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# Production of Hemolysin by a Fish Pathogenic Strain of *Vibrio damsela*

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*Keywords* : *Vibrio damsela*, hemolysin, fish pathogen, yellowtail

**Abstract**

Marine and fish isolates belonging to *Vibrio damsela* exhibited three characteristics consisting of hemolysin, serum resistance, and SDS-decomposition. Complete hemolysis by the cell suspension of *V. damsela* Yp-16 isolated from diseased yellowtail was observed after 30, 45, 90, and more than 300 min incubation for tilapia, eel, yellowtail, and human erythrocytes, respectively.

Hemolysin released by *V. damsela* Yp-16 was inhibited by adding $Mg^{++}$ ion to the reaction mixture with human erythrocytes, but not with tilapia erythrocytes. However, if the bacterial suspension or culture supernatant containing $Mg^{++}$ ion at a high concentration was dialyzed against 1 % NaCl, hemolytic activity for human erythrocytes was recovered substantially.

Hemolysin production in the bacterial cells was repressed by adding antibiotics such as neomycin, chloramphenicol, and polymyxin B to the bacterial suspension.

Partially purified hemolysin from the strain Yp-16 was inactivated completely after heat treatment at above 56℃ for 30 min. Optical temperature of the hemolysin for tilapia erythrocytes was found at 42℃.

*Vibrio damsela* has been reported to be a halophilic bacterium causing skin ulcers in temperature-water fish and wound infections in humans[1-3]. We also reported that an aerogenic bacterium isolated from diseased young fish of yellowtail *Seriola quinqueradiata* fed in aquaculture facilities in Japan was identified as *Vibrio damsela* and that the isolated strain gave rise to death for experimental fish of yellowtail by intramuscular injection of it[4,5]. In the previous paper[4], the isolate was also demonstrated to possess hemolytic activity for erythrocytes of various fish including yellowtail, tilapia, carp, and eel, as well as those of human and sheep.

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Pathogenic bacteria such as *Vibrio cholerae, Vibrio parahaemolyticus* and *Aeromonas hydrophila* are well known to produce various extracellular proteinous substances including hemolysins, cytotoxins, enterotoxins, and proteases which enable them to invade the epithelial cells of host animals[6,7]. Especially, *V. parahaemolyticus* has been demonstrated to produce at least four hemolytic constituents such as a thermostable direct hemolysin, a heat-labile direct hemolysin, phospholipase A, and lysophospholipase[6]. Out of these hemolytic substances, a thermostable hemolysin has been considered to be responsible for human pathogenicity[8,9].

In this paper, we describe the production of hemolysin by a strain of *Vibrio damsela* isolated from diseased yellowtail.

## Materials and Methods

*Organisms and growth conditions*

A selected organism, *Vibrio damsela* Yp-16 was isolated from diseased young fish of yellowtail *Seriola quinqueradiata* and characterized as reported in previous papers[4,5]. Marine *Vibrio* isolates and *Vibrio* standard strains used in this study were also described in previous papers[4,5]. Unless otherwise indicated, test strains were cultured at 25℃ in Z-B II liquid medium containing polypepton 5 g, yeast extract 1 g, and half strength artificial sea water (ASW) 1 ℓ (pH 7.6). Ordinary test tubes and L-shape test tubes containing 10 mℓ Z-B II medium were used for static culture and shaken culture, respectively.

*Preparation of erythrocyte suspensions*

Fish or human blood was mixed with phosphate buffered saline containing $Mg^{++}$ and $Ca^{++}$ ions ($PBS^{++}$) and 1% sodium citrate in a plastic centrifuge tube. The blood mixture was centrifuged at 3,000 rpm for 15 min. A supernatant solution was discarded and blood cells were resuspended in $PBS^{++}$. This process was repeated 3 times to obtain washed erythrocytes. Erythrocytes were reconstituted to a final concentration of 2% (v/v) in $PBS^{++}$.

*Hemolytic activity*

Hemolytic activity was determined by two methods. One method was made by counting degradated erythrocytes microscopically. One mℓ of the erythrocyte suspension in $PBS^{++}$ ($1.6\times10^6-2.0\times10^6$ cells/mℓ) and one mℓ of the supernatant or cell suspension from bacterial cultures were mixed and incubated at 25℃ for 5 hr. At certain incubation times, the reaction mixture was removed to count erythrocytes on haematocytometer by use of a light microscope or phase contrast microscope (Nikon Diaphoto TMD x200). The other one was to measure optical density of the supernatant containing hemoglobin released from lysed erythrocytes. After the above reaction

mixture was incubated at 30℃ for 30 min, the test tubes were centrifuged at 2,000 rpm for 5 min. The supernatant obtained was transferred to a cuvette and the optical density at 540 nm was measured in a spectrophotometer (Shimadzu MPS-2000).

*Partial purification of hemolysin*

*Vibrio damsela* Yp-16 was grown at 25℃ in 300 mℓ of Z-B II broth under shaking condition. The culture supernatant was separated by centrifugation of the culture two times at 5,000 rpm for 20 min. Proteinous substances were precipitated between 45 −60 % ammonium sulfate saturation, solved in 5 mℓ of $PBS^{++}$ and then dialyzed overnight against $PBS^{++}$.

## Results

*Hemolysis and serum sensitivity*

Table 1 shows hemolysis, SDS decomposition, and serum sensitivity of test organisms. Hemolysis was estimated after incubation of test strains on agar plates containing 10 % human blood (v/v). Serum sensitivity of test strains was determined

**Table 1.** Specific characters of *Vibrio* isolates

| Strain | Hemo-lysis | Serum resist. | Halo (Z/SPT) | Strain | Hemo-lysis | Serum resist. | Halo (Z/SPT) |
|---|---|---|---|---|---|---|---|
| Yp-1 | ++ [1] | R [2] | − [3] | VR-1 | − | S | − |
| Yp-5 | ++ | S | − | VR-2 | ++ | | − |
| Yp-16 | ++ | R | ++ | VR-3 | − | Rw | − |
| Va-B | + | S | − | VR-4 | − | | − |
| V-7 | ++ | S | − | VR-5 | ++ | S | − |
| TV-7t | ++ | S | + | VK-8 | ++ | S | − |
| VD-39 | ++ | R | ++ | VK-9 | ++ | | − |
| VV-62 | ++ | S | + | VT-11 | ++ | | − |
| Dy-1 | ++ | R | ++ | DS-21 | ++ | R | ++ |
| Dy-3 | ++ | | ++ | DS-23 | − | S | − |
| Dy-6 | ++ | | ++ | DS-24 | − | S | − |
| DR-1 | ++ | R | ++ | DS-25 | − | S | − |
| DR-2 | ++ | R | ++ | DS-26 | − | S | − |
| DR-3 | ++ | R | ++ | DS-27 | + | R | ++ |
| DR-5 | − | S | − | DS-28 | + | Rw | − |
| DR-7 | − | S | − | DS-30 | − | S | − |
| DK-10 | ++ | S | − | DS-31 | − | S | − |
| DK-15 | ++ | S | − | DS-32 | − | S | − |
| DN-21 | + | Rw | ++ | DS-45 | − | S | − |
| DR-24 | ++ | R | ++ | DS-49 | − | S | − |
| DR-25 | + | R | ++ | DS-50 | − | Rw | − |
| DR-30 | ++ | S | − | DS-58 | − | Rw | − |

[1] Hemolysis on human blood agar, ++>+>−.

[2] Resistance against human serum (1/2 dilution).
R, resistant ; Rw, weak resistant ; S, sensitive.

[3] Ratio of halo diameter on Z/SPT agar, ++>+>−.

against human serum (1/2 dilution). SDS decomposition by test strains was characterized by a turbid halo around the periphery of the colony on Z/SPT agar plates containing 0.1 % SDS described in previous paper[5]. Marine *Vibrios* possessing a halo production on Z/SPT agar exhibited hemolysis and serum resistance against human blood at the same time. *Vibrio damsela* standard strain VD-39 (ATCC 33539) also showed these three characters. Consequently, the halo production on Z/SPT agar could turn out to be a diagnostic character for *Vibrio damsela.*

*Hemolytic activity for different erythrocytes*

As shown in Fig. 1, complete hemolysis was observed after 30, 45, 90, and more than 300 min for tilapia, eel, yellowtail, and human erythrocytes, respectively. When $Mg^{++}$ ion (70 mM) was added to the bacterial cell suspension in 1 % NaCl solution, hemolysis was retarded significantly for all kinds of erythrocytes. Fig. 2 illustrates

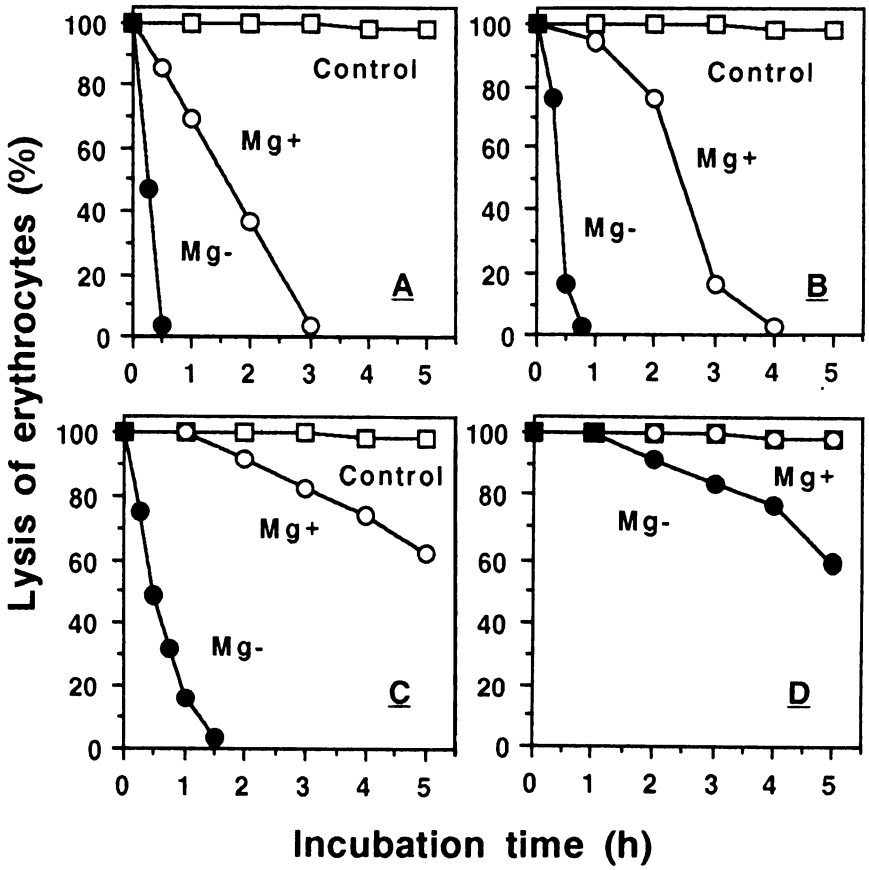


**Fig. 1.** Hemolysis of various erythrocytes by cell suspension of *V. damsela* Yp-16.
A, erythrocytes of tilapia ; B, eel ; C, yellowtail ; D, human.
$Mg^{+}$, bacterial cells suspended in 1% NaCl with 70 mM $MgCl_2$ ;
$Mg^{-}$, bacterial cells suspended in 1% NaCl.

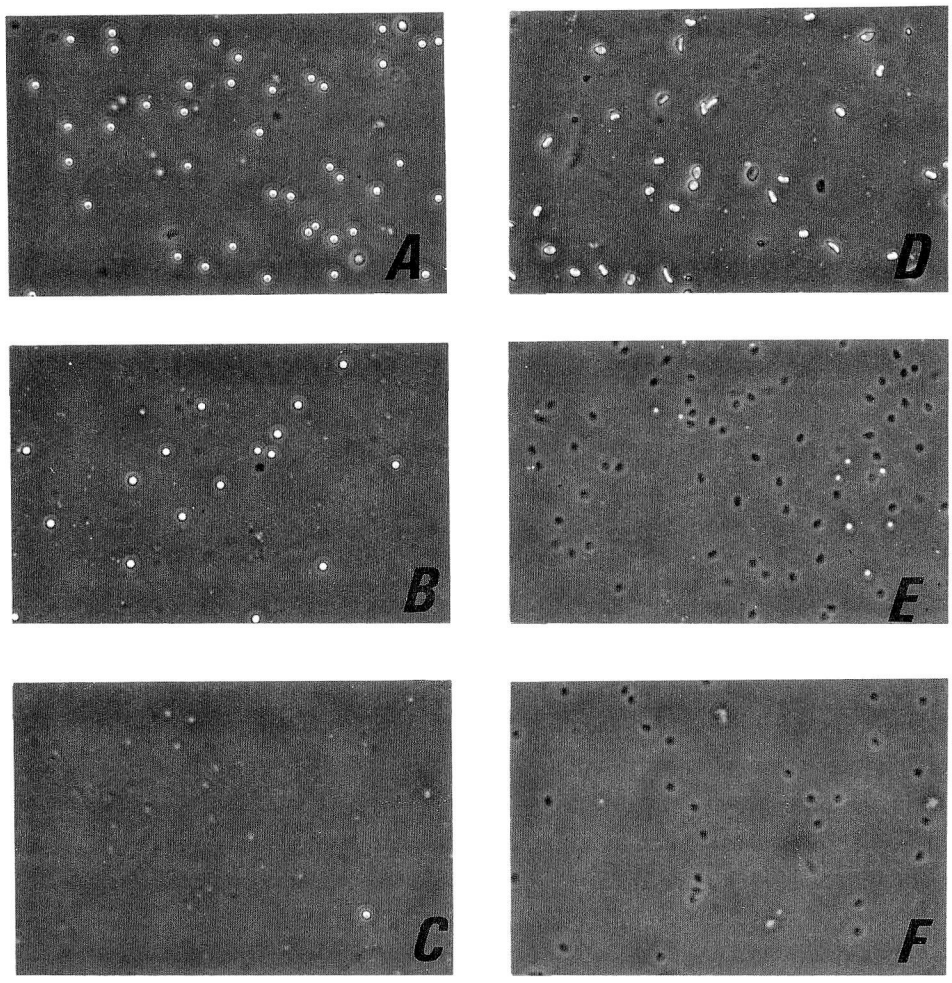


**Fig. 2.** Phase-contrast micrographs of erythrocytes incubated with a bacterial cell suspension.
A, human erythrocytes at 0 time ; B, at 20 min ; C, at 60 min ;
D, tilapia erythrocytes at 0 time ; E, at 5 min ; F, at 30 min.

the degradation process of human and tilapia erythrocytes by using a phase-contrast microscope. In 5 or 20 min incubation of tilapia or human erythrocytes with *V. damsela* Yp-16 cell suspension, refractive erythrocyte cells began to decrease and ghost cells or cell debris increased in the field of observation.

*Effect of $Mg^{++}$ ion of suspension solution on hemolysis*

Hemolytic activity in bacterial cell suspension was observed to vary with the composition of suspension solutions. Fig 3. demonstrates hemolytic activities in the supernatant from cell suspension prepared with various kinds of solution. When bacterial cells were suspended at 25℃ for 1 h in 1 % NaCl and 40 mM $MgCl_2$ solution,

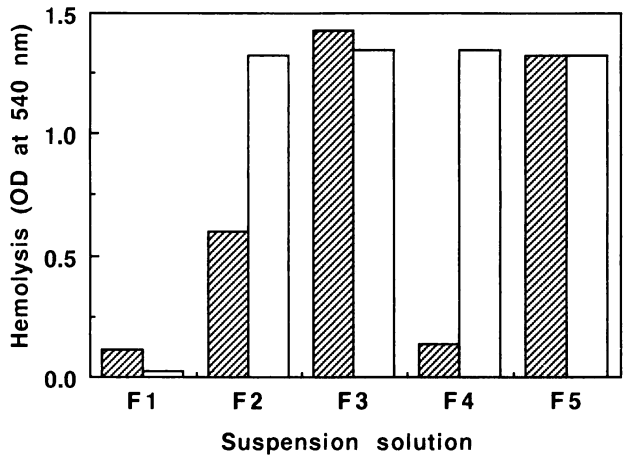


**Fig. 3.** Hemolytic activity in the supernatant from various cell suspensions. F1, 1% NaCl with 40 mM $MgCl_2$ ; F2, F1 after dialysis against 1% NaCl ; F3, 1% NaCl ; F4, F3 added with 40 mM $MgCl_2$ ; F5, F4 after dialysis against 1% NaCl ; ▨, human erythrocytes ; □, tilapia erythrocytes.

hemolytic activity of the supernatant from cell suspension was very low. However, this supernatant was dialyzed against 1 % NaCl solution and as a result hemolytic activity was recovered substantially. On the other hand, hemolytic activity was very high in the supernatant from cell suspension in 1% NaCl solution. On addition of 40 mM $MgCl_2$ to the suspension solution, hemolysis dropped sharply for human erythrocytes but it was not changed for tilapia erythrocytes. Dialysis of the suspension solution added with $MgCl_2$ against 1% NaCl solution brought about a recovery of hemolytic activity for human erythrocytes.

*Effect of antibiotics on hemolysin production*

The bacterial cells harvested were suspended in 1 % NaCl solutions added with various kinds of antibiotics (100 μg/mℓ). After 60 min incubation at 25℃, the cell suspensions were centrifuged to separate the supernatant from cell fractions. As

**Table 2.** Effect of antibiotics on hemolysin production

| Erythrocytes | Fraction | Relative activity (%) for hemolysis | | | | |
|---|---|---|---|---|---|---|
| | | SM[3] | NM | PcA | CP | PB |
| Human | Super.[1] | 93 | 1 | 100 | 1 | 57 |
| | Cells[2] | 99 | 2 | 100 | 32 | 9 |
| Tilapia | Super. | 100 | 1 | 100 | 0 | 40 |
| | Cells | 97 | 3 | 100 | 32 | 6 |

[1] Super., Supernatant fluid from bacterial cell suspensions.
[2] Cells, precipitated cells from bacterial cell suspensions.
[3] SM, streptomycin ; NM, neomycin ; PcA, ampicillin ; CP, chloramphenicol ; PB, polymyxin B.

shown in Table 2, streptomycin (SM) and ampicillin (PcA) did not affect hemolysin production of the tested strain whereas neomycin (NM) repressed it completely. In the case of chloramphenicol (CP), hemolytic activity was not observed in the supernatant fraction and 30 % activity was detected in the cell fraction. On the other hand, polymyxin B (PB) caused hemolytic activity to reduce to about 50 % and to 5−10% for the supernatant and cell fractions, respectively.

*Hemolysin production in bacterial culture*

Hemolysin production was compared between static and shaken tube cultures as illustrated in Fig. 4. For each case, straight test tubes and L-shape test tubes containing 10mℓ Z-B II broth were used. In static cultures the culture supernatant showed very low in hemolytic activity for human erythrocytes compared with the cell frac-

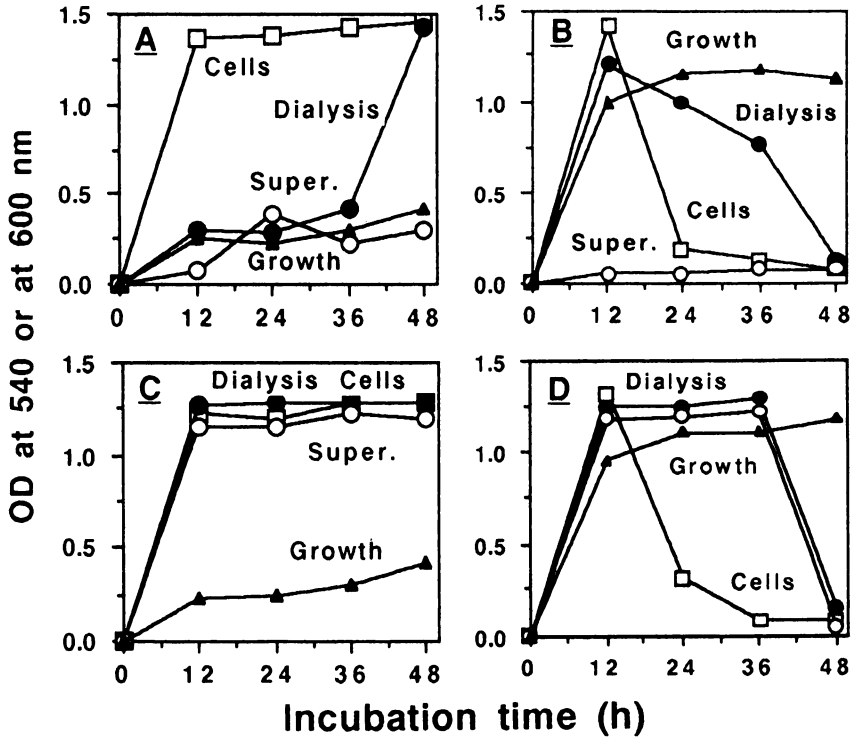


Fig. 4. Time course of hemolysin production in static and shaken cultures. Hemolysis was determined at 540 nm and the bacterial growth was determined at 600 nm spectrophotometrically.
A, for human erythrocytes in static culture ; B, for human erythrocytes in shaking culture ; C, for tilapia erythrocytes in static culture ; D, for tilapia erythrocytes in shaking culture.
Super., supernatant from a bacterial culture ; Dialysis, dialysis of the supernatant against 1% NaCl ; Cells, cell suspension from a bacterial culture.

tion, but both fractions were not different in hemolytic activity for tilapia erythrocytes. In shaken cultures, dialysis of the culture supernatant against 1 % NaCl solution gave rise to increase in hemolytic activity for human erythrocytes. Bacterial growth in shaken cultures was more rapid than in static cultures, therefore hemolytic activities of both in the supernatant and cell fractions decreased earlier in shaken cultures than in static cultures. In fact, hemolytic activity in the cell fraction dropped after 12 h in shaken culture, whereas that in the supernatant fraction decreased after 36 h.

*Effect of temperature on hemolytic activity*

Fig. 5 illustrates an Arrhenius plot for hemolytic activity in a partially purified fraction. The reaction rate of hemolysis increased with temperature until 42 ℃ and level off from 42° to 56 ℃. At above 56 ℃ it exhibited a rapid decrease probably because of heat inactivation of hemolysin. Hemolysin released by *V. damsela* Yp-16 into the culture supernatant began to be inactivated by heat treatment of it at above 30 ℃ for 30 min and was inactivated completely at 56℃ as shown in Fig. 6.

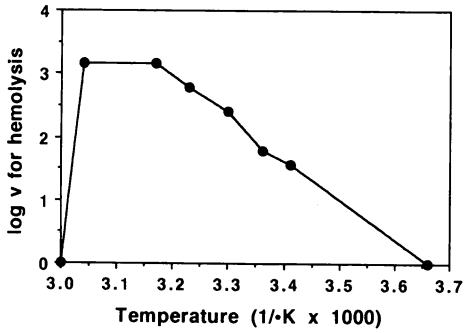


Fig. 5. An Arrhenius plot for hemolytic reaction rates.
Reaction rates were measured in optical density at 540 nm per min.

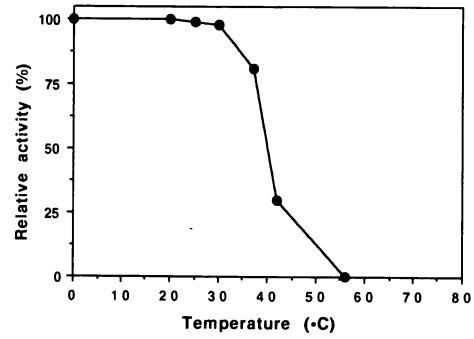


Fig. 6. Effect of temperature on the stability of hemolysin.
Hemolysin in $PBS^{++}$ was heated at each temperature for 30 min.

## Discussion

*Vibrio damsela* has been isolated from fish, marine animals, and humans with skin ulcers or wound infections. Most of the pathogenic strains were found to give cytotoxic and hemolytic effects to the host animal cells. Marine and fish isolates belonging to *V. damsela* showed three characteristics consisting of hemolysis, serum resistance, and SDS-decomposition at the same time. Hemolysin produced by *V. damsela* Yp-16 isolated from diseased yellowtail was inactivated completely after treatment at 56℃ for 30 min, therefore it should be referred to a heat-labile one. Kreger[10] also demonstrated that the hemolytic activity of *V. damsela* cytolysin was inactivated by

heat treatment at 56℃ for 30 min. The rate of hemolysis by the hemolysin of *V. damsela* Yp-16 was optimal at 42℃ and at pH 7–8, in accord with that reported by Kothary and Kreger[11]. In the case of *Vibrio parahaemolyticus*, it has been reported that the organism produce a thermostable direct hemolysin (TDH) and a heat-labile direct hemolysin (LDH). Zen-Yoji *et al.*[12] described that TDH from *V. parahaemolyticus* was inactivated by heating at 60℃ for 5 min but not significantly inactivated by heating at 100℃ for 30 min.

Hemolysin released by *V. damsela* Yp-16 was inhibited by adding $Mg^{++}$ ion to the reaction mixture with human erythrocytes, but not with tilapia erythrocytes. However, if the bacterial suspension or culture supernatant containing $Mg^{++}$ ion at a high concentration was dialyzed against 1 % NaCl, hemolytic activity for human erythrocytes was recovered substantially. Furthermore, the recovered hemolytic activity was inhibited again by adding $Mg^{++}$ ion to the reaction mixture with human erythrocytes, but not with tilapia erythrocytes. Identically the hemolysin solution added with at least more than 10 mM of $Mg^{++}$ ion was reactivated for human erythrocytes by dialysis of it against 1 % NaCl solution. These facts suggest that hemolysin (s) released from the bacterial cells into the supernatant binds specific receptors of the erythrocyte membrane and that the binding process is inhibited by $Mg^{++}$ ion at a high concentration for human erythrocytes but not for tilapia erythrocytes. The agglutination of human erythrocytes with exohemagglutinins (HA) from marine vibrios was reported to be inhibited by L-fucose and D-arabinose[13].

On the other hand, Kreger[10] described that the yield of cytolysin in the culture supernatant of *V. damsela* was optimal in medium containing 1.3 % NaCl and was significantly reduced in culture medium containing above 2 % NaCl. However, he remarked that it was not known whether the decreased yield of cytolysin in culture medium containing above 2 % NaCl was caused by $Na^{+}$ ion-mediated inhibition of cytolysin production or inhibition of cytolysin release. Further work is needed to ascertain the chemical structure of hemolysin receptors of fish and human erythrocytes and the mechanism of inhibition of *V. damsela* hemolysin with $Mg^{++}$ ion.

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