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Identification of Hemolysin as one of the Important Virulent Factors in *Vibrio anguillarum* V7

Youngchool Choe and Gajin Jeong*

Department of Microbiology, Research Center for Molecular Microbiology
Seoul National University, Seoul 151-742, Korea

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We have identified hemolysin rendering virulency of *Vibrio anguillarum* grown at 23℃ which was evaluated on human RBCs. Hemolysin itself was separated as a single band on non-denaturing gel electrophoresis. Vibrio hemolysin was destroyed by trypsin and proteinase K and was heat labile. Optimal pH for activity was around pH 6 while pI of the molecule was recognized as 5.7, with relative distance (R_f) on non-denaturing gel was 0.7. Addition of EDTA and $FeCl_3$ drew the possibility that the production of hemolysin was mainly induced to overcome iron deficiency inside host animals upon infection.

Key words: *Vibrio anguillarum*, hemolysin, iron deficiency

Vibrio anguillarum is a well known pathogenic microorganism infecting not only marine (2, 12, 18, 25, 26) but also fresh water fishes (13, 28), especially causing vibriosis in salmonoid (1, 10, 14). This disease can be characterized with organ hemorrhage and tissue necrosis with high mortality (24).

Since Crosa and his colleagues (5) reported the presence of a specific plasmid (pJM1), responsible for the virulency only in highly pathogenic strains of *V. anguillarum* such as NCMB0572, LS173, 775, ES1, 133S, RG 75834, and V1, outer membrane proteins (6) and many other possible causative factors were discussed.

There were several studies such as one on endotoxin as the possible virulent factor (14). They injected isolated endotoxin into salmonoid fish finding no significant virulency developed, which implies that it is still obscure if endotoxin is related to vibriosis. We, however, could demonstrate an outer membrane protein from *V. anguillarum* V7 as a possible endotoxin protein for the virulency (22).

Exotoxins have also been frequently reported; heat labile exotoxins with proteolytic activity by Inamura *et al.* (15) from *V. anguillarum* ATCC 19264 or PT81049 (serotype 1) and heat stable one with hemolytic and proteolytic activity by Kodama *et al.* (16) from *V. anguillarum* NCMB1 (serotype 3). We have been studying on virulent factors focusing on possible roles of toxic molecules exerting proteolytic and hemolytic activity in *V. anguillarum* V7 (NCMB6 serotype J01) and V104 (NOAA V775, serotype J03). We now report that the presence and nature of hemolysin from *V. anguillarum* V7.

* To whom correspondence should be addressed.

Materials and Methods

Strains

Vibrio anguillarum strain V-7 (NCMB6, serotype J01) and V-104 (NOAA V-775, serotype J03) were kindly provided by Professor Kimura of Hokkaido University, Japan, in 1987 and maintained in our laboratory thereafter. For the maintenance of the virulence in both strains, a group of gold fish *Cyprinus caprio* L. weighing 4～5 g each was injected intraperitoneally with these strains cultured in tryptic soy broth supplemented with 1.5% NaCl (Merck, Darmstadt, Germany) at 22-23℃. Media for bacterial culture were purchased from Difco (Detroit, MI), unless otherwise mentioned. Through *in vivo* passages, virulent bacteria were recovered from the peritoneal cavity and spleen of dead fish on tryptic soy agar (TSA) supplemented with 1% NaCl at 22℃ for 24～48 hrs, and they were kept at 4℃ for storage. When the strains became avirulent, bacteria thus kept were cultured at 22～23 for 18～24 hrs to restore the virulence through *in*

vivo passages. Reisolates were confirmed of *V. anguillarum* by O/129 test and culture in TCBS medium (5).

Preparation of RBCs

Human blood type O RBCs were useful to evaluate the hemolytic activity of *V. anguillarum* (5). Heparinized normal peripheral blood from healthy volunteers with blood type O was diluted in an equal volume of Hanks' Balanced Salt Solution (HBSS) and loaded onto Lymphocyte Separation Medium (LSM ρ=1.077, Flow Laboratory, North Ryde, N.S.W., Australia). After centrifugation at 1,000×g for 20 min, RBCs were recovered from the sediment, and washed twice in Alserver's solution making the final concentration of 40%, and kept at 4℃ until use. The RBC preparation was washed twice again in 10 mM phosphate buffered saline (PBS) solution before use and finally adjusted to 1% solution (v/v, 1.1×10^8 cells/ml), or 1.5% if necessary, which gave the A_{540} value of 1.0 when mixed with the equal volume of PBS supplemented with 0.02% SDS. Fresh RBCs were stable at room temperature for more than a week.

Hemolytic activity

The method of Cavalieri and Snyder (4) was slightly modified to evaluate the hemolytic activity from growing bacteria. In brief, bacteria were cultured in TSB and human RBC suspension was added to estimate the different hemolytic activities between bacteria passed three times *in vivo* and those passed four times *in vitro* and between bacteria grown at 23℃ and those grown at 32℃. Bacterial cultures were adjusted to A_{600}=0.5 and added to the equal volume of 1.5% human RBC suspension. Reaction mixture was kept at 25℃ for 20 min. After centrifugation, lysed RBCs were quantified by measuring absorbency of supernatant at 540 nm (Shimadzu Model UV-265).

Hemolytic activity in the culture supernatant was measured by Short and Kurtz's method (28) also with some modification. Each of hemolysin preparation in two fold serial dilution was added to the equal volume of 1% RBC suspension. They were gently stirred for 30 min, and tested for hemolytic activity in an hour by measuring absorbency at 540 nm. The maximum dilution to lyse 50% RBCs was converted to 50% hemolysis unit (HU_{50}). RBCs from other animals were compared for the hemolytic activity of *V. anguillarum*; gold fish, rabbit, mouse, sheep, and human. RBCs in 750 μl PBS at 5×10^7 cells/ml were mixed with 250 μl of hemolytic supernatant (64HU_{50}, therefore finally 16HU_{50}/ml) at room temperature, and the intact RBCs were counted on hemocytometer after 20 min. The count of RBCs in control group treated with PBS only did not decrease during the experiment.

Influences of enzymes to hemolysin

Hemolysin at 2 HU_{50}/ml has been reacted each with deoxyribo-nuclease (DNase) type I, ribonuclease, lysozyme, trypsin, and proteinase K at the final concentration of 50 μg/ml, 50 μg/ml, 1 mg/ml, 10 mg/ml, 100 μg/ml, respectively, and incubated at 32℃ for 1 hr. After the reaction, RBCs were added to each reaction mixture and hemolytic activity was estimated. Proteinase K was purchased from Boeringer Mannheim GmbH (Mannheim, Germany), and other reagents from Sigma. Chemicals were purchased from Sigma unless otherwise mentioned.

Heat sensitivity of hemolysin

Hemolysin, at 4 HU_{50}/ml, was kept for 10 min at different temperatures; 18, 25, 37, 56, 70, and 100℃. An equal volume of 1% RBCs was mixed with heat treated hemolysin, and hemolytic activity was evaluated 1 hr later.

Determination of optimal temperature and pH for hemolysin activity

Optimal temperature and pH were determined to maximize hemolysin activity. Human RBC suspension in PBS, 0.5 ml at 1% concentration, was mixed with 0.4 ml PBS, and kept at 4, 16, 25, 32, 37, and 45℃ for 30 min each. After adding 0.1 ml of hemolysin at 10 HU_{50}/ml, making final concentration 1 HU_{50}/ml, hemolytic activity was measured as explained previously (5). For optimal pH, acetate buffer (pH 4, 5), acetate-citrate buffered saline (pH 4, 5, 6), phosphate buffered saline (pH 6, 7, 8), and Tris-HCl buffered saline (pH 7, 8, 9) were used.

Isoelectric focusing

Isoelectric point was decided according to O'Farrell's method (12). Detergent was not added to keep the hemolysin active for the activity staining. For resolving gel, 4.2% of acrylamide and 2.4% ampholyte was added to tertiary distilled water with ammonium persulfate and TEMED at normal concentration, 0.02 and 0.1%, respectively.

Ampholyte was prepared with Bio-lyte (BioRad, Richmond, CA) 5~7 range, 6~9 range, 3~10 range in 3 : 1 : 1 ratio to make gradient between 5~7 range. Overlaying buffer was prepared with Bio-lyte 5~7 range and 3~10 range at 9 : 1 ratio making 5% solution in tertiary distilled water. Sample buffer was made up with glycerol 60%, Bio-lyte 5~7 range 12%, 6~9 range 6%, and 3~10 range 2%, and mixed with the equal volume of sample solution. Each of NaOH, 0.1 N and H_3PO_4, 0.06% solutions was used for catholyte and anolyte, respectively. Total of 10 HU_{50} was analyzed under 400 V for

first 18 hrs and 800 V for 2 hrs, and pH was measured after fixing gel slices in 10 mM KCl solution for 30 min.

Effects of EDTA and $FeCl_3$ on the production of hemolysin

Effects of EDTA and ferric chloride on hemolysin production were estimated. In the presence of EDTA at the final concentration of 0.025, 0.05, and 0.25 mM, and $FeCl_3$ at 0.5, 1, 2.5, and 5 mM, *V. anguillarum* was cultured in BHI (0.85% NaCl) with shaking at 23℃ for 24 hrs with the equal volume of inoculum. Production of hemolysin was evaluated by the method mentioned above. In addition, bacterial growth itself was also measured to rule out the possibility of low level of hemolysin due to poor growth. Total amount of bacteria after incubation was measured by A_{600}.

Results and Discussion

Identification of hemolysin molecule

Hemolytic activity was evaluated on blood agar plate. Aiming at purification of hemolysin from *V. anguillarum,* concentrated culture supernatant was analyzed on non-denaturing gel electrophoresis. Gel slice of 5 mm width was laid on blood agar plate with human RBCs, and kept at R.T. for 20 min. Figure 1 shows hemolysis on blood agar by diffused hemolysin separated with electrophoresis.

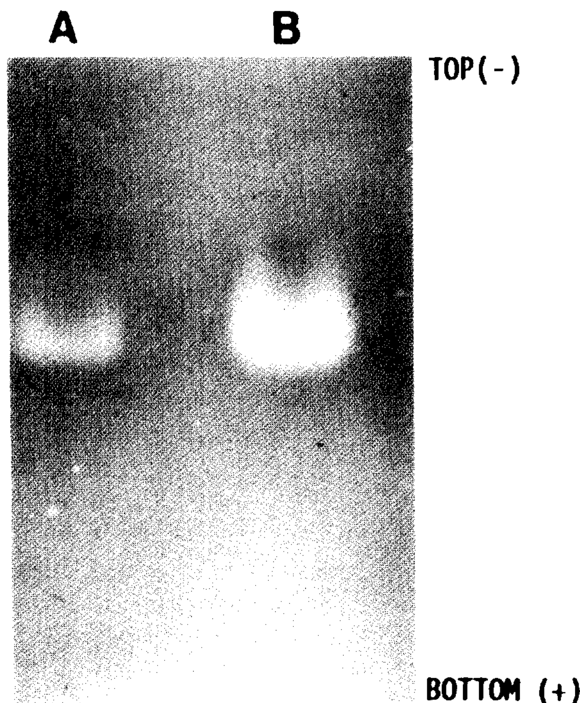

Fig. 1. The hemolysin molecules isolated on non-denaturing polyacrylamide gel. Hemolysin was separated on 7.5% native resolving gel, and gel slices were transferred to agar blood plate, after washing in PBS, where hemolysin was recongnized by lysed RBCs by diffused hemolysin. A, V7 culture lysate adjusted to 5 mg/ml PBS, B, V7 hemolysin from culture supernatant.

Hemolysin was initially separated on SDS-polyacrylamide gel, but determination of m.w. of hemolysin from *V. anguillarum* was hindered by SDS itself lysing RBCs. It should be purified first for m.w. estimation.

Nature of Hemolysin

Several different enzymes were treated to hemolysin which was partially purified and adjusted to 2 HU_{50}/ml. Only trypsin and proteinase K depleted hemolytic activity almost completely, while the activity was not affected at all by lysozyme, DNase, or RNase (Table 1). Hemolysin after trypsin or proteinase K treatment could not exert hemolysis with human RBC even reacted for 24 hrs. This implies that active site of vibrio hemolysin at least is protein in nature, as was shown in hemolysin from *E. coli,* and activity is not affected by biopolymers such as nucleotide or peptidoglycan (28, 30). Enzymes themselves did not destroy RBCs during evaluation.

Heat sensitivity

When hemolysin was kept at different temperatures, it was revealed that it was heat labile losing whole activity irreversibly when kept at the temperature above 56℃ (Figure 2). Most of hemolysin previously reported are heat sensitive. Hemolysin from *E. coli,* for example, was not stable if kept at 56℃ for 10 min (21, 24, 29). *Pseudomonas aeruginosa* has heat labile toxin with phospholipase C activity (10), and *Leptospira interrogans* also produces heat labile one with sphingomyelinase C (31). Some microbial toxins, however, are very heat stable as can be seen in hemolysins such as thermostable direct hemolysin (TDH) from *Vibrio parahemolyticus* (22) and a glycolipid, 2-*o*-*β*-hydroxydecanoyl-*β*-hydroxy-deca-

Table 1. Sensitivity of Vibrio hemolysin to various enzymes

Enzymes[a]	Concentration[b]	Relative activity (%)[c] after enzyme treatment
Trypsin	10 mg/ml	0.53
Proteinase K	100 μg/ml	0.68
Lysozyme	1 mg/ml	100.0
DNase	50 μg/ml	100.5
RNase	50 μg/ml	101.4

[a] RBCs for hemolysis evaluation was not affected by any enzymes used in this study, and hemolysin was incubated with each enzyme for 1 hr prior to activity estimation.
[b] Final concentration in reaction mixture.
[c] Compared with control (100.0), where no enzyme was added.

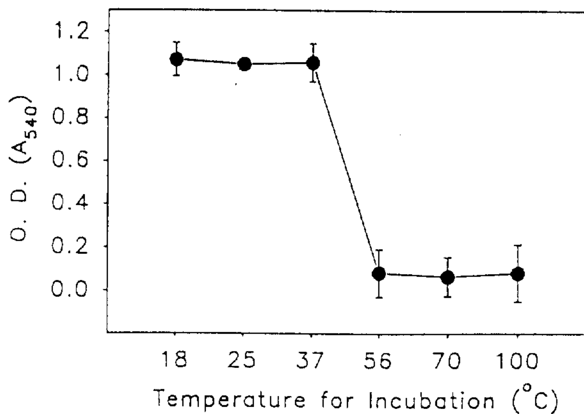

Fig. 2. Heat stability of Vibrio hemolysin at different temperature. Hemolysin, at 4 HU_{50}/ml, was kept for 10 min at different temperature, 18, 25, 37, 56, 70, and 100℃. Equal volume of 1% RBCs was mixed with heat treated hemolysin, and hemolytic activity was evaluated 1 hr later.

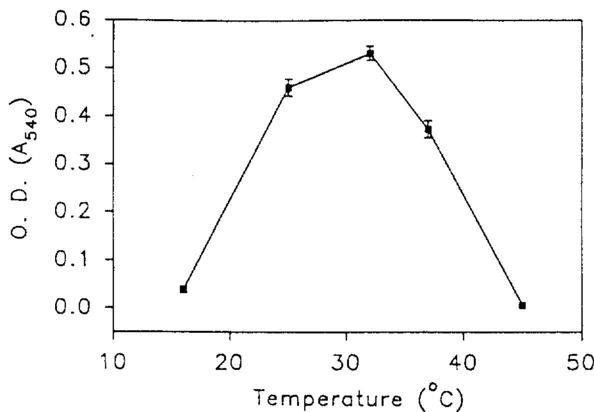

Fig. 3. Activity changes of Vibrio hemolysin at different temperature. Hemolysin, at final concentration of 1 HU_{50}/ml was added to each reaction mixture and incubated at given temperature for 30 min prior to activity estimation.

noate, from *Pseudomonas aeruginosa* (3).

Hemolysin from *V. anguillarum* strain V7 was stable for 1~2 weeks when kept at 4℃. Freezing at −20℃ also makes hemolysin lose half activity when thawed for use. Even at R.T., vibrio hemolysin gradually but very quickly lost its activity leaving about 1/8 activity after 20 hrs at R.T., even though it was quite stable for the first few hours.

Optimal reactive condition for hemolysin activity

Hemolytic activity at 32℃ was highest, as shown in Figure 3. Optimal temperature for hemolytic activity performed by *E. coli* hemolysin was 40~45℃, slightly higher than its optimal temperature for growth, 37℃. Hemolytic activity induced by bacterial cells was stronger at 25℃ than at 32℃. From the observation that cell free hemolysin destroys RBCs more easily at higher temperature

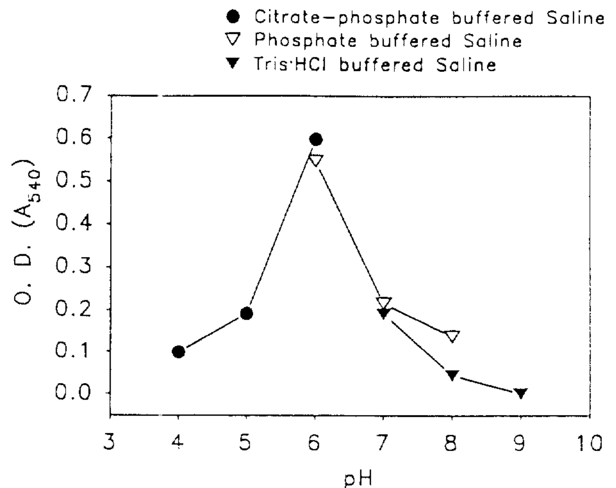

Fig. 4. Activity changes of Vibrio hemolysin at different pH. Hemolysin, at final concentration of 0.5 HU_{50}/ml was added to each reaction mixture adjusted to indicated pH and incubated at R.T. for 30 min prior to activity estimation.

than at 25℃, it is believed that increased membrane fluidity of RBCs at high temperature makes it easy for hemolysin to penetrate into plasma membrane unless its structure is not damaged at the given temperature.

Optimal pH for hemolytic activity was estimated to be 6 (Figure 4). RBCs were rather unstable at pH below 4.0 and above 9.0. Even if the activity was high at pH 6, most experiments were carried out at pH 7, simply because it is the physiological pH for RBCs.

Isoelectric focusing

Isoelectric focusing has been carried out for Vibrio hemolysin. Non-denaturing gel slice after electrophoresis was put on blood agar plate where hemolytic activity could be detected in 2~4 hrs. The pI was determined to be around 5.7, suggesting that Vibrio hemolysin is rather acidic (data not shown). Since the hemolytic activity was not evaluated using the same gel as was used for pI estimation, R_f was roughly estimated 0.7 even if it may not be accurate.

Hemolysin from *E. coli* was of pI 4.0~5.0 (28), while it was reported to precipitate at isoelectric point of pH 4.6 (24). It was also known that at the time of hemolysin production, pH of the media temporarily was lowered. The optimal pH for Vibrio hemolysin, however, was pH 6.

In general, outer surface of bacterial cellular membrane is charged negatively due to some polar head group such as sialic acid. The fact that optimal pH was 6 while pI was 5.7 for Vibrio hemolysin may stand for that reaction of hemolysin with cell membrane could be made easier due to increase in hydrophobicity with low net

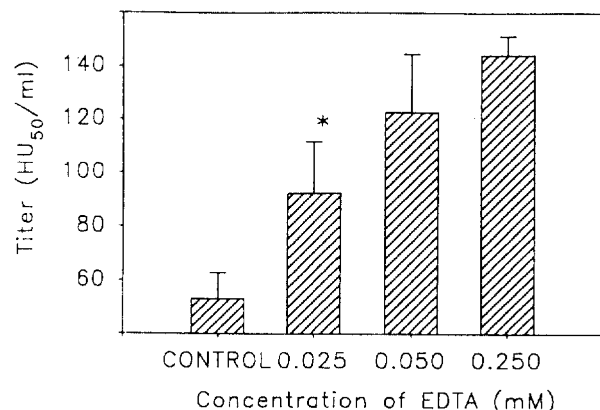

Fig. 5. The effect of metal-chelating agent EDTA on the induction of hemolysin production. EDTA, at the given concentration, did not destroy RBCs or inhibit cellular growth in culture.

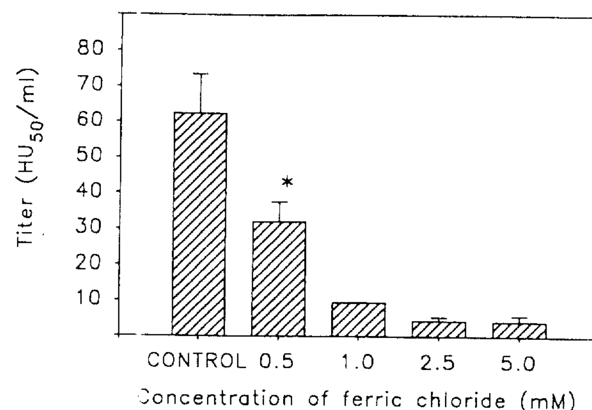

Fig. 6. The effect of ferric chloride on the induction of hemolysin production. Neither destruction of RBCs nor growth inhibition was detected at the given concentration of $FeCl_3$.

charge.

Effects of EDTA and $FeCl_3$ for Hemolysin Production

At the concentration over 0.5 mM, EDTA inhibited bacterial growth meanwhile growth was completely blocked at 5 mM or higher. But when EDTA was given at lower concentration than 0.5 mM, production of hemolysin increased in proportion to the amount of EDTA added (Figure 5). Up to 0.25 mM of EDTA concentration, total number of bacterial cells was slightly less than that of control group, implying total production of hemolysin is not directly dependent on the mass of bacterial cells but rather on the presence of EDTA.

When measured with the addition of $FeCl_3$, hemolysin production decreased as the concentration of $FeCl_3$ increased. No difference in biomass could be detected until the concentration increased up to 5 mM, and the mass of bacterial cells of experimental groups was bigger, instead, in comparison with that of control group. This also supports the concept that hemolysin was produced in relation with $FeCl_3$ rather than the number of cells. On the hemolysin from *E. coli,* it was reported that Fe ion plays an important role inhibiting hemolysin production at higher concentration than 100 mM (Figure 6) (20, 32). Crosa *et al.* (8) also found that whenever *V. anguillarum* has to overcome iron deficiency, it produces some factors which might comprise hemolysin we have been studying. In conclusion, we do believe that hemolysin production was induced to overcome iron deficiency which was caused, in return, by the addition of EDTA at least in this experimental study. Even growing in host animals, *V. anguillarum* would destroy RBCs overcoming iron deficiency caused by lactoferrin or transferrin of host animals.

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