Thesis for Degree of Master of Engineering

Purification, Characterization and Molecular Cloning of *Vibrio fluvialis* Hemolysin Associated with Cytotoxic activity on RTG-2 Fibroblast Cells of Rainbow Trout

by

Jeong-Hyun Han

Department of Biotechnology and Bioengineering

The Graduate School

Pukyong National University

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무지개 송어의 섬유상 세포인 RTG-2의 독성과 연관된 *Vibrio fluvialis* 용혈효소의 정제, 특성, 그리고 클로닝

Adviser: In-Soo Kong

by

Jeong-Hyun Han

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Jeong-Hyun Han

Approved as to style and content by :

Chairman

Member

Member

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Jeong-Hyun Han

Department of Biotechnology and Bioengineering, Graduate School

Pukyong National University

Abstract

Vibrio fluvialis, an etiological agent of enteric disease, produces and secretes a hemolysin which is thought to be an important factor in the pathogenesis of disease. Hemolysin of *V. fluvialis* (VFH) was purified from culture supernatants by ammonium sulfate precipitation and successive column chromatographies on DEAE-cellulose and Mono Q. The estimated molecular weight was 79 kDa and N-terminal amino acid

sequences were determined. The purified protein demonstrated hemolytic activity on many mammalian erythrocytes and rabbit erythrocytes were the most sensitive to VFH. Activity of the native VFH was inhibited by the addition of Zn^{2+}, Ni^{2+}, Cd^{2+} and Cu^{2+} ions at low concentrations. Pores formed on rabbit erythrocytes were approximately 2.8-3.7 nm in diameter, as demonstrated by osmotic protection assay. The cytotoxicity of VFH was evaluated on RTG-2 cells of rainbow trout (*Oncorhynchus mykiss*). At 50 μg/ml, purified VFH caused significant cell death after 9 h incubation.

I. Introduction

Vibrio species cause systemic infections known as vibriosis in fish, and are linked to human disease from the consumption of seafoods such as oyster, shrimp, and cooked fish [1, 2].

Vibrio fluvialis was originally described by Lee *et al* [3] and its association with human disease was heightened after isolation from many patients with apparently inflammatory diarrhea in Bangladesh [4]. The symptoms of enteric disease attributed to *V. fluvialis* are similar to those caused by *V. cholerae*. Patients typically have watery diarrhea with vomiting, abdominal pain, moderate to severe dehydration and often fever. A notable difference from cholera is the frequent occurrence of bloody stools in infections due to *V. fluvialis* [4]. From the enzyme-linked immunosorbent assay, Chikahira *et al* has reported that several *V. fluvialis* strains isolated from environmental and human sources produced an enterotoxin which is immunologically indistinguishable from cholera toxin (CT) [5].

V. fluvialis produces several toxin substrates that may be important in pathogenesis. They include an enterotoxin-like substance, protease, cytotoxin, and hemolysin [6]. Endotoxin activity of *V. fluvialis* has been

demonstrated in vitro using chinese hamster ovary (CHO) cells. Lockwood *et al* reported that at least four biologically active substances could be found from culture supernatants of *V. fluvialis* strain 5489 [7]. CHO elongation factor, CHO killing factor (CKF), and cytolysin active against rabbit erythrocytes were identified when the bacterium was grown without lincomycin. Finally, CHO cell rounding toxin, which is known to be a protease, was found. CKF was internalized and cell death was induced by distruption of cellular function [6]. These were all heat labile and crude concentrates of each factor caused fluid accumulation in infant mice. Of many virulence factors produced from *V. fluvialis*, hemolysin was also thought to be important. However, the role and biological properties of hemolysin from *V. fluvialis* have not been studied.

In general, hemolysin has been proven to be an important virulence factor in the pathogenic processes of many clinical microorganisms by causing hemorrhagic septicemia and diarrhea. Hemolysin may also play a multifunctional role in disease. It can lyse erythrocytes and a variety of other cell types including mast cells, neutrophiles, and polymorphonuclear cells. Also, it may enhance virulence by causing tissue damage or by dissolving material that normally would prevent spreading of the pathogen throughout the tissue. *V. cholerae* hemolysin interacted with immobilized phospholipids and cholesterol, and formed oligomers in vesicles

constituted from phospholipids alone [8]. The thermostable hemolysin (TDH) from *V. parahaemolyticus* exhibited enterotoxic and cytotoxic effects on both human and rat cell monolayers [9]. Several extracellular hemolysin genes have been cloned from *V. cholerae* [10,22], *V. parahaemolyticus* [11], and *V. anguillarum* [12]. More recently, the hemolysin gene of *V. mimicus*, which is known to be atypical non-O1 *V. cholerae*, was isolated and the DNA sequence of this gene was reported in our laboratory [13]. We found that these *Vibrio* hemolysin sequences have exhibited the difference in gene structure.

In this paper, we describe the purification procedure for extracellular hemolysin of *V. fluvialis*, and characterization of the purified hemolysin. In addition, we attempted to invetigate the cytotoxic effects on RTG-2 cells.

II. Materials and methods

1. Bacterial strains, and growth condition

V. fluvialis (ATCC 33809) strain was obtained from KCTC (Korean Collection for Type Cultures) . Cells were grown at 37℃ on solid or in liquid brain heart infusion (BHI) medium (Difco). *E.coli* XL1-Blue [*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*F' *proAB*$^{+}$ *lacI*^{q} *lac*ZΔM15 Tn*10* (Tet^{r})] was used as a host propagate plasmid construct and was grown in Luria-Bertani broth (LB) medium. Transformation of plasmid DNA into *E.coli* was performed with $CaCl_2$-treated cells.

2. Purification of VFH

The culture was cultivated at 37℃ for 24 h with shaking, and the culture supernatant was collected by centrifugation at 7,000 × g for 30 min. Solid ammonium sulfate was added to 80% saturation (591 g/l). After 8 to 10 h, the resulting precipitate was collected by centrifugation at 7,000 × g for 30 min at 4℃, and dissolved in small volume of 10mM Tris-HCl buffer (pH 7.5). The suspension was centrifuged at 7,000 × g for 20 min to remove insoluble residue. Dialysis was performed overnight against the same buffer, and used as crude VFH.

Crude VFH preparation was applied to a DEAE-cellulose column equilibrated with 10mM Tris-HCl buffer (pH 7.5), and eluted with the same buffer containing 0.5M NaCl at a flow rate of 1 ml/min. Fractions containing VFH were collected and concentrated by freeze drying. Concentrated VFH was finally applied to a Mono-Q HR 10/10 column (Pharmacia) which was equilibrated with 10mM Tris-HCl buffer (pH 7.5), and eluted with a linear gradient of 0 to 0.5M NaCl in the same buffer at a flow rate of 0.5 ml/min. Hemolytic activity of chromatographically fractioned samples were detected on blood agar plate. N-terminal amino acid sequences were determined by standard Edman degradation on a model ABI 491 microsequencer.

3. Assay of hemolytic activity

Hemolytic activity was determined with 4% rabbit erythrocytes as described [14]. Briefly, rabbit erythrocytes were washed with 10mM Tris-buffered saline (TBS: pH 7.5) three to four times and adjusted to a final concentration of 4% (v/v) in TBS. A sample (0.2 ml) of VFH suspension diluted with TBS was mixed with rabbit erythrocytes (0.2 ml) and incubated at 37℃ for 1 h. Reaction mixtures were centrifuged at 1,000 × g for 5 min and the amount of hemoglobin released from disrupted erythrocytes was determined by absorbance at 540 nm of the supernatants.

One hundred percent hemolysis is defined as the optical density at 540 nm of hemoglobin released from erythrocytes that have been lysed by 0.1% Triton X-100. One hemolysin unit (HU) was defined as the amount of hemolysin eliciting 50% hemoglobin release.

4. Osmotic protection experiments

For these experiments, 0.2 ml of 4% rabbit erythrocyte suspensions containing an osmotic protectant was mixed with 0.2 ml of VFH solution (2 HU). Glucose, sucrose, maltotriose, inulin and PEG 4000 were used as the osmotic protectants at a final concentration of 30 mM. Dextran 10 and PEG 6000 were used at a final concentration of 15 mM. The mean hydrated diameters of glucose, sucrose, maltotriose, inulin, PEG 4000, dextran 10, PEG 6000 were 0.72, 0.9, 1.2, 2.8, 3.66, 4.7, and 5.66 nm, respectively (15). Protection from hemolysis was calculated as follows: % protection = (1 – hemolytic rate in the presence of saccharide / hemolytic rate without osmotic protectant) × 100.

5. Effect of divalent cations on hemolytic activity

Inhibitory activity of cations on hemolysis was determined by addition of divalent cations as the chloride forms in TBS. A 0.1 ml volume of the cation solution was mixed with 0.1 ml of VFH (2 HU) and 0.2 ml of the

rabbit erythrocyte suspension. The mixture was incubated at 37℃ for 1 h, and immediately subjected to the hemolytic activity assay.

6. Effect of temperature on activity of VFH

Hemolytic activity of the VFH was assayed at 37℃, with 2 HU routinely. To determine the effect of temperature on hemolytic activity of VFH, a suspension of the washed 4% rabbit erythrocytes (0.2 ml) was incubated with VFH (2 HU in 0.2 ml of TBS) for 5 to 60 min at 15, 20 , 25, 30, 35, 37, and 42℃, and the supernatants were obtained and measured the absorbance at 540 nm.

7. Determination of cytotoxicity by lactate dehydrogenase (LDH) release assay

Release of LDH from rainbow trout fibroblast cells, RTG-2, after treatment with VFH was measured to determine the permeability of the membrane and cell death by hemolysin. RTG-2 cells of *Oncorhynchus mykiss* (CCL-55; American Type Culture Collection) were grown in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 0.2% fungizone, and 0.035% sodium biscarbornate per liter. Single cell suspensions were obtained from 70 to

80% confluent cultures by incubation with 0.25% trypsin. Cells were transferred to 48 multiwell plate (300 μl, 3×10^5 cells/well) and incubated until nearly confluent (5). Purified VFH was addded to RTG-2 cell cultures at a various concentration.

The amount of LDH release was determined with a Vitros DT II chemistry system (Johnson & Johnson). Briefly, LDH in the supernatants catalyze the conversion of pyruvate and NADH to lactate and NAD^+ producing a change in the reflection density. The change in reflection density (340 nm) was monitored at 37℃, and the rate of change was then used to calculate enzyme activity. Results were expressed as changes in optical density compared to that of a 100% lysis control.

III. Results

1. Purification of VFH

Hemolysin(VFH) was purified from culture supernatants as indicated in Materials and methods. The purification results are shown in Table 1. Ammonium sulfate precipitation followed by dialysis resulted in 1.6-fold increase of specific activity. The protein was then adsorbed onto DEAE-cellulose, and the hemolytic activity was recovered as one peak with the majority eluting at 0.5 M NaCl. Subsequently, the pooled active fractions were applied to a Mono Q FPLC column. The level of hemolytic activity from the Mono Q fraction was 10,000 U/mg; VFH was purified approximately 49-fold, and yielding 0.6%. The SDS-PAGE analysis of the purified VFH revealed a single band of 79 kDa (Fig. 1). The N-terminal amino acid sequences of the purified protein was determined to be Asp-Ile-His-Asp-Pro-Val.

2. Susceptibilities of mammalian erythrocytes to VFH

Of the six mammalian erythrocytes including human erythrocytes, the purified VFH exhibited the highest activity on rabbit erythrocytes, and the lowest hemolytic activity was observed with sheep and human

Purification stage	Vol of purifying Solution (ml)	Total amt of protein Recovered (mg)	Total hemolytic activity (HU)	Sp act (HU/mg)	Relative Activity	Yield of Activity (%)
Collection of culture supernatant	1,000	170	33,660	198	1	100
Ammonium sulfate precipitation (80%)	85	62	19,310	311	1.6	57.4
DEAE-cellulose chromatography	50	2	1,910	955	4.8	5.7
Mono-Q column chromatography	1	0.02	200	10,000	49.3	0.6

TABLE. 1. Purification of extracellular hemolysin produced by *V. fluvialis*

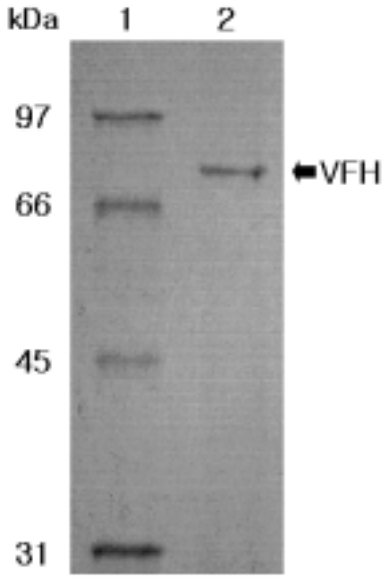


Fig. 1. SDS-PAGE of purified hemolysin from culture supernatant of *Vibrio fluvialis*. Lane 1, molecular weight markers; Lane 2, purified VFH.

erythrocytes (Table 2). This is in contrast to studies with TDH from *V. parahaemolyticus* where mouse erythrocytes were the most sensitive, and chicken and sheep erythrocytes were the least sensitive [16]. Shinoda *et al* reported that horse erythrocytes were the most sensitive to hemolysin (VMH) from *V. mimicus* [17]. In case of El Tor hemolysin from *V. cholerae*, rabbit erythrocytes were more sensitive, while horse erythrocytes were the least sensitive [18]. Consequently, rabbit red blood cells were used to assay hemolytic activity because of their documented sensitivity.

3. Effect of temperature on hemolytic activity of VFH

As shown in Fig. 2, there were significant differences in hemolytic activity when VFH was incubated at various temperatures (15℃ to 42℃). The lysis of erythrocyte by the purified VFH was temperature dependent and the optimum temperature was 37℃. Hemolysis commenced from afew minutes and completed by 60 min at 37℃, whereas no hemolysis occurred at 4℃. The observation that VFH did not lyse rabbit erythrocytes at 4℃ prompted a series of experiments to determine whether VFH binds to rabbit erythrocytes at 4℃. To further demonstrate that the binding of VFH to erythrocyte was temperature dependent, rabbit erythrocytes were

Erythrocyte source	Specific activity (HU/mg protein)	Relative Activity (%)
Rabbit	9,770	100
Chicken	7,700	77
Mouse	7,320	73
Rat	4,200	42
Sheep	730	7
Human	430	4

TABLE 2. Hemolytic activities of the VFH on the erythrocytes from various sources

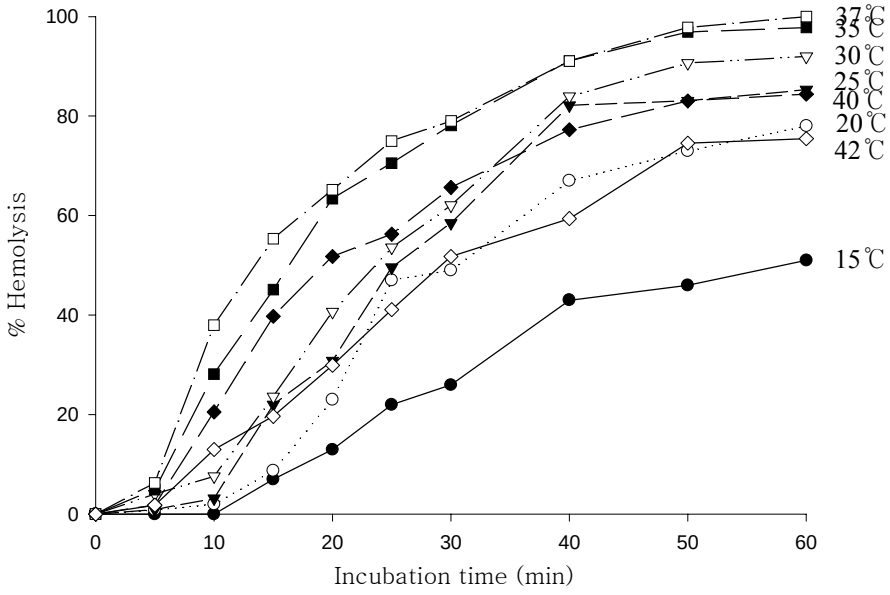


Fig. 2. Kinetics of erythrocyte lysis by VFH as a function of temperature. Washed rabbit erythrocytes were incubated VFH of 2 HU for 5 to 60 min and hemolysis was determined by absorbance at 540 nm

incubated with 2 HU VFH at 4℃ for 60 min, subsequently washed with cold TBS to remove unbound VFH and resuspended in TBS. When the resuspended erythrocytes were incubated at 37℃ for 1 h, hemolysis occurred (data not shown). This indicated that VFH had bound to erythrocytes at 4℃. There are also strong evidences in *Vibrio* spp. hemolysins showing that the binding step of hemolysin appeared to be temperature independent, but the lysis was temperature dependent [14, 19, 20].

4. Inhibitory effect of divalent cations

Cations possibly function for protection from the increase in the intracellular osmotic pressure through blockage of the influx of extracellular water via pores [21]. The effect of several cations on hemolytic activity of the purified VFH was tested. Hemolytic activity was assayed after divalent cations, including Zn^{2+}, Ni^{2+}, Cu^{2+}, Cd^{2+}, Mn^{2+}, Co^{2+}, Mg^{2+}, and Ca^{2+} were added to the reaction mixture at various concentrations. Erythrocyte suspensions were incubated with VFH at 37℃ for 1 h. The concentration of VFH was adjusted to result in approximately 50% lysis of erythrocytes. With increasing concentration of cations, progressive inhibition of hemolysis was observed. Hemolysis by

VFH was prevented by divalent cations in decreasing order, Zn^{2+}> Ni^{2+}> Cu^{2+} > Cd^{2+} and collective results are depicted in Table 3. It is noteworthy that monovalent cations such as Cs^{+} and Li^{+} had no effect on hemolysis (data not shown). Evidence that these cations inhibit membrane destruction was obtained from the following result. Erythrocytes were incubated with VFH and the divalent cation at 4℃ for 1 h, followed by centrifugation. The sedimented erythrocytes were washed twice and incubated at 37℃ for 1 h in fresh TBS. Marked hemolysis was observed, indicating that the divalent cations did not inhibit the binding of hemolysin to erythrocytes.

5. Inhibitory effect of osmotic protectants on hemolysis

To assess whether hemolysis is differentially affected by osmotic protectants, we estimated hemolytic activity in the presence of saccharides, PEG solutes and dextran. When rabbit erythrocytes were mixed with VFH in the presence of 30 mM of glucose, sucrose, maltotriose and inulin, hemolysis occurred. However, there was a significant inhibition of hemolysis (>90% protection from hemolysis) by PEG 4000 and complete osmotic protection was afforded by dextran 10 and PEG 6000, respectively (Fig. 3). The inhibitiory effects were dependent on the molecular diameter

Divalent cation [b]	ID_{50} (mM) [c]
Zn^{2+}	0.18
Ni^{2+}	0.2
Cd^{2+}	0.7
Cu^{2+}	1
Mn^{2+}	>25
Co^{2+}	>25
Mg^{2+}	>25
Ca^{2+}	>25

TABLE 3. Inhibitory effect of divalent cations on the hemolytic activity[a]

[a] VFH (2HU) and rabbit erythrocytes (4%) were incubated at 37℃ for 1 hr in the presence of the cation. Thereafter, hemoglobin was quantified by measuring absorbance at 540 nm.

[b] The chloride salt of each cation was used.

[c] ID_{50} is defined as concentration of divalent cation that inhibits 50% of hemolytic activity

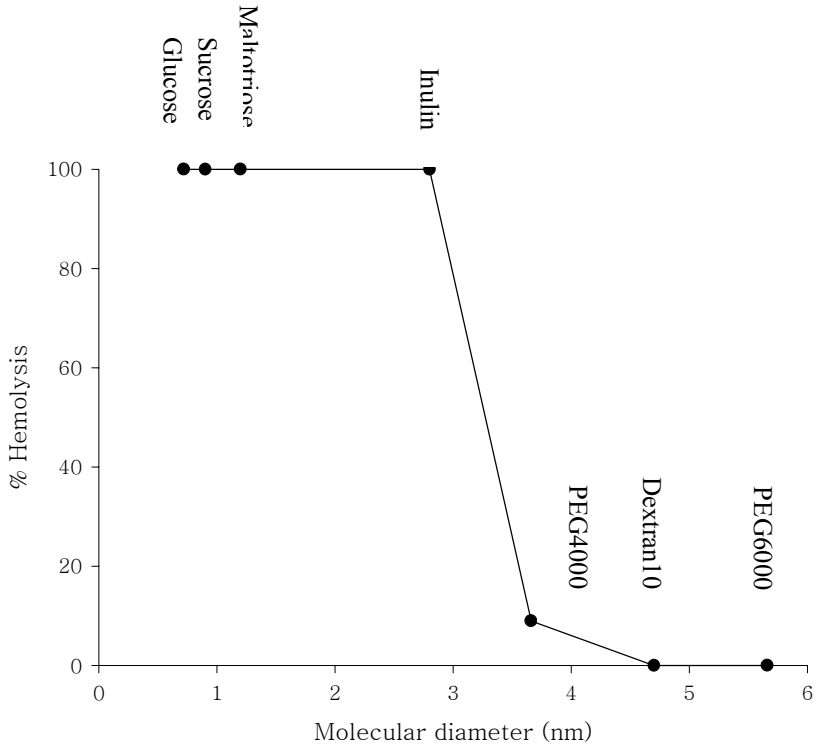


Fig. 3. Inhibitory effect of osmotic protectants on hemolysis. Rabbit erythrocyte (4%, v/v) suspensions containing 30 mM glucose, 30 mM maltotriose, 30 mM inulin, 30 mM PEG 4000, 15 mM dextran 10, and 15 mM PEG 6000 were incubated with VFH (2 HU) at 37℃.

of the colloids and the extent of protection from hemolysis by saccharides suggested that hemolysis was osmotically protected by mean hydrated diameters of 2.8 nm to 3.7 nm.

6. Cloning of *V. fluvialis* hemolysin gene

Approximately 3,000 *E. coli* transformants from the genomic library of *V. fluvialis* were screened on blood agar plates and one colony showing clear halo formation was selected. Plasmid DNA was isolated from this colony. Restriction enzyme analysis revealed that the plasmid, named pVFH460, contained insert of 4.6 kb.

IV. Discussion

Hemolysin production has been shown to be associated with virulence for many bacterial species. In spite of the importance of hemolysin as a potential virulence factor of *V. fluvialis*, virtually nothing is known about its role and biological properties associated with virulent activities. In this report, we purified hemolysin from *V. fluvialis* and cloned the corresponding gene. The cloned gene revealed that the 4.6 kb DNA fragment. The molecular weight determined by SDS-PAGE, and N-terminal sequences of the purified VFH determined that the cleavage of the signal peptide in VFH

In the case of *V. cholerae*, the hemolysin was initially synthesized as an 82 kDa protein and processed to 79 kDa protoxin by cleavage of the signal peptide during secretion through the inner membrane. The protoxin is then processed into 65 kDa active mature hemolysin in the periplasm and culture medium. Specifically, the preprotoxin synthesized in the cytoplasm is secreted into the periplasm, and the protoxin is secreted into the culture medium by two step processing [22]. Shinoda *et al* suggested that the two step processing might exist in the production of mature VMH, as found in *V. cholerae* hemolysin [23].

Binding of VFH appeared to be temperature independent, but lysis step showed temperature dependency. These data are similar to the mechanism of *V. cholerae* cytolysin that does bind to the erythrocytes at 4℃ and induces lysis of erythrocytes in a temperature independent [20]. The temperature dependence of the cell disruption step seems to be due to the requirement for high temperature for increasing the membrane fluidity to allow transmembrane pore formation.

The hemolysis by VFH was inhibited by addition of Zn^{2+}, Ni^{2+}, Cd^{2+} and Cu^{2+} at low concentration of ID_{50}. Of the 8 divalent cations we examined, four (Mn^{2+}, Co^{2+}, Mg^{2+}, Ca^{2+}) had a higher concentraion (>25mM) of ID_{50}. Except for Co^{2+}, the inhibitory patterns described for this toxin similar basically from those observed for *V. cholerae* and *V. metschnikovii* [20, 25].

Hemolysin is a membrane damaging agent that serves as important virulence factor. With further examination, we determined the action of VFH involves the formation of pores in erythrocytes membranes, and the diameter of the pores was estimated to be 2.8 to 3.7 nm using the osmotic protectants. This size seems larger than those formed by other *Vibrio* hemolysins such as *V. cholerae* [20, 24], *V. parahaemolyticus* [14], *V. metschnikovii* [25], *V. mimicus* [17], and *V. vulnificus* [26].

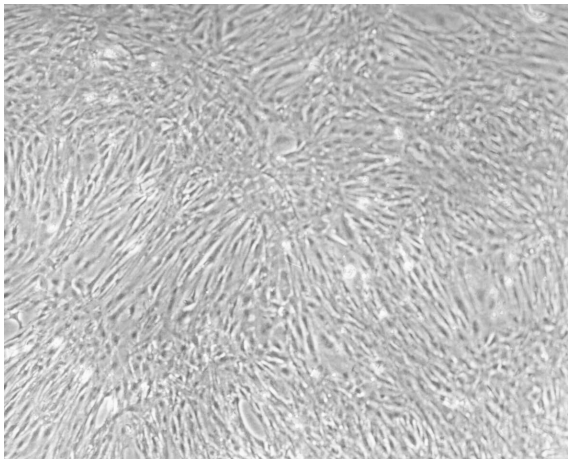
It has been reported that microbial hemolysins from *V. cholerae*,

Serratia marcescens, and *V. parahaemolyticus* have cytotoxicity or vacuolating activity on mammalian cells [27,28,29]. *V. cholerae* hemolysin exhibited vacuolating activity on nucleated mammalian cells at low concentrations of toxin, but was lethal to cells in high concentrations *S. marcescens* hemolysin (ShlA) caused irreversible vacuolation and subsequent lysis of cells. Vacuolation by ShlA was observed only in epithelial cells, and not in fibroblasts. One explanation for this difference may be that fibroblasts are more efficient than epithelial cells in repairing the lesions caused by ShlA (5). The pathogenicity of *V. parahaemolyticus* has been suggested to be linked mostly to TDH. Raimondi *et al* reported novel information about the toxin's potential mechanism in the intestine and a hypothesis about its role in human disease by using human and rat cell monolayer cultures [9]. At high TDH concentrations, the number of channels increased and caused massive ionic influx. These effects overwhelmed the cell's capacitiy to compensate and resulted in irreversible cell swelling, cell rounding, and death.

So far, the action of VFH on cultured fish cells has not been published. For better understanding of the role of this VFH in the first source of infection, we examined the cytotoxic effects on RTG-2 cell lines, the fibroblast cells of rainbow trout gonad. As shown in Fig 4, the fibroblast cells changed into round form at 4 h after addition of the purified VFH (50

μg/ml). Cytotoxicity was evaluated by LDH release from RTG-2 cells after addition of the purified VFH with various concentrations. VFH (50 μg/ml) caused the release of approximately 80% of the total cellular LDH from RTG-2 cells, while 10 μg/ml of VFH caused the release of only 16% of the LDH (Fig. 5). Exposure to low concentrations of VFH caused no significant change in the general morphology of the monolayer. In contrast, monolyers treated with 50, 40, 30, and 20 μg of VFH per ml showed complete or partial significant rounding and detachment of cells. These results suggest that VFH can cause morphological changes of fibroblast and subsequently lysis cells.

A



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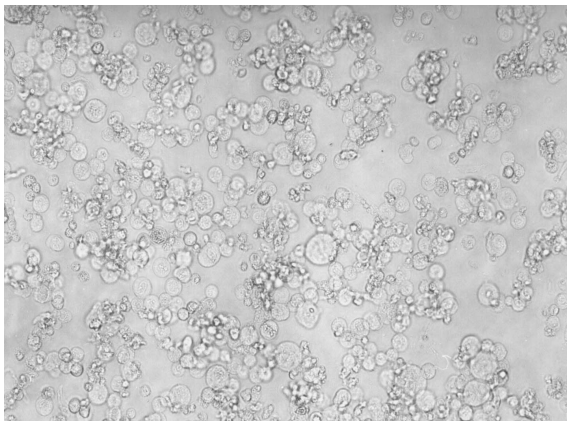

Fig. 4. Morphological changes of RTG-2 cells after treatment with VFH. (A) Morphology of normal RTG-2 cells (magnification, × 100). (B) Morphology of rounded cells (magnification, × 100).

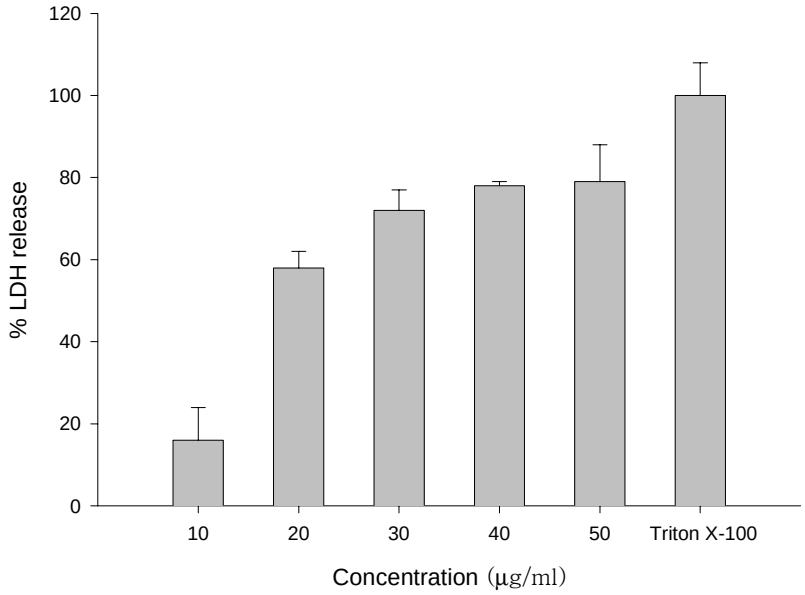

Fig. 5. LDH release on the cytotoxicity of RTG-2 cells after VFH treatment. The percentage of LDH retention by cells incubated with VFH is expressed as the percentage of LDH retained by control cells. Results are expressed as mean ± standard deviation of at least three independent determinations.

V. Summary

1. Hemolysin of *V. fluvialis* (VFH) was purified from culture supernatants by ammonium sulfate precipitation and successive column chromatographies on DEAE-cellulose and Mono Q. The estimated molecular weight was 79 kDa and N-terminal amino acid sequences were determined.
2. The purified protein demonstrated hemolytic activity on many mammalian erythrocytes and rabbit erythrocytes were the most sensitive to the VFH.
3. Activity of the native VFH was inhibited by the addition of Zn^{2+}, Ni^{2+}, Cd^{2+} and Cu^{2+} ions at low concentrations.
4. By osmotic protection assay, pores formed on rabbit erythrocytes were approximately 2.8-3.7 nm in diameter.
5. The cytotoxicity of VFH was evaluated on RTG-2 cells of rainbow trout (*Oncorhynchus-mykiss*). At 50 μg/ml, Purified VFH caused significant cell death after 9 h incubation.

VI. Acknowledements

본 논문이 이루어지도록 언제나 지도와 채찍과 사랑으로 이끌어주신 공인수 교수님께 머리 숙여 감사드립니다. 학문에의 길로 이끌어 주시고 항상 관심과 애정을 보내주신 공재열 교수님, 홍용기 교수님, 이형호 교수님, 김중균 교수님, 박남규 교수님, 김성구 교수님께 감사드립니다.

그 동안의 연구실 생활에서 부족한 후배를 아껴주시고 많은 도움을 주신 윤수철, 김구택, 하정철, 김영옥, 박기재, 박효진, 김대경, 이종희, 김현국, 최선영, 이상봉, 신승렬, 신승렬, 진철호 선배님께 진심으로 감사드립니다. 그리고 옆에서 많은 도움을 주었던 동기들 남현, 윤혁이와 언제나 묵묵히 맡은 일을 하던 실험실 후배들인 제현, 선희, 선회, 은미, 승하, 나리, 막내인 나영이에게도 고마운 마음을 전합니다.

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VII. References

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Purification, Characterization and Molecular Cloning of *Vibiro fluvialis* Hemolysin Associated with Cytotoxic activity on RTG-2 Fibroblast Cells of Rainbow Trout

by
Jeong-Hyun Han
Department of Biotechnology and Bioengineering
Pukyong National University

Abstract

Vibrio fluvialis, an etiological agent of enteric disease, produces and secretes a hemolysin which is thought to be an important factor in the pathogenesis of disease. Hemolysin of *V. fluvialis* (VFH) was purified from culture supernatants by ammonium sulfate precipitation and successive column chromatographies on DEAE-cellulose and Mono Q. The estimated molecular weight was 79 kDa and N-terminal amino acid sequences were determined. The purified protein demonstrated hemolytic activity on many mammalian erythrocytes and rabbit erythrocytes were the most sensitive to VFH. Activity of the native VFH was inhibited by the addition of Zn^{2+}, Ni^{2+}, Cd^{2+} and Cu^{2+} ions at low concentrations.

Pores formed on rabbit erythrocytes were approximately 2.8-3.7 nm in diameter, as demonstrated by osmotic protection assay. The cytotoxicity of VFH was evaluated on RTG-2 cells of rainbow trout (*Oncorhynchus mykiss*). At 50 μg/ml, purified VFH caused significant cell death after 9 h incubation.

Key Word : *Vibrio fluvialis*, hemolysin, cytotoxic activity

무지개 송어의 섬유상 세포인 RTG-2의 독성과 연관된 *Vibrio .fluvialis* 용혈효소의 정제, 특성, 그리고 클로닝

부경대학교 생물공학과
한정현

V. fluvialis 는 장내 감염을 일으키는 질병요소로서 병원성 질병의 중요한 요소로서 여겨지는 용혈효소를 생산하고 방출한다. *.V. fluvialis* 의 용혈효소는 배양액 상층을 $(NH4)_2SO_4$ 를 처리하여 침전 시킨 후 DEAE-cellulose 와 Mono-Q 컬럼을 이용하여 정제 하였다. 예상되어지는 분자량은 79kDa 이며 N-터미널 아미노 말단 서열을 결정하였다. 정제된 용혈효소(VFH)를 여러 포유류의 적혈구에서 실험한 결과 토끼의 적혈구가 가장 민감하게 반응을 나타내었다. VFH 의 활성은 Zn^{2+}, Ni^{2+}, Cd^{2+}, Cu^{2+} 이온들의 낮은 농도에서 저해되며, 삼투압 방해 실험을 통해 토끼의 적혈구에 형성되는 구멍의 사이즈는 대략 2.8 에서 3.7 nm 였다. 무지개 송어의 RTG-2 세포에 대해 VFH 의 독성실험을 하였는데, 정제된 VFH (50ug/ml)을 넣은 후 9 시간 뒤에 세포의 죽음을 일으켰다.

Key Word : *Vibrio fluvialis*, hemolysin, cytotoxic activity