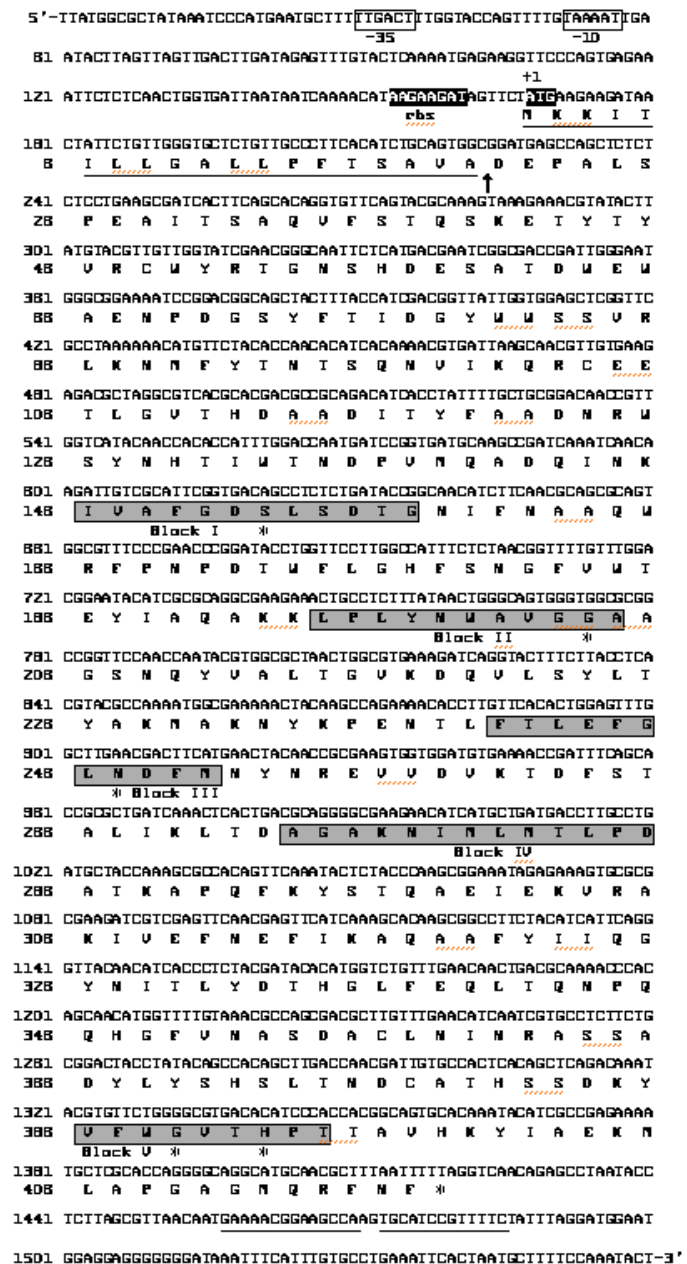
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5'-TTATGGCGCTATAAATCCCATGAATGCTTTTTGACTTTGGTACCAGTTTTGTAAAATTGA
                                    -35                   -10
81 ATACTTAGTTAGTTGACTTGATAGAGTTTTGTACTCAAAATGAGAAGGTTCCCAGTGAGAA
                                                         +1
121 ATTCTCTCAACTGGTGATTAATAATCAAAACATAAGAAGATAGTTCTATGAAGAAGATAA
                                        rbs              M  K  K  I  T
181 CTATTCTGTTGGGTGCTCTGTTGCCCTTCACATCTGCAGTGGCGGATGAACCCGCTCTCT
 8    I  L  L  G  A  L  L  P  F  T  S  A  V  A  D  E  P  A  L  S
241 CTCCTGAAGCGATCACTTCAGCACAGGTGTTCAGTACGCAAAGTAAAGAAACGTATACTT
 28   P  E  A  I  T  S  A  Q  V  F  S  T  Q  S  K  E  T  Y  T  Y
301 ATGTACGTTGTTGGTATCGAACGGGCAATTCTCATGACGAATCGGCGACCGATTGGGAAT
 48   V  R  C  W  Y  R  T  G  N  S  H  D  E  S  A  T  D  W  E  W
361 GGGCGGAAAATCCGGACGGCAGCTACTTTACCATCGACGGGTTATTGGTGGAGCTCGGTTC
 88   A  E  N  P  D  G  S  Y  F  T  I  D  G  Y  W  W  S  S  V  R
421 GCCTAAAAAAACATGTTCTACACCAACACATCACAAACGTGATTAAGCAACGTTGTGAAG
 88   L  K  N  M  F  Y  T  N  T  S  Q  N  V  I  K  Q  R  C  E  E
481 AGACGCTAGGCGTCACGCACGACGCGCAGACATCACCTATTTGCTGCGGACAACCGTT
 108  T  L  G  V  T  H  D  A  A  D  I  T  Y  F  A  A  D  N  R  W
541 GGTCATACAACCACACCATTGGACCAATGATCCGGTGATGCAAGCCGATCAAATCAACA
 128  S  Y  N  H  T  I  W  T  N  D  P  V  N  Q  A  D  Q  I  N  K
801 AGATTGTCGCATTCGGTGACAGCCTCTCTGATACCGGCAACATCTTCAACGCCAGCGCAGT
 148  I  V  A  F  G  D  S  L  S  D  T  G  N  I  F  N  A  A  Q  W
                      Block I       *
881 GGCGTTTCCCGAACCCGGATACCTGGTTCCTTGGCCATTTCTCTAACGGTTTTGTTTGGA
 188  R  F  P  N  P  D  T  W  F  L  G  H  F  S  N  G  F  V  W  T
721 CGGAATACATCGCGCAGGCGAAGAAACTGCCCTCTTTATAACTGGGCAGTGGGTGGCCGG
 188  E  Y  I  A  Q  A  K  K  L  P  L  Y  N  W  A  V  G  G  A
                              Block II             *
781 CCGGTTCCAACCAATACGTGGCGCTAACTGGCGTGAAAGATCAGGTACTTTCTTACCTCA
 208  G  S  N  Q  Y  V  A  L  T  G  V  K  D  Q  V  L  S  Y  L  T
841 CGTACGCCAAAATGGCGAAAAACTACAAGCCAGAAAACACCTTGTTCACACTGGAGTTTG
 228  Y  A  K  M  A  K  N  Y  K  P  E  N  T  L  F  T  L  E  F  G
901 GCTTGAACGACTTCATGAACTACAACCGCGAAGTGGTGGATGTGAAAACCGATTTCAGCA
 248  L  N  D  F  M  N  Y  N  R  E  V  V  D  V  K  T  D  F  S  T
                 Block III
981 CCGCGCTGATCAAACTCACTGACGCGAGGGGCGAAGAACATCATGCTGATGACCTTGCCTG
 288  A  L  I  K  L  T  D  A  G  A  K  N  I  L  N  T  L  P  D
                            Block IV
1021 ATGCTACCAAAGCGGCCACAGTTCAAATACTCTACCCAAGCGGGAAATAGAGAAAGTGCGCG
 288  A  T  K  A  P  Q  F  K  Y  S  T  Q  A  E  I  E  K  V  R  A
1081 CGAAGATCGTCGAGTTCAACGAGTTCATCAAAGCACAAGCGGCCTTCTACATCATTCAGG
 308  K  I  V  E  F  N  E  F  I  K  A  Q  A  A  F  Y  I  I  Q  G
1141 GTTACAACATCACCCTCTACGATACACATGGTCTGTTTGAACAACTGACGCAAAACCCAC
 328  Y  N  I  L  Y  D  T  H  G  L  F  E  Q  L  T  Q  N  P  Q
1201 AGCAACATGGTTTTGTAAACGCCCAGCGACGCTTGTTTGAACATCAATCGTGCCTCTTCTG
 348  Q  H  G  F  V  N  A  S  D  A  C  L  N  I  N  R  A  S  S  A
1281 CGGACTACCTATACAGCCACAGCTTGACCAACGATTGTGCCACTCACAGCTCAGACAAAT
 388  D  Y  L  Y  S  H  S  L  T  N  D  C  A  T  H  S  S  D  K  Y
1321 ACGTGTTCTGGGGCGTGACACATCCCACCACGGCAGTGCACAAATACATCGCCGAGAAA
 388  V  F  W  G  V  T  H  P  T  I  A  V  H  K  Y  I  A  E  K  N
                 Block V     *      *
1381 TGCTCGCACCAGGGGCAGGCATGCAACGCTTTAATTTTTAGGTCAACAGAGCCTAATACCC
 408  L  A  P  G  A  G  N  Q  R  F  N  F  *
1441 TCTTAGCGTTAACAATGAAAACGGAAGCCAAGTGCATCCGTTTTCTATTTAGGATGGAAT

1501 GGAGGAGGGGGGGATAAATTTCATTTGTGCCTGAAATTCACTAATGCTTTTCCAAATACT-3'
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Figure 2. DNA and predicted amino acid sequences of the *VpIA* gene and its flanking regions. The sequences of putative promoters are boxed and ribosome binding sites (rbs) are shown in a black box. A transcription terminator-like sequence was underlined. Putative signal sequence was also underlined with a cleavage site indicated by an arrow. The conserved blocks for lipolytic enzymes are shown in shaded boxes and the residues constituting an active site were marked with asterisks.

acid sequence level. The ORF carried conserved sequences (Block I-V, Figure 3) frequently observed among various lipases/acyltransferases in their active sites (Thornton et al., 1988; Brumlik and Buckley, 1996). The catalytic signature for lipolytic enzymes, GDSL, was observed in Block I and the residues constituting the

active site are all conserved except one in Block V, in which a D residue was substituted with a G at residue 386 (Figure 3). The significance of the substitution is yet to be elucidated. Based on the results of the comparative analysis, the cloned DNA fragment was likely to carry a putative hemolytic PLase gene (*VpIA*). Southern blot analysis confirmed that the insert was originated from the chromosomal DNA of *V. vulnificus* (data not shown).

Expression of the *VpIA* gene in *E. coli*

The *VpIA* gene cloned on pHPLC3 was subcloned onto an expression vector, pET22b(+), since it expressed the PLase activity poorly in *E. coli* when *p*NPPC was used as the substrate (Supplementary data). The structural *VpIA* gene amplified by PCR was placed under the control of T7 promoter and fused to six histidine residues at the carboxyl terminus on the expression vector. The resulting recombinant DNA was designated as pVPL10 and *E. coli* carrying the subclone formed even clearer and larger zones on both blood and lecithin agar plates than those carrying pHPLC3 (Figure 1). However, purification of *VpIA* from the supernatant of the *E. coli* cell lysate using a Ni-NTA column was not successful, since the protein induced by the addition of IPTG was precipitated forming inclusion body (Figure 4a, lanes 3 and 4). Therefore, in order to recover active *VpIA* from the insoluble fraction of the cell lysate, the proteins in the precipitate was solubilized in the presence of 8 M urea (Figure 4a, lane 5) and mixed with Ni-NTA agarose before being packed into a column. Then, the enzyme was refolded before eluted by sequential removal of urea as described above. Renatured *VpIA* was present in the soluble fraction and purified to an apparent homogeneity (Figure 4a, lane 6).

Inclusion body formation of PLases that were overexpressed in heterologous systems has been reported previously for PLase C from *Bacillus cereus* (Tan et al., 1997), YpIA from *Yersinia enterocolitica* (Hatic et al., 2002), and human pancreatic PLase (Han et al., 1997). However, when *VpIA* was expressed without the signal sequence, the mature protein (ca. 45 kDa) did not form inclusion body and was purified from the soluble fraction of the cell lysate in one step using the Ni-NTA column (Figure 4b). The two PLases expressed from pVPL10 and pVPL11 showed identical enzymatic properties.

Substrate specificity of *VpIA*

Substrate specificity of purified VpIA was analyzed using 0.5% (w/v) phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and soyalecithin homogenized in 50 mM Tris-maleic acid-NaOH buffer (pH 7.0) at 37℃ for 1 h. TLC analysis of apolar lipids in the reaction mixtures indicated that the

	Accession Number	Block I		Block II		Block III		Block IV		Block V
Vibrio vulnificus	vv2_1483	IVAFGDSLSDTG	(36)	LPLYNWAVGGAA	(34)	FTLEFGLNDFM	(22)	AGAKNIMLMTLPD	(101)	FWGVTHPT
Aeromonas hydrophila	P10480	IVMFGDSLSDTG	(37)	LTIANEAEGGPT	(37)	VILWVGANDYL	(26)	NGAKQILLFNLPD	(128)	FWDQVHPT
Vibrio parahaemolyticus	Q99289	VVALGDSLSDTG	(36)	LPLYNWAVGGAA	(34)	FTLEFGLNDFM	(22)	AGAKNFMLMTLPD	(101)	FWNVTHPT
Xenorhabdus uminescens	P40601	LYVFGDSLSDGG	(34)	KGGTNYAAGGAT	(31)	YVHWIGGNDVD	(29)	AGAGLVIVPTVPD	(155)	FADDFHPT
Pseudomonas putida	P40604	MIVFGDSLSDAG	(48)	PDGNNWAVGGYT	(45)	YYLTGGGNDFL	(26)	GGARYIMVWLLPD	(94)	FNDLVHPT
Arabidopsis thaliana	P40602	VFFFGDSVFDTG	(62)	LTGVSFASGGAG	(55)	AIVVGGSNDLI	(35)	YGARRIGVIGTPP	(101)	FWDGVHPT
Brassica napus	P40603	VFFFGDSIFDTG	(68)	LTGVSFASGGAG	(45)	AIVVAGSNDLI	(35)	YGARRIGVIGTPP	(101)	FWDGLHPS
Escherichia coli	P29679	LLILGDSLS-AG	(27)	ISGDTSQQGLAR	(12)	VLVELGGNDGL	(36)	YGRRYNEAFSAIY	(26)	QDDGIHPN
Vibrio mimicus	Q07792	LLVLGDSLS-AG	(30)	ISGDTTGNGLAR	(12)	VLIELGANDGL	(36)	YGKRYSDMFYDIY	(26)	MDDGLHPK

Figure 3. Comparison of conserved blocks among various lipolytic enzymes harboring the GDSL motif. Amino acid residues that are conserved in at least six of the enzymes are presented in shade and those constituting the catalytic sites are underlined. Numbers in parentheses represent the spacing between the conserved blocks (Brumlik and Buckley, 1996).
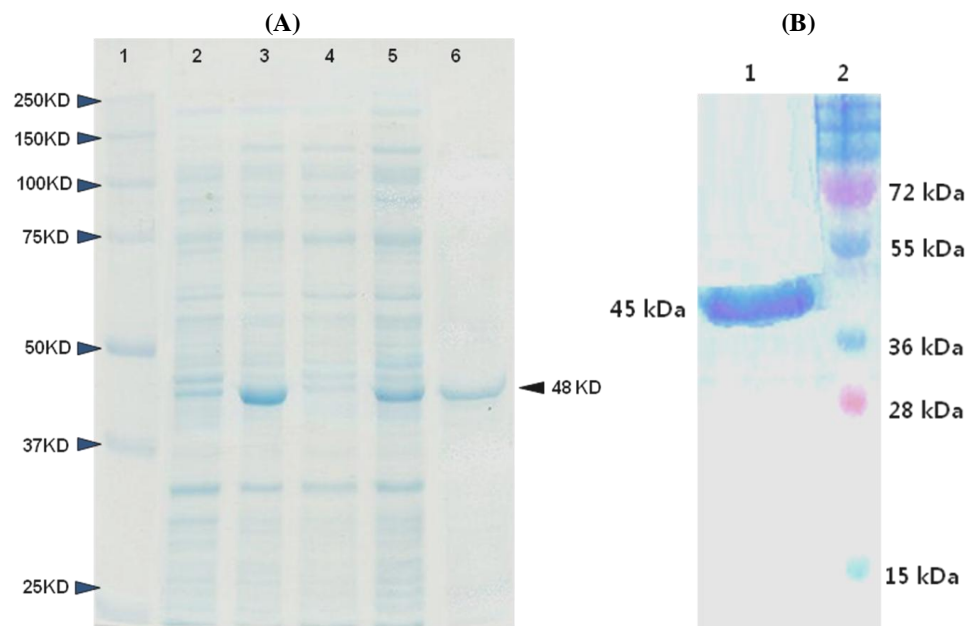

Figure 4. SDS-PAGE analysis of the purified VplA enzymes from *E. coli* BL21 harboring pVPL10 (A) or pVPL11 (B). Proteins were subjected to 10% SDS-polyacrylamide gel and visualized by staining Coomassie-blue. A). Lane 1 was loaded with standard markers; lane 2, *E. coli* cell lysate without induction; lane 3, *E. coli* cell lysate that had been induced by 0.5 mM IPTG; lane 4, supernatant of the induced *E. coli* cell lysate; lane 5, denaturated VplA using 8 M urea; lane 6, purified VplA using a Ni-NTA column. B) Lane 1 was loaded with purified VplA without the signal sequence; lane 2, standard markers.
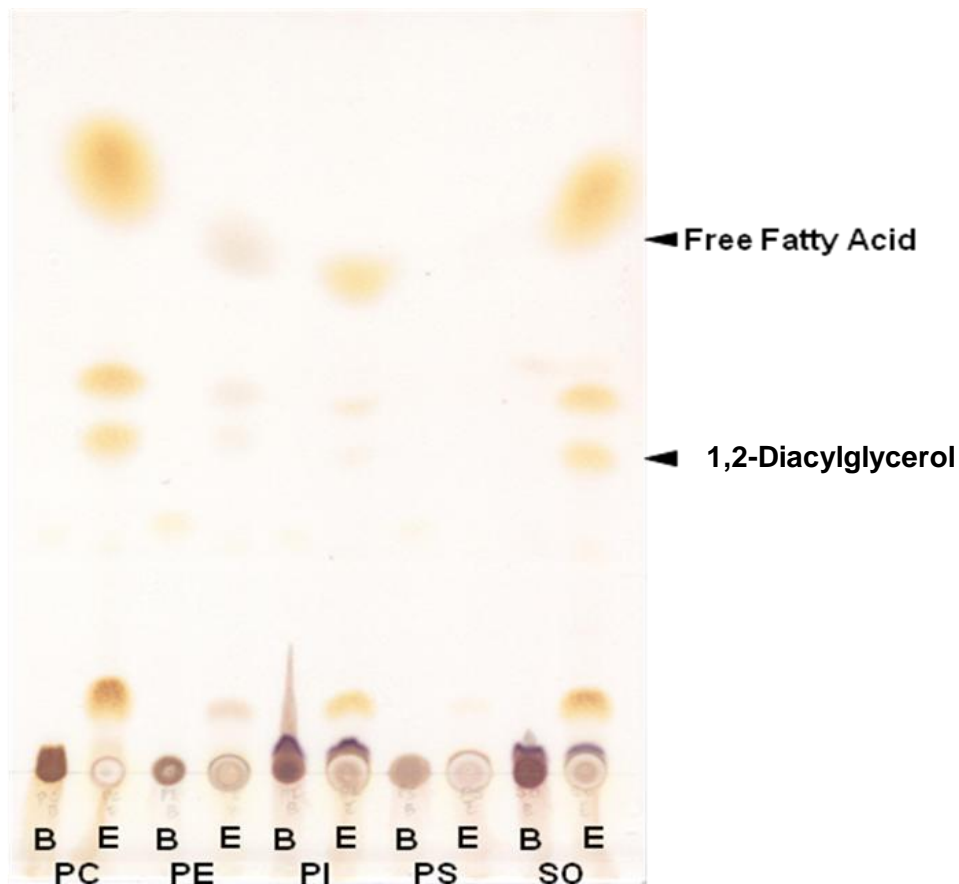
Figure 5. Substrate specificity of VplA analyzed by TLC. VplA was incubated with 0.5% of each substrate homogenized in 50 mM Tris-HCl (pH 7.0) at 37°C for 30 min and the reaction mixture was subjected to TLC. PC represents phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SO, soya-lecithin. Lanes B were spotted with reactions carried out in the absence of VplA; lanes E, those carried out in the presence of the enzyme.

substrates were hydrolyzed mainly to free fatty acids at various degrees, suggesting that VplA might be a PLase A (Figure 5; Flieger et al., 2000). PLase A has been known to hydrolyze phospholipids into lysophosphatidylcholine and fatty acid (Dessen, 2000). However, significant amounts of 1,2-diacylglycerol were also released, implying that it also had PLase C activity (Flieger et al., 2000). VplA preferentially hydrolyzed various phospholipids in the order of phosphatidylcholine > soya-lecithin > phosphatidylinositol phosphatidylethanolamine > phosphatidylserine. VplA could also hydrolyze *p*NPPC, an artificial substrate that was specific for PLase C (Figure 6) (Kuroshima and Hayano, 1982). The results, therefore, indicated that VplA seemed to be a unique lecithin-dependent PLase A with PLase C activity.

However, VplA did not share significant homology with any PLase C that has been documented. PLase A has been reported from various pathogenic bacteria such as *Mycobacterium tuberculosis* (Parker et al., 2007),

Legionella pneumophila (Broich et al., 2006) and *P. aeruginosa* (Kirschnek and Gulbins, 2006) as a potent virulence factor. Recently, Koo et al. (2007) reported that a hemolytic PLase A of *V. vulnificus* could have a crucial role in the pathogenesis of the bacterial infection using a cytolysin-negative mutant strain, CVD707. However, it is not clear yet whether the PLase A enzyme they reported is a protein identical to VplA, since they did not identify the gene responsible for the activity.

Enzymatic properties of *VplA*

Temperature and pH optima of purified VplA were investigated using 50 mM *p*NPPC as the substrate. VplA had the highest activity at 45°C with a broad temperature range for activity between 25 and 80°C in 50 mM Tris-HCl buffer (pH 7.0; Figure 6a). It is interesting to note that PLase from *V. vulnificus*, a mesophile, is a thermophilic
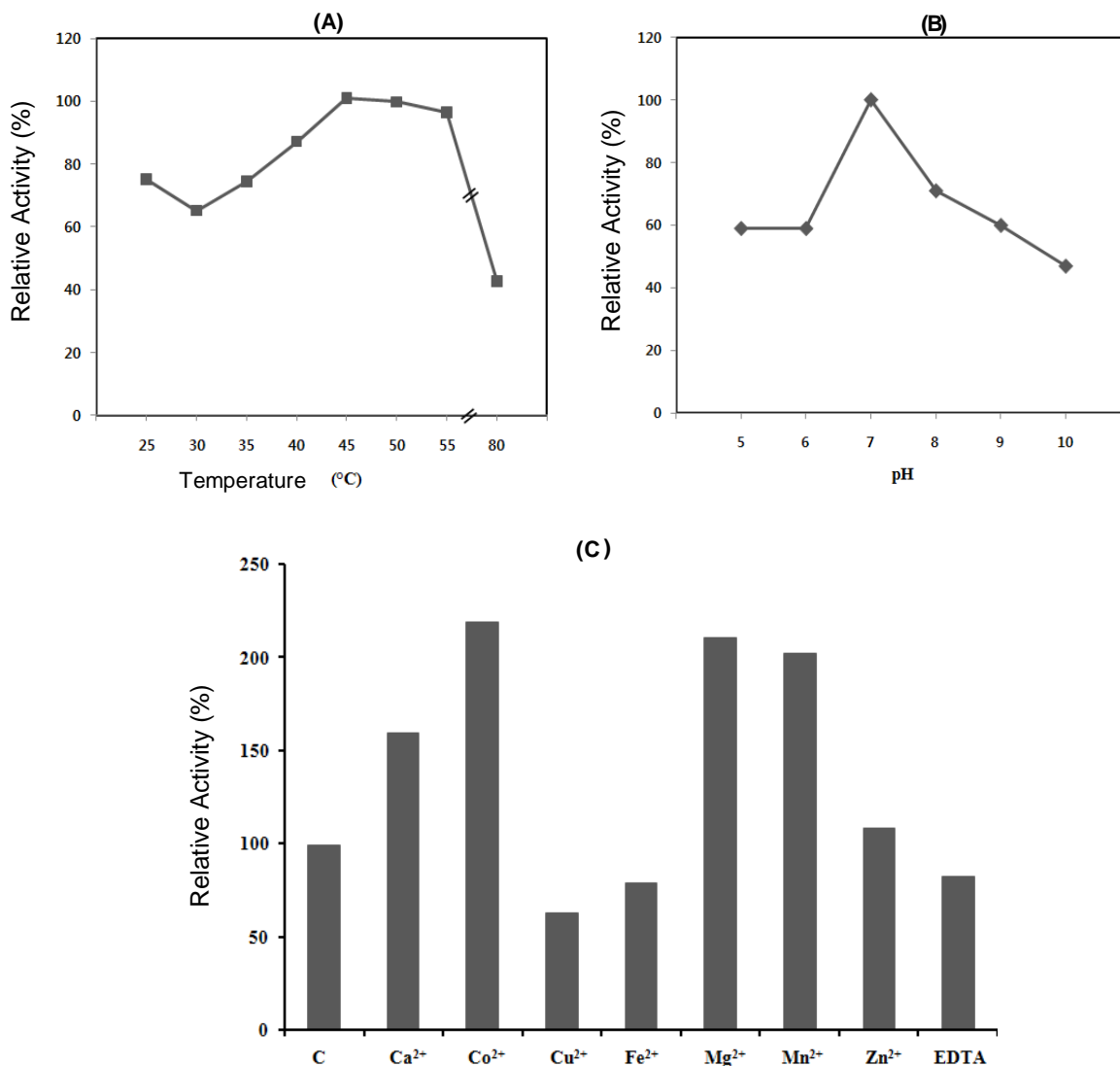
Figure 6. Effects of various factors on the activity of VplA. (A) Effect of temperature on the enzyme activity was analyzed using 50 mM pNPPC in 50 mM Tris-HCl (pH 7.0) for 30 min. (B) Effect of pH on the activity was analyzed using 50 mM pNPPC in 50 mM sodium acetate for pH 5.0 and 6.0; 50 mM Tris-HCl for pH 7.0-9.0; 50 mM CHES for pH 10.0. The reactions were carried out at 45°C for 30 min. (C) Effect of various divalent cations and EDTA (5 mM) on the activity was analyzed using 50 mM pNPPC in 50 mM Tris-HCl (pH 7.0) at 45°C for 30 min.

enzyme. The enzyme showed enzyme activity between pH 5 to 10, being most active at neutral pH in 50 mM Tris-HCl buffer (Figure 6b) at 45°C. These results indicated that VplA has quite stable structure for maintaining the activity under various conditions. To our knowledge, PLase A that was active at broad high temperature and pH ranges has not been reported from *Vibrio* spp. The effect of various metal ions on the activity of VplA was also tested in 50 mM Tris-HCl buffer (pH 7.0) at 45°C. The activity of the enzyme was enhanced by divalent cations such as Ca^{2+}, Co^{2+}, Mg^{2+}, and Mn^{2+}; inhibited severely by Cu^{2+} and Fe^{2+}; but not affected significantly by either Zn^{2+} or EDTA (Figure 6c).

Previously, extracellular PLase A_2 and Lyso-PLase of *V. vulnificus* E4125 have been characterized by Testa et al. (1984). However, the physico-chemical properties of PLase A_2 were different from those of VplA in that it was larger in molecular mass (≥80 kDa), heat-labile, and active at pH 5.0 to 5.5. The enzyme was not affected by Ca^{2+}, either. Lyso-PLase was heat-resistant with molecular mass of larger than 80 kDa. Both PLases reported by the group did not seem to be identical to VplA. Moreover, the activity of hemolytic and membranous PLase A reported by Koo et al. (2007) was dependent on Ca^{2+} and exhibited the highest activity at 10 mM. From all these results, *V. vulnificus* was likely to

possess multiple or strain specific PLases with various activities and functions. Further analysis constructing the *VplA* mutant might prove the existence of multiple PLases in the pathogenic bacterium.

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