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Study on the Hemolysin from Marine *V. vulnificus*

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A halophilic *V. vulnificus* is an estuarine microorganism that has been associated with fatal wound infection and life-threatening septicemia. Hemolysin is defined as toxic substance produced by various species of bacteria including *V. vulnificus*. Hemolysin from marine *V. vulnificus* was purified and the effect of pH, temperature, metal ion on the activity of hemolysin, and thermostability of hemolysin were tested in this study. Hemolysin lysed the sheep red blood cell and the optimum pH was 8.0, the optimum temperature was 40℃, and K^{+} increased but Mn^{2+} decreased the hemolytic activity of hemolysin, but hemolysin was unstable to heat.

Key words : hemolysin, pH, temperature, metal ion

1. Introduction

Several new vibrio species have been recognized as possible causes of human diseases. They are slightly halophilic bacteria and inhabit marine and estuarine area that has been associated with severe and frequently fatal wound infections and life-threatening septicemia, particulary in immunocompromised individuals or in patients with pre-existing hepatic diseases. *Vibrio vulnificus*(Roland, 1970) causes two types of infection : septicemia and wound infections(Azuma et al., 1984 ; Blake et al., 1980). This vibrio produces many substances associated with pathogenicity, one of which is a hemolysin that can cause disruption of the membrane of most mammalian erythrocyte(Gray and Kreger, 1985). It possesses cytotoxic effects against Chinese hamster ovary cells, has the ability to accelerate vascular permeability in guinea-pig skin, and shows a lethal action against mice(Kreger and Lockwood, 1981). An antigenic protein, *V. vulnificus* hemolysin, is produced in maximal amounts at the mid- to late-exponential growth phase. A radiolabeled probe to the hemolysin gene provides specific confirmation of both environmental and clinical isolates of *V. vulnificus*(Wright et al., 1985). Hemolysin production has been reported in all *V. vulnificus* isolates tested by numerous investigators, including 12 isolates tested by Johnson and Calia(1981). In colloid-osmotic hemolysis, the initial pore size of the erythrocyte membrane formed by the hemolysin is small. The effective diameter of the pore size formed by E. coli α-hemolysin is about 2-3 nm(Bhakdi et al., 1986) and that of lesions present in the membrane after treatment with *S. aureus* α-toxin is 2.5 nm(Fussle et al., 1981). It is generally considered that positively-charged ions cannot pass through the membrane. However, pore formed by the hemolysin can allow such ions to permeate the

cells. If hemoglobin, with an effective diameter of 4.8 nm, cannot pass through the pore formed by the hemolysin, its release in hemolysis could have occurred by cell lysis.

The present paper showed the purification of hemolysin from marine *V. vulnificus* and examined the effect of pH, temperature, metal ion on the activity of hemolysin, and thermostability of it.

2. Materials and Methods

2.1 Reagent and apparatus

The following reagents and apparatuses were used. Phenyl-Sepharose CL-4B, 10mM Tris/HCl buffer(pH 7.5), 0 - 0.5M NaCl, liquid chromatography(LC), anion-exchange column(5 x 50 mm), and spectrophotometer were used for the purification of hemolysin. Sheep blood, Alserver solution, phosphate buffered saline, Tris buffered saline, 0.9% NaCl, 0.1% bovine serum albumin, and incubator were used for the assay of hemolysis. 50mM Sodium citrate buffer, 50mM sodium acetate buffer, and 50mM sodium phosphate buffer were used for knowing the effect of pH on the hemolytic activity. 25mM Metal(K, Ca, Mg, and Mn) cations were used for estimating the effect of metal ions on the hemolytic activity.

2.2 Purification of hemolysin

Crude hemolysin was obtained from the culture filtrate by ammonium sulfate fractionation and hemolysin was purified from the crude preparation by column chromatography on phenyl-Sepharose CL-4B by the method of Gray and Kreger (1985) and by liquid chromatography(LC). The vibrio was cultivated in heart infusion broth at 37℃ for 16 h and the culture supernatant was obtained by centrifugation at 7,000 x g for 30 min. Ammonium sulfate was added to the supernatant to 60% saturation. The precipitate was collected by centrifugation, washed once with 60% saturated ammonium sulfate solution, and dissolved in small amount of 20mM phosphate buffer. The solution was dialyzed against the buffer overnight and centrifuged at 7,000 x g for 30 min, and the brown colored supernatant was further fractionated by LC. LC was performed as follows. The partially purified hemolysin obtained from the hydrophobic column chromatography was loaded on anion-exchange column(5 x 50 mm) equilibrated with 10mM Tris/HCl buffer(pH 7.5)(Miyoshi et al., 1986). The column was washed with 10mM Tris buffer followed by gradient elution with 0 - 0.5M NaCl solution in 10mM Tris buffer. Hemolysin was obtained as a single peak eluting at around 0.2M NaCl. The amount of hemolysin was measured by the absorbance at 280 nm using spectrophotometer. One hemolytic unit was defined as the amount of hemolysin which elicited 50% hemolysis upon incubation at 37℃ for 1h.

2.3 Assay of hemolytic action

Hemolytic action was assayed by measuring the amount of hemoglobin released from sheep red blood cell(SRBC) suspension treated with hemolysin (Shinoda et al., 1985). Sheep blood was collected from sheep in modified Alserver solution and centrifuged at 1,500 rpm for 10 min. The obtained SRBC was washed with distilled water and used. SRBC was added to the phosphate buffered saline (final concentration was 1.0%). One mL of the serially diluted hemolysin solution was mixed with 1 mL of 1.0% sheep erythrocyte suspension and incubated at 37℃. After 1 h incubation, the reaction mixtures were centrifuged at 1,000 x g for 5 min and absorbance of the supernatant

at 540 nm was measured. The hemolysis was observed after incubation at 37℃ for 24 h.

2.4 Effect of pH on the activity of hemolysin

Hemolytic activity over a pH range of 3.0 - 11.0 was tested. Hemolysin was assayed in 50mM sodium citrate buffer(pH 3.0 and 4.0), 50mM sodium acetate buffer(pH 5.0, 6.0, and 7.0), and 50mM sodium phosphate buffer(pH 8.0, 9.0, 10.0, and 11.0).

2.5 Effect of temperature on the activity of hemolysin

Sample of hemolysin preparation was added to assay mixture and the hemolytic activity was measured by incubating the mixture over a temperature range of 20 - 60℃ at optimum pH.

2.6 Effect of inorganic salt and metal ion on the activity of hemolysin

The effect of inorganic salt on the production of hemolysin from *V. vulnificus* was investigated and the residual activity was measured to know the effect of metal ion on the activity of hemolysin by adding 25mM metal cations.

2.7 Measurement of the thermostability of hemolysin

The purified hemolysin was diluted with glycine-NaOH buffer and heated for 10 min at each temperature. Erythrocyte suspension was added and centrifuged, and then the residual hemolytic activity was tested.

3. Results and Discussion

3.1 Optimum pH of hemolysin

Activity of hemolysin in buffers of varying pH values after bacterial cells were cultured for 9 h was given in Fig. 1. The

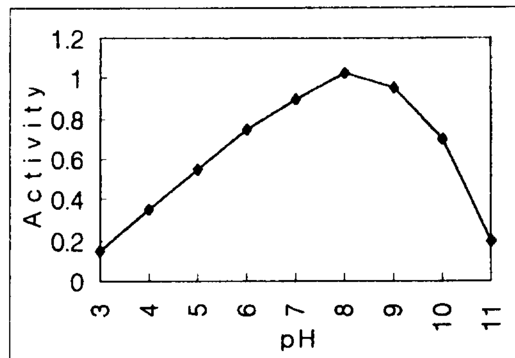

Fig. 1. Effect of pH on hemolytic activity of hemolysin.

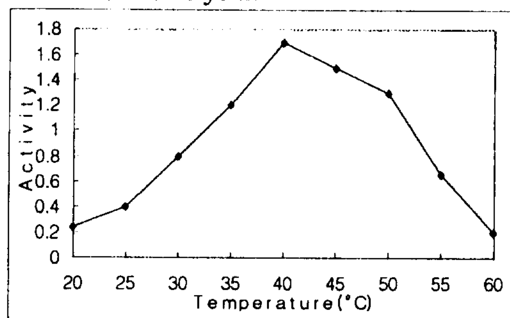

Fig. 2. Effect of temperature on hemolytic activity of hemolysin.

hemolysin was active for pH in the range of pH 5.0 to 10.0 and the maximal activity was found around pH 8.0. Because hemolysin is more hydrophobic in acidic condition, the molecule has a tendency to form aggregate or complex with other molecule at this pH. This seemed to be the reason that hemolysin was co-eluted with colored substances. Under the conditions for the second chromatography hemolysin was more soluble, because pH was 8.0 and a detergent was also added. At this step, binding capacity of the column for hemolysin at pH 8.0 was adequate because the major protein of the preparation was hemolysin. Thus fully purified hemolysin was obtained by the brief method.

3.2 Optimum temperature

V. vulnificus was known to be well

Table 1. Effect of inorganic salt on the production of hemolysin

inorganic salt	activity (unit/mL)	growth (A_{660})	pH
$BaCl_2$	4.7	2.14	7.8
$CaCl_2$	6.3	2.51	7.5
KCl	9.1	2.63	8.2
$MgCl_2$	5.2	2.15	8.3
$MnCl_2$	1.2	0.40	7.2
$(NH_4)_2SO_4$	5.6	2.03	8.6

Table 2. Effect of metal ion on hemolytic activity of hemolysin

metal ion[a)]	relative hemolytic activity[b)]
-	100
K^+	115
Ca^{2+}	96
Mg^{2+}	94
Mn^{2+}	20

a) The chloride salt of each metal ion was used.
b) A_{540} with metal ion / A_{540} without metal ion X 100

Table 3. Thermostability of hemolysin

heat treatment	dilution ratio						
	2	4	8	16	32	64	128
untreatment	45	36	12	4	+	-	-
56°C, 10min	0.5	0.3	-	-	-	-	-
100°C, 10min	-	-	-	-	-	-	-

+ : partial hemolysis
− : no hemolysis

grown at higher temperature and the production of hemolysin was investigated at each temperature. As shown in Fig. 2, the highest activity was obtained when incubated at 40℃.

3.3 Effect of inorganic salt and metal ion

Table 1 showed the effect of inorganic salt on the production of hemolysin. KCl produced the largest amount of hemolysin, but $MnCl_2$ the smallest. The effect of metal ion on the activity of hemolysin was shown in Table 2. Monovalent metal ion, K^+ increased the hemolytic activity and divalent Ca^{2+} and Mg^{2+} had no effect, but divalent Mn^{2+} decreased the activity.

3.4 Thermostability of hemolysin

Hemolysin did not have the hemolytic activity when it was heated for 10 min at 100℃ as shown in Table 3. The residual activity was shown until only it was diluted four fold at 56℃, so hemolysin was thought to be unstable to heat.

Hemolysis by hemolysin followed a two-step process : a temperature-independent membrane-binding step and a temperature-dependent cell disruption step (Shinoda et al., 1985 ; Gray and Kreger, 1985). In the latter step, both K^+ and hemoglobin release were observed above 15℃, but not below 10℃. The temperature-dependent cell disruption step was further divided into two steps : a change in membrane permeability(transmembrane-pore formation) and an erythrocyte-bursting stage. When the erythrocyte membrane was observed by electron microscopy after complete hemolysis, 10 - 40 nm pore was observed. This suggested that large membrane pore was finally formed in the erythrocyte-bursting step. *Vibrio vulnificus* hemolysin is a unique hemolysin with cholesterol-binding ability like streptolysin O, but with a different mode of action in the release of hemoglobin. Exact role of *V. vulnificus* hemolysin in the *V. vulnificus* infection and precise mechanism of cell lysis by hemolysin still remain to be investigated.

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해양 *V. vulnificus*의 Hemolysin에 관한 연구

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호염기성 *V. vulnificus*는 연안에 존재하는 미생물로서 치명적인 상처 감염과 생명을 위협하는 패혈증과 관련이 있다. Hemolysin은 *V. vulnificus*를 포함하는 여러 가지 세균종에 의해 생성되는 독성 물질이다. 본 연구에서는 해양 *V. vulnificus*에서 hemolysin을 정제한 후 hemolysin의 활성에 미치는 pH, 온도 및 금속 이온의 영향을 조사하였다. Hemolysin은 sheep red blood cell을 용혈시켰으며 hemolysin의 최적 pH는 8.0, 최적 온도는 40℃이었으며 K^+ 이온은 hemolysin의 활성을 증가시켰으나 Mn^{2+}는 감소시켰다. 그러나 hemolysin은 열에 불안정하였다.