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Molecular Cloning and Characterization of Alkaliphilic Phospholipase B (VFP58) from *Vibrio fluvialis*

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Abstract *Vibrio fluvialis*, an enteropathogenic bacterium, produces a phospholipase which is thought to be an important factor in the pathogenesis of disease. In this study, the phospholipase gene (*vfp*) was identified from *V. fluvialis* (KCTC 2473) and its sequence was determined. The entire open reading frame was composed of 1,689 nucleotides and 563 amino acids. The phospholipase gene (*vfp*) was overexpressed in *Escherichia coli* as a his-tag fused protein. This recombinant protein (rVFP58) was solubilized with 6 M urea and purified by Ni-NTA affinity chromatography. The action mode of rVFP58 was determined by TLC and GC-MS, and it showed phospholipase B activity, which had both phospholipase A and lysophospholipase activities. The rVFP58 showed a maximum activity at pH around 9–10 and temperature of about 40°C, and it was stable under alkaline condition over pH 9. The cytotoxicity of rVFP58 was evaluated, using a fish cell line, CHSE-214, and was found to cause significant cell death after 14 h of exposure to 250 μg of the protein.

Key words: *Vibrio fluvialis*, alkaliphilic phospholipase B, cytotoxicity

Vibrio fluvialis is a Gram-negative pathogenic bacterium [19],, which causes intestinal diseases such as watery diarrhea, abdominal pain, fever, and occasionally, bloody excrement. This strain was isolated from aquatic environments and humans with severe diarrheal disease. Pathogenicity of *V. fluvialis* is known to be due to hemolysin [15, 17], cytolysin [23, 24], protease [2], cytotoxin [24], enterotoxin-like substance [29, 34], and phospholipase [40]. Specifically, hydrolytic enzymes such as proteases, lipase [3] and phospholipases play important roles in the pathogenesis for host tissue invasion.

Phospholipase is an enzyme that catalyzes the hydrolysis of phospholipids. There are 5 different types of phospholipases; phospholipase A_1 (PLA_1) hydrolyzes the acyl group of glycerophospholipid attached to the *sn*-1 position, while phospholipase A_2 (PLA_2) hydrolyzes the acyl group attached to the *sn*-2 position. Phospholipase B (PLB) catalyzes the hydrolysis of acyl group of glycerophospholipid at the *sn*-1 and *sn*-2 positions of phospholipid and then displays both PLA_1 or PLA_2 and lysophospholipase activities. Phospholipase C (PLC) catalyzes the deacylation of glycerophospholipid at the *sn*-3 position, and phospholipase D (PLD) hydrolyzes phospholipid into a water-soluble head group and phosphatidic acid of glycerophopholipid. To date, it has been reported that phospholipases contribute to bacterial escape from host cells after intracellular multiplication [5, 11], the destruction of macrophages and epithelial cells [1, 22, 28, 41], the generation of signal transducers such as lysophosphatidylcholine [33], and the destruction of lung surfactant [13, 16].

PLB was first identified from *Mycobacterium phlei* [30]. Thereafter, PLB enzymes have been reported in microorganisms such as *Fusobacterium necrophorum, Candida albicans*, and *Saccharomyces cerevisiae* [12, 25, 39]. Recently, it has been known that PLB plays an important role in the pathogenesis [28, 41]. Destruction of phospholipids by PLB and subsequent change of membrane constituents are regarded to be major virulence mechanisms in infection. Many studies also showed that PLB is involved in survival, growth, and replication within macrophages [8], and in the destruction of lung tissue and production of eicosanoids, which modulate phagocytic activity [31].

Although we regard PLB as important pathogenic factor, PLB from *Vibrio* sp. has not yet been studied in detail. In this study, we identified a gene from *V. fluvialis* with PLB

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activity and overexpressed it in *E. coli* to investigate its biochemical characteristics. We also describe herein that this protein has a high cytotoxic activity on CHSE-cells, originated from chinook salmon.

MATERALS AND METHODS

Reagents

DNA restriction enzymes were purchased from Promega Co. (U.S.A.) and media for cultivation of bacteria were from Difco (U.S.A.). L-α-phosphatidylcholine (PC, P-5394), L-α-lysophosphatidylcholine (LPC, L-4129), 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (P-9648), 1-O-hexadecyl-2-[(cis)-9-octadecanoyl]-rac-glycero-3-phosphocholine (P-6159), 4-nitro-3-(octanoyloxy)-benzoic acid (N-1646), *p*-nitrophenylphosphorylcholine (NPPC, N-5879), and *Clostridium perfringenes* PLC were obtained from Sigma (U.S.A.), and silica GEL-60 TLC plates and organic solvents were from Merck (Germany). The rest of the reagents were purchased from Sigma (U.S.A.) and were of the highest purity.

Bacterial Strains, Plasmids, and Culture Condition

V. fluvialis strain was obtained from Korea Collection for Type Cultures (KCTC 2473). The bacterial strains and plasmids used in this study are described in Table 1. *V. fluvialis* was grown in brain heart infusion (BHI) medium, and *E. coli* was incubated in Luria-Bertani (LB) medium at 37°C.

Cloning of the Phospholipase Gene from *V. fluvialis*

The cloning vector pGEM-4Z was used to generate genomic DNA library from *V. fluvialis*. To construct the DNA library, chromosomal DNA was isolated from *V. fluvialis* and completely digested with *Hin*dIII. The resulting fragments were inserted into the multiple cloning site of pGEM-4Z. Ligation was performed overnight by T4 DNA ligase at 16°C and introduced into competent *E. coli* XL1-Blue cells prepared with 100 mM $CaCl_2$. To screen for phospholipase gene, the colonies which have phospholipase activity on yolk plate were selected. One clone, containing a 5.8-kb insert, showed phospholipase activity on yolk plate and was named pVFP25, and the sequence of this plasmid was determined.

Construction of the Expression System

PCR was performed by using the pVFP25 plasmid DNA with specific primers to amplify the open reading frame (ORF) of the phospholipase gene. These specific primers were designed with *Nde*I and *Hin*dIII each terminal (vfp58up-GGCCCATATGAGTAGCCCCCGC; *Nde*I site is underlined, vfp58rp-GGCCAAGCTTAGAGCTCAGGTTTAA; *Hin*dIII site is underlined). The amplified DNA fragment and overexpression vector pET-22b(+) were digested with *Nde*I and *Hin*dIII, and then ligated by using T4 DNA ligase (Promega Co.). Recombinant plasmid was named pVFP58 and transformed into *E. coli* BL21(DE3).

Overexpression and Purification of Phospholipase (rVFP58)

E. coli BL21(DE3), harboring a pVFP58 plasmid, was cultivated in 1 l of LB medium supplemented with ampicillin (100 μg/ml) at 37°C to an optical density of 0.6 at 600 nm. The expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The cells were incubated for an additional 4 h at 37°C and then were harvested by centrifugation at 5,000 ×*g* for 15 min at 4°C. Pellets were resuspended in 50 ml of 20 mM Tris-HCl buffer (pH 7.5). The cell suspension was sonificated for 5 min, and the lysed bacterial cells were centrifuged at 10,000 ×*g* for 20 min at 4°C. The insoluble pellet was suspended in 20 mM Tris-HCl (pH 7.5) containing 6 M urea and incubated at 4°C overnight. The dissolved suspension was followed by dialysis in 20 mM Tris-HCl buffer (pH 8.0) and then applied directly to Ni-NTA resin equilibrated with 60 mM $NiSO_4$. The proteins were eluted by 1 M imidazole buffer. To recover the enzyme activity, refolding was done by dialysis [10, 37, 42]. Concentration of the purified proteins was measured

Table 1. Bacterial strains and plasmids used in this study.

Bacterial strains/ plasmids	Relevant characteristics[a]	Reference or source
Strains		
V. fluvialis	KCTC2473 corresponded to ATCC33809	KCTC[b]
E. coli XL1-Blue	*RecA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F' *proAB lacI*^{q}*ZΔM15* Tn*10*(Tet^r)]	Stratagene
E. coli BL21(DE3)	F'*ompT hsdS*$_B$(r_B^- m_B) *gal dcm* (DE3)	Novagen co.
Plasmids		
pGEM-4Z	Cloning vector; Ap^r	Promaga co.
pET-22b(+)	His-tag fusion expression vector; Ap^r, T7 promoter	Novagen co.

[a]Ap^r, ampicillin resistant; Tet^r, tetracycline resistant.
[b]KCTC, Korea Collection for Type Cultures.

by Bradford method [4]. All purification procedures were performed at 4°C, and the purified proteins were analyzed by SDS-PAGE.

Thin-Layer Chromatography (TLC) and Gas Chromatography-Mass Spectrophotometry (GC-MS)

To determine the action mode of the rVFP58 toward phospholipids, TLC and GC-MS were performed with several substrates, such as L-α-PC, 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine, 1-O-hexadecyl-2-[(cis)-9-octadecanoyl]-rac-glycero-3-phosphocholine, and L-α-LPC, as described by Lee *et al.* [20]. GC-MS analysis was performed with a QP-5050A (Shimazu, Japan) with polydimethylsiloxane capillary column HP-5MS (30 m× 0.32 mm I.D., 0.25 μm film thickness) at 200°C. Samples were injected in the split mode with a ratio of 1:15 at injector temperature of 250°C. The oven was temperature programmed to increase from 180 to 260°C at 5°C/min. Helium (24.4 kPa) was used as the carrier gas. MS operating conditions were as follows; electron impact energy was 70 eV, and spectra were registered in a mass range of 50 to 550 atomic mass units with 2.94 scans per second.

Measurement of Phospholipase Activity

Phospholipase activity of the rVFP58 was measured by the method of Cho and Kezdy [7]. Three milliliters of reaction buffer (10 mM Tris-HCl, 100 mM NaCl, 10 mM $CaCl_2$; pH 9) were mixed with 50 μl of substrate stock solution [4-nitro-3-(octanoyloxy)-benzoic acid, 3.1 mM in acetonitrile]. Enzyme suspension was removed by centrifugation at 4°C for 1 min. Enzyme assays in duplicate samples were carried out at 37°C. The enzyme activity was calculated by the change of absorbance at 410 nm over time per mg of the rVFP58 enzyme. The change in absorbance of 0.2 at 410 nm was equivalent to 155 nmol. The optimal condition and stability were determined at various ranges of temperature and pH.

Cytotoxicity Assay of the rVFP58 on CHSE-124 Cell Line

Cytotoxicity of the rVFP58 was investigated by measuring the amount of lactate dehydrogenase (LDH) released from chinook salmon embryo (CHSE) cells. The CHSE-214 cell line was inoculated in MEM containing penicillin G and streptomycin, and cultivated at 17°C for 24 h. Various concentrations of the enzyme in 100 μl (0–50 μg) were added and incubated at the same temperature. Cytotoxicity was determined by the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega). Cytotoxicity calculations were based on the following formula; cytotoxicity (%)=100× $(A_{sample}-A_{spontaneous})/(A_{total}-A_{spontaneous})$, where A_{sample} is the optical density (OD) of the treated cells, $A_{spontaneous}$ is OD of untreated cells, and A_{total} is OD of treated cells lysed for maximal LDH release with 1% final concentration of Triton X-100. The OD of blank wells was substracted from all other readings.

Nucleotide Sequence Accession Number

The nucleotide sequence of the *vfp* ORF has been deposited in the GenBank database under accession number AY676063.

RESULTS

Cloning of Phospholipase Gene (*vfp*) from *V. fluvialis* and Purification of Overexpressed rVFP58 Protein in *E. coli*

To construct the DNA library, chromosomal DNA of *V. fluvialis* was digested with *Hin*dIII and ligated with pGEM-4Z. The ligation product was transformed into *E. coli* XL1-Blue, and then colonies were picked off the egg yolk agar plate to select the colony with phospholipase activity. Plasmid pVFP25 purified from a clone contained a 5.8-kb insert which had phospholipase activity (data not shown). We determined the nucleotide sequence of the phospholipase gene (*vfp*) through the sequence analysis, and found that the phospholipase gene (*vfp*) from *V. fluvialis* was composed of 1,690 nucleotides and 563 amino acids. The putative ribosome-binding sequence (GAGATA) was found 9-nt upstream of the proposed translation-initiation codon ATG (data not shown).

The amplified *vfp* gene by PCR was inserted into pET-22b(+) vector and transformed into *E. coli* BL21(DE3). Overexpression of the rVFP58 was induced by adding IPTG. Inclusion body was denatured using 6 M Urea, and subsequently was refolded by dialysis. Purification of the rVFP58 tagging with 6-His residues at the C-terminus was

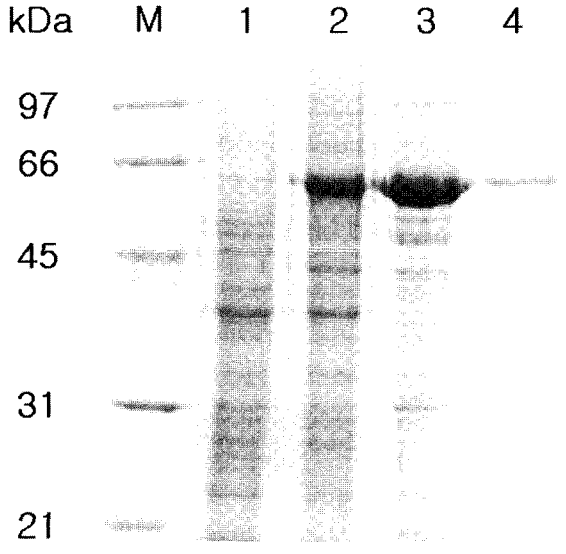

Fig. 1. SDS-PAGE of rVFP58 during purification.
M, molecular size marker; lane 1, crude extract of cells with pET-22b(+); lane 2, cell cultured for 4 h after induction; lane 3, inclusion body isolation; lane 4, his-tag column elution fraction.

Table 2. Purification steps for the rVFP58 from *E. coli.*

Purification step	Total protein (mg)	Specific activity*	Total activity (U)	Yield (%)	Purification fold
Cell homogenate	83	2.9	244.8	100.0	1.0
Refolded inclusion body	10	7.5	75.0	30.6	2.5
Ni-NTA affinity chromatography	4	8.9	34.9	14.2	3.0

*nmoles product/min/mg protein.

performed with Ni-NTA resin and verified by SDS-PAGE (Fig. 1). The purified recombinant protein showed a single band on SDS-PAGE, and its estimated molecular size was about 65 kDa. This molecular size coincided well with the value from the deduced amino acid sequence. When inclusion body was refolded, the specific activity increased by 2.5-fold, while recovery was 30.6%. Finally, the rVFP58 was purified 3.0-fold, and 4 mg of the enzyme was obtained. The specific activity of purified rVFP58 was 8.9 U/mg (Table 2).

TLC and GC-MS

To determine the phospholipase type of the rVFP58, we tested the rVFP58 with several substrates. First, we checked for PLC activity through a liquid assay, using specific substrate, *p*-NPPC, while using the PLC of *C. perfringens* as the control. The result did not show any PLC activity. Therefore, we investigated whether the rVFP58 has a PLA activity that cleaved free fatty acid residue of phospholipids, and analyzed the free fatty acid released from phospholipids, using TLC and GC-MS, as described by Lee *et al.* [20]. Here, PC was used as a substrate and was incubated with the enzyme for 10 h. As shown in Fig. 2A, the quantity of PC (R_f 0.57) decreased in comparision with the initial concentration, but the LPC spot (R_f 0.2) was not detected, resulting from cleavage of one fatty acid residue from PC. To identify released free fatty acid (FFA) from the reactant, 1-palmitoyl-2-linoleoyl-sn-glycero-3-PC was used as a substrate, and the result detected a FFA spot at around R_f 0.25 (Fig. 2B, lane 1). Subsequently, the FFA spot around R_f 0.25 was scraped from the TLC plate and eluted with chloroform. Eluted sample was then analyzed with GC-MS to investigate the site of cleavage in PC. As shown in Fig. 2C, palmitic acid and linoleic acid peaks appeared at retention times 12.8 and 15.5 min, and molecular masses of peaks were 270 and 294, respectively. When the mass of methyl group was subtracted from those values, the resulting values were well matched with the exact molecular weights of palmitic acid and linoleic acid. This result suggests that the rVFP58 has the ability to cleave fatty acid of the sn-1 and sn-2 sites in PC. However, it is possible that the linoleic acid at the sn-2 site was transferred to the sn-1 site after the

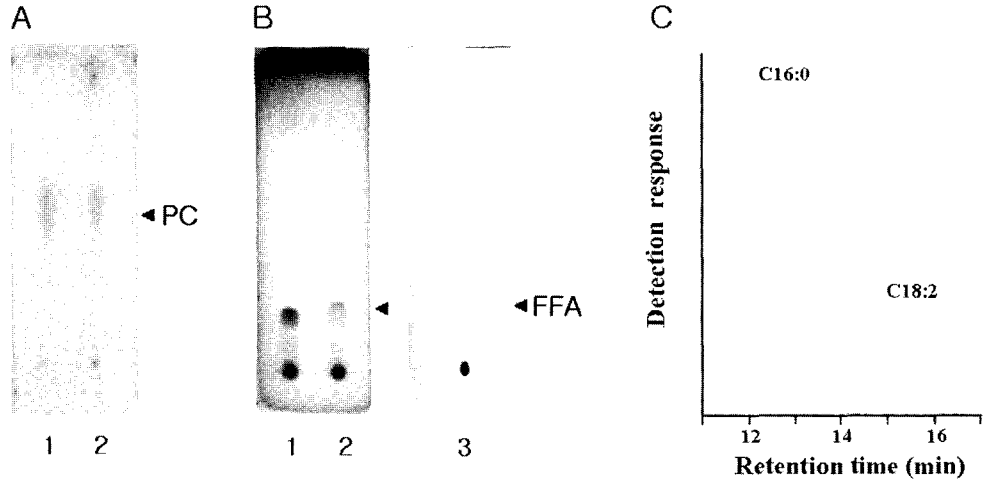

Fig. 2. TLC and GC-MS.

The reaction products, after 14 h incubation of various substrates with 1 mg rVFP58, were eluted in the chloroform. Each was separated on TLC and sprayed with 50% sulfuric acid, followed by heating at 115°C for 30 min. (A) The reaction products of PC without (control) or with the VFP58. Lane 1: PC; Lane 2: PC with the rVFP58. The R_f of PC was 0.57. (B) Products of the enzymatic hydrolysis with the rVFP58 and synthetic phospholipids. Lane 1, 1-Palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine hydrolysis product; lane 2, 1-O-hexadecyl-2-[(cis)-9-octadecanoyl]-rac-glycero-3-phosphocholine hydrolysis products; lane 3: L-α-LPC hydrolysis products. The FFA spot (R_f 0.25) was detected. (C) Reaction product of 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine with 1 mg of VFP58 was separated on TLC plate. It was scraped out and methylated, and then analyzed with GC-MS. Palmitic acid peak, C16:0; Linoleic acid peak, C18:2.

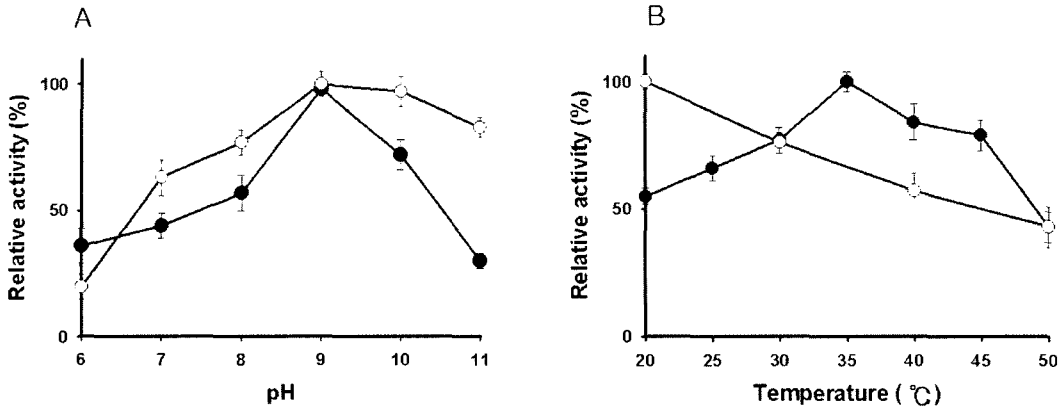

Fig. 3. Effects of pH and temperature on the rVFP58 activity and stability.
(A) The optimal pH for the rVFP58 activity was determined, using different pHs from 6 to 11. To investigated the pH stability of the rVFP58, the rVFP58 was preincubated in the different pHs at 4°C for 2 h, and residual activity was then measured under standard condition. The buffers used were 10 mM citric acid-sodium citrate at pH 6, 10 mM Tris-HCl from pH 7 to 9, and 10 mM sodium carbonate. NaOH from pH 10 to 12. -●-: Optimal pH; -○- pH stability. (B) The optimal temperature of the rVFP58 activity was assayed under various temperatures for 1 h. To investigate the thermostability, the rVFP58 was preincubated at temperatures of 20, 30, 40, 50, and 60°C for 2 h and residual activity was assayed under standard condition. -●-: Optimal temperature; -○- Thermostability.

palmitic acid at the sn-1 site was cleaved from PC. To clarify that the fatty acid at the sn-2 site was not generated by this transition, 1-O-hexadecyl-2-[(cis)-9-octadecanoyl]-rac-glycero-3-PC, which contains an alkyl ether linkage at the sn-1 site, was used as a substrate instead of PC. When we analyzed the free fatty acid on TLC (Fig. 2B, Lane 2), the result confirmed that the rVFP58 cleaved fatty acid of sn-1 and sn-2 in PC.

To examine whether the rVFP58 has LPCase activity, L-α-LPC was reacted with the enzyme suspension. As shown in Fig. 2B (lane 3), the FFA spot could be identified by TLC, in support of the result in Fig. 2A which did not show a LPC spot. These results indicate that the rVFP58 is a PLB enzyme.

Measurement of Phospholipase Activity

The optimal pH for enzyme activity was determined, using different pH values ranging from 6 to 11. As shown in Fig. 3A, the rVFP58 had maximal activity at pH 9 and 80% of the activity was still retained at pH 10. The pH stability was studied by preincubating the rVFP58 in the pH ranges of 6- 12 at 4°C for 2 h and then by assaying the residual enzyme activity was assayed at pH 9.

To examine the optimal temperature, the rVFP58 was incubated at different temperatures. To check the thermostability, the enzyme was preincubated at 20- 60°C for 2 h, and then the residual activity was assayed. As shown in Fig. 3B, the rVFP58 showed the highest activity at 35°C, and 80% of the activity were retained at around 30°C and 40°C. Enzyme activity was stable at 20- 30°C, but it drastically decreased over 40°C. This enzyme was relatively unstable at temperature higher than 40°C. Taken together, the rVFP58 was alkaliphilic and unstable at high temperature.

Assay of Cytotoxicity on CHES-214 Cell Line

Distinctive morphological difference was observed between cells with or without the rVFP58 in the culture medium. When the amount of rVFP58 was increased, significant morphological change was also detected (Fig. 4).

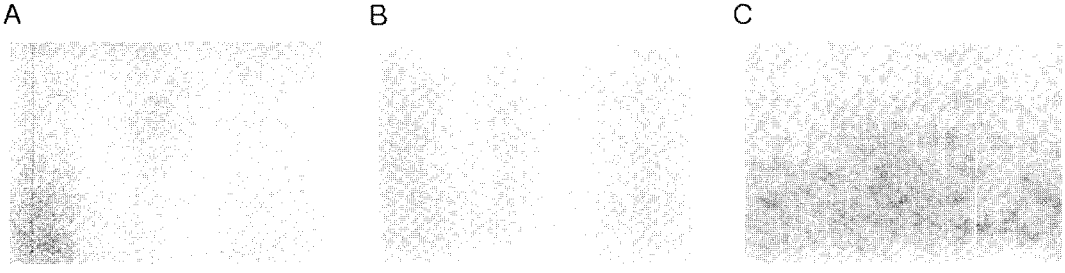

Fig. 4. Morphological changes of CHSE-214 cells after treatment with the rVFP58.
(A) Morphology of normal CHSE-214 cells (magnification, ×100). (B) Morphology of CHSE-214 cells treated with 50 μg of rVFP58 protein. (C) Morphology of CHSE-214 cells treated with 100 μg of rVFP58 protein.

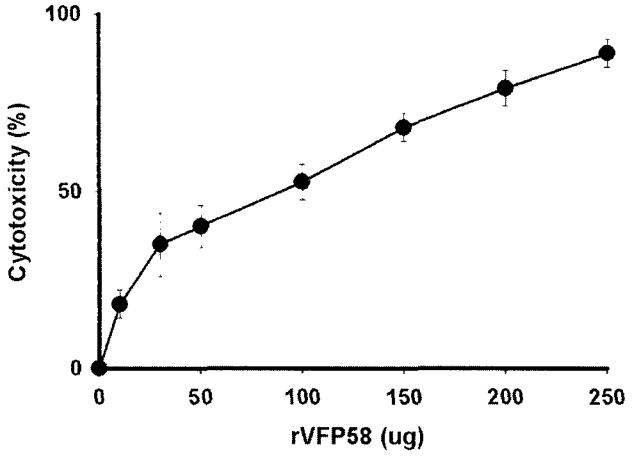

Fig. 5. Cytotoxicity of the rVFP58 on CHSE-214 cells.
Cells were cultured as described in Materials and Methods, and cytotoxicity of CHSE-214 cells was assayed by measuring the amount of LDH released from the lysed cells. The released LDH was measured, using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit. Results represent means±standard deviations of triplicates of the cytotoxicity at each concentration of the rVFP58 (0 to 250 μg) on CHSE-214.

Cytotoxic effect on CHSE-214 cell line was quantified by measuring the amount of LDH released from the lysed cells. As shown in Fig. 5, increasing amounts of the rVFP58 proportionally induced the cytotoxic effect on CHSE-214 cells. Compared to the control, the level of cytotoxicity at 10 μg increased by 30%, and was 80% at higher than 200 μg of rVFP58. These findings indicate that CHSE cell line injury was dependent on the concentration of rVFP58.

DISCUSSION

V. fluvialis is a halophilic bacterial pathogen that has been isolated from humans with diarrhea or from river and estuarine water, marine mollusks, crustaceans, and fish. Illness caused by this pathogen is usually associated with consumption of raw or improperly cooked seafood. This pathogen produces several toxins such as hemolysin, protease, and cytotoxin that may be important in pathogenesis. These toxic substances influence infection to the host cell. Phospholipase is also well known as a pathogenic factor produced from a number of pathogenic bacteria, contributing to destruction of lung surfactant [13, 16], increased vascular permeability [27], colonization of host tissues [9, 36], stimulation of the inflammatory response [18], and generation of signal transducers [32]. Phospholipase is classified by the difference of action site toward phospholipids; PLA_1, PLA_2, PLB, PLC, and PLD. A large part of the studies on the function of phospholipases in the development of infections have been carried out with PLA and PLC [14, 35, 38]. PLB is an enzyme that displays both PLA and LPL activities, and can release fatty acids from phospholipids at both the *sn*-1 and *sn*-2 positions. It is likely that the phospholipid degradation activity of PLB will damage host cell membranes, leading to cell lysis. Few microbial PLBs have been characterized, particularly in terms of their contribution to pathogenesis. To date, most phospholipases in *Vibrio* sp. have been identified as PLA. It has been reported that *V. parahaemolyticus* and *V. mimicus* produce PLA_2 enzyme with hemolytic activity [20, 35]. A PLA with high cytotoxicity was also found from *V. cholerae* [35]. However, PLB from *Vibrio* sp. has not been studied in detail. In this study, we isolated a gene encoding PLB from *V. fluvialis* and characterized its biochemical properties with a recombinant PLB. We also showed that PLB of *V. fluvialis* could be a possible virulence factor in pathogenesis.

PLBs have been reported in various fungi [13]. According to Chen *et al.* [6], PLB of *Cryptococcus neofomans* has a molecular size of about 80 kDa, and its activity was stable at acidic pHs with optimal pH of 3.5 to 4.5. It also did not show the change of enzyme activity in the presence of calcium ion. A 84-kDa PLB of *C. albican* showed maximal activity at pH 6 and the enzyme activity was not dependent on divalent cations such as Ca^{2+} and Mg^{2+} [26]. A *Penicillium notatum* PLB had high activity in acidic pHs [14]. These fungal PLBs had common enzymatic properties, showing high activities under acidic conditions and having molecular size larger than 80 kDa with calcium-independent activities. However, the enzymatic characteristic of PLB from *V. fluvialis* was distinguishable from those of fungi. The VFP58 of *V. fluvialis* has 65 kDa of molecular size that is smaller than those of fungal PLBs (Fig. 1) and it has a high activity in alkaline pH. As seen in Fig. 3, the VFP58 had maximal activity in pH 9 and more than 80% of the activity was retained at pH 12, and it was relatively stable at around pH 8–10. The VFP58 enzyme also showed a calcium-dependent pattern whose activity increased in proportion to calcium concentration (data not shown). Also, the VFP58 of *V. fluvialis* showed maximal activity at 35°C and was relatively stable at lower than 40°C. Thus, this high enzyme activity at lower temperature may be associated with its growth environment as a marine bacterium.

Cytotoxicity is one of the common characteristics attributed to bacterial virulence factors. Therefore, we investigated the cytotoxic effect of *V. fluvialis* PLB, using the CHSE-214 cell line, by measuring the amount of LDH released from the lysed cells. When the fish cell line CHSE-214 was treated with different amounts of the VFP58, the morphological change of CHSE-214 cell line occurred in proportion to the amount of VFP58. Thus, the enzymatic activity of the *V. fluvialis* PLB on membrane phospholipids could result in CHSE-214 cell lysis. Therefore, our results led us to suggest that the VFP58 in *V. fluvialis* may act as a virulence factor. In *C. albicans*, PLB contributes to the pathogenicity by abetting the fungus in damaging and traversing host cell membranes [21]. In general, phospholipase

toxicity has been linked to cytolytic activity and is presumed to be due to phospholipase acting directly upon membrane phospholipids and membrane destruction [35]. The cytolytic activity varies greatly amongst bacterial phospholipases. In our previous study, the cytotoxicity of PhlA from *V. mimicus* on CHSE-214 cell line was 90%, when exposed to 40 μg [20]. In this study, the cytotoxicity was 90%, when 200 μg of VFP58 were used on CHSE-214 cell line, thus cytotoxicity of PhlA from *V. mimicus* was higher than that of VFP58 from *V. fluvialis*. The higher cytotoxicity of PhlA from *V. mimicus* may be due to hemolytic activity as well as phospholipase activity. However, we could not detect hemolytic activity in the VFP58 protein.

In conclusion, we have shown for the first time that a PLB in *V. fluvialis* has high activity under alkaline condition and also cytotoxic activity on the CHSE cell line. Although the PLB is regarded as an important virulence factor, we did not attempt to identify the role of *V. fluvialis* phospholipase in infection. In order to understand the role of the virulence factor of *V. fluvialis* PLB, further *in vivo* experiments with animals need to be done.

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References

1. Aronson, J. F. and L. W. Johns. 1977. Injury of lung alveolar cells by lysolecithin. *Exp. Mol. Pathol.* **27:** 35-42.
2. Alam, M., S. Miyoshi, and S. Shinoda. 1995. Production of antigenically related exocellular elastolytic proteases mediating hemagglutination by vibrios. *Microbiol. Immunol.* **39:** 67-70.
3. Baek, S. H., J. Y. Kwak, S. H. Lee, T. Lee, S. H. Ryu, D. J. Uhlinger, and J. D. Lambeth. 1997. Lipase activities of p37, the major envelope protein of vaccinia virus. *J. Biol. Chem.* **19:** 32042-32049.
4. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.
5. Camilli, A., L. G. Tilney, and D. A. Portnoy. 1993. Dual roles of plcA in *Listeria monocytogenes* pathogenesis. *Mol. Microbiol.* **8:** 143-157.
6. Chen, S. C., L. C. Wright, R. T. Santangelo, M. Muller, V. R. Moran, P. W. Kuchel, and T. C. Sorrell. 1997. Identification of extracellular phospholipase B, lyso phospholipase, and acyltransferase produced by *Cryptococcus neoformans*. *Infect. Immun.* **65:** 405-411.
7. Cho, W. and F. J. Kezdy. 1991. Chromogenic substrates and assay of phospholipases A_2. *Methods Enzymol.* **195:** 75-79.
8. Cox, G. M., H. C. McDade, S. C. Chen, S. C. Tucker, M. Gottfredsson, L. C. Wright, T. C. Sorrell, S. D. Leidich, A. Casadevall, M. A. Ghannoum, and J. R. Perfect. 2001. Extracellular phospholipase activity is a virulence factor for *Cryptococcus neoformans*. *Mol. Microbiol.* **39:** 166-175.
9. Dorrell, N., M. C. Martino, R. A. Stabler, S. J. Ward, Z. W. Zhang, A. A. McColm, M. J. Farthing, and B. W. Wren. 1999. Characterization of *Helicobacter pylori* PldA, a phospholipase with a role in colonization of the gastric mucosa. *Gastroenterology* **117:** 1098-1104.
10. Lee, J. H., Y. D. Cho, J. J. Choi, Y. J. Lee, H. S. Hoe, H. K. Kim, and S. T. Kwon. 2003. High-level expression in *Escherichia coli* of alkaline phosphatase from *Thermus caldophilus* GK24 and purification of the recombinant enzyme. *J. Microbiol. Biotechnol.* **13:** 660-665.
11. Lim, S. K., J. W. Choi, M. H. Chung, E. T. Lee, Y. H. Khang, S. D. Kim, and D. H. Nam. 2002. Production and characterization of extracellular phospholipase D from *Streptomyces* sp. YU100. *J. Microbiol. Biotechnol.* **12:** 189-195.
12. Fifis, T., C. Costopoulos, and J. A. Vaughan. 1996. Evidence for phospholipase B activity in *Fusobacterium necrophorum* cultures and its association with hemolysin/leucocidin activities. *Vet. Microbiol.* **49:** 219-233.
13. Flieger, A., S. Gongab, M. Faigle, H. A. Mayer, U. Kehrer, J. Mussotter, P. Bartmann, and B. Neumeister. 2000. Phospholipase A secreted by *Legionella pneumophila* destroys alveolar surfactant phospholipids. *FEMS Microbiol. Lett.* **188:** 129-133.
14. Ghannoum, M. A. 2000. Potential role of phospholipase in virulence and fungal pathogenesis. *Clin. Microbiol. Rev.* **13:** 122-143.
15. Han, J. H., J. H. Lee, Y. H Choi, J. H. Park, T. J. Choi, and I. S. Kong. 2002. Purification, characterization and molecular cloning of *Vibrio fluvialis* hemolysin. *Biochim. Biophys. Acta* **1599:** 106-114.
16. Holm, B. A., L. Keicher, M. Y. Liu, J. Sokolowski, and G. Enhorning. 1991. Inhibition of pulmonary surfactant function by phospholipases. *J. Appl. Physiol.* **71:** 317-321.
17. Kothary, M. H., H. Lowman, B. A. McCardell, and B. D. Tall. 2003. Purification and characterization of enterotoxigenic El Tor-like hemolysin produced by *Vibrio fluvialis*. *Infect. Immun.* **71:** 3213-3220.
18. Kume, N., M. I. Cybulsky, and M. A. Jr. Gimbrone. 1992. Lysophosphatidycholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. *J. Clin. Invest.* **90:** 1138-1144.
19. Lee, J. V. and P. Shread. 1978. The taxonomy of group F organisms: Relationships to *Vibrio* and *Aeromonas*. *J. Appl. Bacteriol.* 45 ix.
20. Lee, J. H., S. H. Ahn, S. H. Kim, Y. H. Choi, K. J. Park, and I. S. Kong. 2002. Characterization of *Vibrio mimicus* phospholipase A (PhlA) and cytotoxicity on fish cell. *Biochem. Biophys. Res. Commun.* **25:** 269-276.

21. Leidich, S. D., A. S. Ibrahim, Y. Fu, A. Koul, C. Jessup, J. Vitullo, W. Fonzi, F. Mibod, S. Nakashima, Y. Nozawa, and M. A. Ghannoum. 1998. Cloning and disruption of caPLB1, a phospholipase B gene involved in the pathogenicity of *Candida albicans*. *J. Biol. Chem.* **273:** 26078-26086.
22. Lindahl, M., A. R. Hede, and C. Tagesson. 1986. Lysophosphatidylcholine increases airway and capillary permeability in the isolated perfused rat lung. *Exp. Lung Res.* **11:** 1-12.
23. Lockwood, D. E., A. S. Kreger, and S. H. Richardson. 1982. Detection of toxins produced by *Vibrio fluvialis*. *Infect. Immun.* **35:** 702-708.
24. Lockwood, D. E., S. H. Richardson, A. S. Kreger, M. Aliken, and B. Mc Creedy. 1983. *In vitro* and *in vivo* biologic activities of *Vibrio fluvialis* and its toxic products, pp. 87-99. *In* S. Kawahara and N. E. Pierce (eds.), *Advances in Research on Cholera and Related Diarrheas*. Vol. 1. KTK Scientific Publishers. Tokyo, Japan.
25. Merkel, O., M. Fido, J. A. Mayr, H. Pruger, F. Raab, G. Zandonella, S. D. Kohlwein, and F. Paltauf. 1999. Characterization and function *in vivo* of two novel phospholipases B/lysophospholipases from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **274:** 28121-28127.
26. Mirbod, F., Y. Banno, M. A. Ghannoum, A. S. Ibrahim, S. Nakashima, Y. Kitajima, G. T. Cole, and Y. Nozawa. 1995. Purification and characterization of lysophospholipase-transacylase (h-LPTA) from a highly virulent strain of *Candida albicans*. *Biochim. Biophys. Acta* **1257:** 181-188.
27. Muckle, C. A. and C. L. Glyes. 1983. Relation of lipid content and exotoxin production to virulence of *Corynebacterium pseudotuberculosis* in mice. *Am. J. Vet. Res.* **44:** 1149-1153.
28. Mukherjee, P. K., K. R. Seshan, S. D. Leidich, J. Chandra, G. T. Cole, and M. A. Ghannoum. 2001. Reintroduction of the *PLB1* gene into *Candida albicans* restores virulence *in vivo*. *Microbiology* **147:** 2585-2597.
29. Niewoehner, D. E., K. Rice, A. A. Sinha, and D. Wangensteen. 1987. Injurious effects of lysophosphatidylcholine on barrier properties of alveolar epithelium. *J. Appl. Physiol.* **63:** 1979-1986.
30. Nishibuchi, M., R. J. Seidler, D. M. Rollins, and S. W. Joseph. 1983. Vibrio factors cause rapid fluid accumulation in suckling mice. *Infect. Immun.* **40:** 1083-1091.
31. Nishijima, M., Y. Akamatsu, and S. Nojima. 1974. Purification and properties of a membrane-bound phospholipase A_1 from *Mycobacterium phlei*. *J. Biol. Chem.* **249:** 5658-5667.
32. Noverr, M. C., G. M. Cox, J. R. Perfect, and G. B. Huffnagle. 2003. Role of *PLB1* in pulmonary inflammation and cryptococcal eicosanoid production. *Infect. Immun.* **71:** 1538-1547.
33. Prokazova, N. V., N. D. Zvezdina, and A. A. Korotaeva. 1998. Effect of lysophosphatidylcholine on transmembrane signal transduction. *Biochemistry (Moscow)* **63:** 31-37.
34. Rahim, Z. and K. M. Aziz. 1996. Factors affecting production of haemolysin by strains of *Vibrio fluvialis*. *J. Diarrhoeal Dis. Res.* **14:** 113-116.
35. Sanyal, S. C., R. K. Agarwal, E. Annapurna, and J. V. Lee. 1980. Enterotoxicity of group F *Vibrios*. *Jpn. J. Med. Sci. Biol.* **33:** 217-222.
36. Schmiel, D. H. and V. L. Miller. 1999. Bacterial phospholipases and pathogenesis. *Microbes Infect.* **1:** 1103-1112.
37. Shim, E. J., S. H. Jeon, and K. H. Kong. 2003. Overexpression, purification, and biochemical characterization of the thermostable NAD-dependent alcohol dehydrogenase from *Bacillus stearothermophilus*. *J. Microbiol. Biotechnol.* **13:** 738-744.
38. Singh, S., K. M. Cisera, J. D. Turnidge, and E. G. Russell. 1997. Selection of optimum laboratory tests for the identification of *Moraxella catarrhalis*. *Pathology* **29:** 206-208.
39. Songer, J. G. 1997. Bacterial phospholipase and their role in virulence. *Trends Microbiol.* **5:** 156-161.
40. Sugiyama, Y., S. Nakashima, F. Mirbod, H. Kanoh, Y. Kitajima, M. A. Ghannoum, and Y. Nozawa 1999. Molecular cloning of a second phospholipase B gene, *caPLB2*, from *Candida albicans*. *Med. Mycol.* **37:** 61-67.
41. Timpe, J. M., M. M. Holm, S. L. Vanlerberg, V. Basrur, and E. R. Lafontaine. 2003. Identification of a *Moraxella catarrhalis* outer membrane protein exhibiting both adhesin and lipolytic activities. *Infect. Immun.* **71:** 4341-4350.
42. Yoon, S. C., J. H. Lee, S. H. Ahn, E. M. Lee, E. M. Park, and I. S. Kong. 2003. Purification and comparison of properties of the C-terminus truncated agarase of *Pseudomonas* sp. W7. *J. Microbiol. Biotechnol.* **13:** 767-772.