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ISOLATION AND PARTIAL PURIFICATION OF THERMOLABILE DIRECT HEMOLYSIN OF *VIBRIO PARAHAEMOLYTICUS*[1]

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SUMMARY Thermolabile direct hemolysin was isolated from the culture filtrate of *Vibrio parahaemolyticus*. It was partially purified by successive column chromatographies on DEAE-cellulose and DEAE-Sephadex A-50. The partially purified hemolysin was thermolabile and its activity was destroyed by heating at 70 C or 100 C for 10 min. Its activity was not enhanced by addition of lecithine. Sheep erythrocytes were less sensitive than human erythrocytes to the hemolysin and horse erythrocytes were insensitive.

The Arrhenius effect, demonstrated with staphylococcal α-toxin, was observed with crude hemolysin of *Vibrio parahaemolyticus*. The possible mechanism of this effect was discussed.

INTRODUCTION

Vibrio parahaemolyticus was first isolated at autopsy from a case of food-poisoning and its hemolytic character was demonstrated (Fujino et al., 1953). Kawamura (1964) first studied the nature of the hemolysin produced by *V. parahaemolyticus* and reported that it was not a protein and was heat-stable. On the other hand, Kato et al. (1966) reported that the hemolysin of *V. parahaemolyticus* had the nature of a protein and was free from polysaccharide and lipid. They also reported a relation between the production of the hemolysin and the pathogenicity of this organism. Zen-Yoji et al. (1971) recently reported the purification of heat-stable hemolysin of *V. parahaemolyticus*.

We (Fujino et el., 1969) and Yanagase et al. (1970) reported the existence of a thermolabile hemolysin in the culture filtrate of *V. parahaemolyticus*. This paper describes the isolation and the partial purification of this thermolabile direct hemolysin and possible differences between the hemolysin reported here and those reported by other workers are discussed.

MATERIALS AND METHODS

1. *Strain and cultivation of organisms*

Vibrio parahaemolyticus WP-1 (04: K12), isolated from a patient with gastroenteritis by Dr. M. Hori of Wakayama Prefecture Public Health Research In-

1 Part of this work was presented at the 4th Symposium on *Vibrio parahaemolyticus* at Miyazaki on Oct. 31, 1969.

stitute, was used throughout. The medium used for cultivation of the organism had the following composition; sodium chloride 30 g, peptone (Difco) 10 g, $Na_2HPO_4 \cdot 12H_2O$ 5 g, glucose 5 g, in 1000 ml of distilled water. The pH was adjusted to 7.6–7.8. One ml of culture in this medium was inoculated into 2000 ml of the same medium in a 6500 ml Erlenmeyer's flask and incubated at 37 C for 15 hr with shaking (60 cycles/min).

2. *Preparation of crude hemolysin from the culture filtrate of the bacteria*

Solid ammonium sulfate (45 g per 100 ml) was added to the culture filtrate and the resulting precipitate was dissolved in a small amount of phosphate buffer (0.01 M, pH 7.0). This solution was dialyzed overnight against the same buffer. Then it was centrifuged at 10,000 rev/min for 30 min and 0.2 N acetic acid was added in the resulting supernatant fluid to adjust the pH to 3.0. The resulting precipitate was collected by centrifugation and suspended in a small amount of 0.01 M phosphate buffer, pH 7.0.

3. *Determination of hemolytic activity*

To estimate hemolytic activity, 0.25 ml of the fraction was serially diluted two-fold with 0.01 M phosphate buffer (pH 7.0) containing 0.9% sodium chloride. A 0.25 ml aliquot of a 1% suspension of erythrocytes (about 8.3×10^7 cells/ml) was added each tube. Erythrocytes (human, unless other wise specified) were washed three times with saline before use. The mixtures were incubated at 37 C for 2 hr and then kept in the cold (4 C) for about 12 hr. Hemolytic activity was expressed as follows: 3+, complete hemolysis; 2+, + and ±, degrees of partial hemolysis; and −, no hemolysis.

4. *Preparation of DEAE-cellulose and DEAE-Sephadex columns*

DEAE-cellulose (Brown Co., USA, capacity = 0.85 mEq/g) was activated as described by Peterson and Sober (1962) and buffered with 0.01 M phosphate buffer (pH 7.0). DEAE-Sephadex A-50 (Pharmacia, Sweden) was activated and buffered with 0.01 M phosphate buffer (pH 7.0). A column (2.5 cm × 35 cm) was prepared. Columns were prepared by allowing the buffered materials to settle by gravity.

5. *Other materials*

Purified egg yolk lecithine was a gift from Dr. Y. Yanagase. Sheep and horse erythrocytes were supplied by Dr. K. Ukawa, Kannonji Institute of the Researeh Foundation for Microbial Diseases of Osaka University.

RESULTS

1. *Purification of the crude hemolysin fraction by DEAE-cellulose column chromatography*

A 20 ml sample of the crude hemolysin fraction was applied to a DEAE-cellulose column (2.5 cm × 20 cm) previously equilibrated as described above. Unadsorbed protein was washed out with 120 ml of phosphate buffer (0.01 M, pH 7.0) and then the column was eluted with a linear gradient of 0 – 0.7 M NaCl. A total volume of 400 ml of buffer was used for elution and 8 ml fractions were collected. As shown in Fig. 1, hemolytic activity was eluted with 0.35 M – 0.7 M NaCl. Most of the protein was eluted after the hemolysin fractions. The fractions containing the hemolytic activity were pooled and concentrated. The sample was then rechromatographed on DEAE-cellulose twice more in the same way. A typical profile of the 3rd chromatography is shown in Fig. 2. Extensive purification was achieved in this way but hemolytic activity was not consistent eluted with protein. Hemolysin was eluted from DEAE-cellulose with about 0.4 M NaCl (Fig. 1 and 2), so the hemolysin fraction from the 3rd

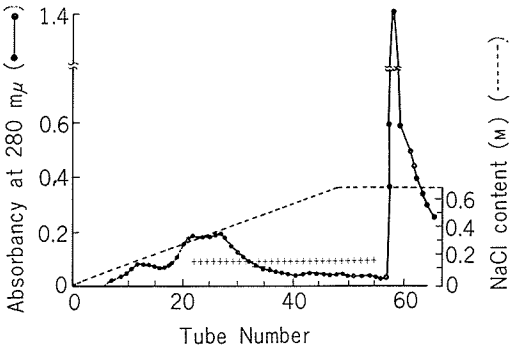

FIGURE 1. *DEAE-cellulose column chromatography of crude hemolysin. 20 ml of crude hemolysin were chromatographed on DEAE-cellulose column (2.5 cm × 20 cm) as described in the text. Eluate was collected in 8 ml fractions. The symbol (+) indicates that hemolytic activity was detected.*

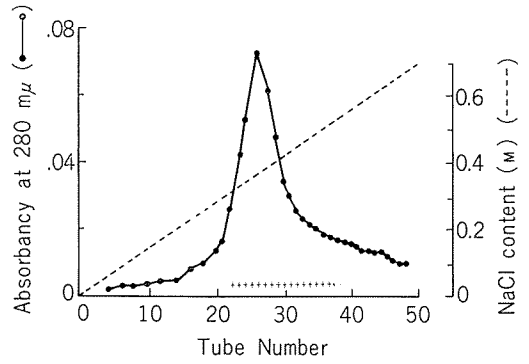

FIGURE 2. *3rd DEAE-cellulose column chromatography of hemolysin. Experimental conditions were as for Fig. 1.*

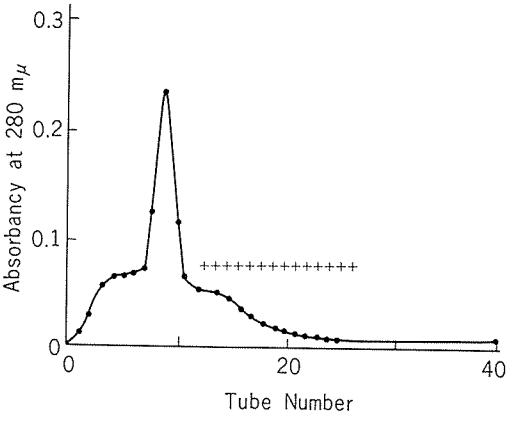

FIGURE 3. *4th DEAE-cellulose column chromatography of hemolysin. Experimental conditions were for Fig. 1.*

DEAE-cellulose column were applied to another DEAE-cellulose column (2.5 cm × 55 cm) and eluted with phosphate buffer (0.01 M, pH 7.0) containing 0.4 M NaCl. As shown in Fig. 3, most of the protein was eluted before the hemolysin.

2. *Purification of hemolysin by DEAE-Sephadex A-50 column chromatography*

The hemolysin fraction from the DEAE-cellulose column was applied to a DEAE-Sephadex A-50 column. Unadsorbed protein was washed out with 80 ml of phosphate buffer (0.01 M, pH 7.0) and then hemolysin was eluted with the same buffer containing 0.4 M NaCl. As shown in Fig. 4, two peaks of hemolytic activity were obtained. The minor and major peaks were designated as H-1 and H-2, respectively.

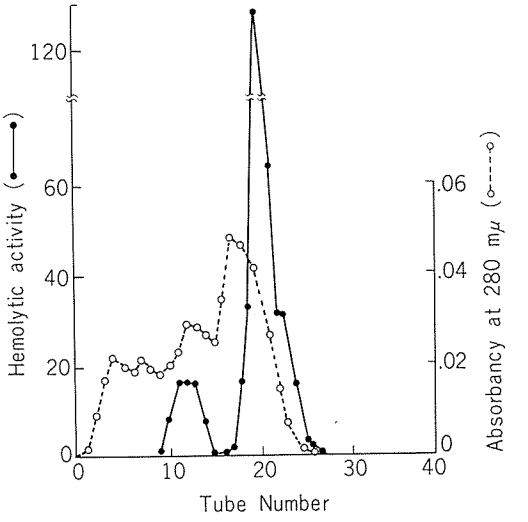

FIGURE 4. *DEAE-Sephadex column chromatography of hemolysin. Preparation of the DEAE-Sephadex procedure are described in the text. Eluate was collected in 8 ml fractions. Hemolytic activity is expressed as the reciprocal of the highest dilution which gave complete hemolysis.*

3. *Heat stability of crude and partially purified hemolysin (H-1 and H-2)*

The heat stability of crude hemolysin was tested and the result is shown in Table 1. As reported previously (Fujino et al., 1969), the fraction caused complete hemolysis even at the high dilution of 1: 128, and most of the hemolytic activity were inactivated by heating at 60 C for 10 min. However, heating the crude hemolysin fraction at a higher temperature, even at 100 C for 10 min, caused little inactivation of the hemolytic activity of the fraction.

Results on the heat stabilities of H-1 and H-2 are shown in Table 2. H-1 lost its hemolytic activity on heating at 70 C for 10 min, and did not recover it on further heating at 100 C for 10 min. On the other hand, heating H-2

by either 70 C for 10 min or 100 C for 10 min did not result in loss of hemolytic activity. These data indicate that the crude hemolysin fraction is a mixture of thermolabile hemolysin (H-1) and thermostable hemolysin (H-2).

4. *Effects of lecithine on the hemolytic activities of H-1 and H-2*

The effects of lecithine on the hemolytic activities of H-1 and H-2 are shown in Table 3. H-1 and H-2 showed the same hemolytic activities with and without. This suggests that these hemolysins are not indirect, but direct hemolysins.

5. *Sensitivities of sheep and horse erythrocytes to H-1 and H-2*

The sensitivities of erythrocytes of other animals to the direct hemolysins isolated were studied, the results are shown in Table 4.

TABLE 1. *Heat stability of crude hemolysin*

	Dilution						
	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256
Untreated	3+	3+	3+	3+	3+	3+	3+
40 C, 10 min	3+	3+	3+	3+	3+	3+	3+
50 C, 10 min	3+	3+	3+	3+	3+	3+	3+
60 C, 10 min	2+	+	±	−	−	−	−
70 C, 10 min	2+	+	±	−	−	−	−
80 C, 10 min	3+	3+	2+	+	±	−	−
90 C, 10 min	3+	3+	3+	2+	2+	+	±
100 C, 10 min	3+	3+	3+	3+	3+	3+	2+

TABLE 2. *Heat stabilities of H-1 and H-2*

		Dilution							
		1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256
H-1	Untreated	3+	2+	±	−	−	−	−	−
	70 C, 10 min	±	−	−	−	−	−	−	−
	100 C, 10 min	±	−	−	−	−	−	−	−
H-2	Untreated	3+	3+	3+	2+	+	+	±	−
	70 C, 10 min	3+	3+	3+	2+	+	+	±	−
	100 C, 10 min	3+	3+	3+	2+	+	+	−	−

TABLE 3. *Effect of lecithine on hemolytic activities of H-1 and H-2*

	Dilution							
	1 : 1	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128
Lecithine (+)								
H-1	3+	3+	+	−	−	−	−	−
H-2	3+	3+	3+	+	+	±	−	−
Lecithine (−)								
H-1	3+	2+	+	±	−	−	−	−
H-2	3+	3+	3+	+	+	±	±	−

Sheep erythtocytes were sensitive to both H-1 and H-2, although they were less sensitive than human erythrocytes. On the other hand, horse erythrocytes were insensitive to both direct hemolysins. It was also shown that H-1 was thermolabile and H-2 was thermostable with sheep erythrocytes.

TABLE 4. *Sensitivity of sheep and horse erythrocytes to H-1 and H-2*

Source of Erythrocytes	Hemolysin	Treatment	Dilution							
			1 : 1	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128
Sheep	H-1	Untreated	+	−	−	−	−	−	−	−
		70 C, 10 min	−	−	−	−	−	−	−	−
		100 C, 10 min	−	−	−	−	−	−	−	−
	H-2	Untreated	3+	2+	2+	+	±	−	−	−
		70 C, 10 min	3+	2+	2+	+	−	−	−	−
		100 C, 10 min	3+	2+	2+	+	−	−	−	−
Horse	H-1	Untreated	−	−	−	−	−	−	−	−
	H-2	Untreated	−	−	−	−	−	−	−	−

DISCUSSION

Several workers have attempted to isolate hemolysin from *Vibrio parahaemolyticus*. Kawamura (1964) reported that the hemolytic activity of this organism was not destroyed by heat and that the hemolysin did not have a protein nature. Kato et al. (1966) isolated a protein from the culture filtrate of *V. parahaemolyticus* which was hemolytic to human, but not horse erythrocytes. They found that this hemolysin was not destroyed by heating at 100 C for 30 min. Recently, Zen-Yoji et al. (1971) and Obara (1971) isolated a hemolysin produced by *V. parahaemolyticus* and purified it extensively. They reported that it was a protein and was stable on heating at 100 C for 10 min.

In contrast to these thermostable hemolysins, we have previously reported the existence of a thermolabile hemolysin in the culture filtrate of *V. parahaemolyticus* (Fujino et al., 1969), which we isolated from the culture filtrate by ammonium sulfate fractionation. This hemolysin was inactivated by heating at 60 C for 10 min. In the present work, we purified this thermolabile hemolysin and separated a thermolabile hemolysin from a thermostable one by successive column chromatographies.

Yanagase et al. (1970) reported the existence of thermolabile hemolysin in *V. parahaemolyticus*. They found that the hemolytic activity was significantly enhanced by the addition of lecithine to the reaction mixture. However, in the present work the hemolytic activity of the thermolabile hemolysin was not enhanced under our experimental conditions by addition of lecithine. This suggests that our thermolabile hemolysin differs from that reported by Yanagase et al. (1970). This difference may be due to a difference in the culture conditions of the organism, as suggested previously (Yanagase et al., 1970).

It was surprising to find that the crude hemolysin fraction was inactivated by heating at 60 – 70 C for 10 min, but not by heating at 100 C for 10 min (Table 1). This was also found after considerable purification of the hemolysin, after the 3rd DEAE-cellulose column chromatography. A similar phenomenon has been reported for staphylococcal α-toxin (Arrhenius, 1907) and has been described as the Arrhenius effect (Arbuthnott, 1970). Several workers studied this phenomenon with staphylococcal α-toxin, but its cause is unknown. Landsteiner and von Rauchenbichler (1909) proposed that the inactivation at low temperature was due to formation of an inactive complex consisting

of toxin and a component of the culture filtrate (Arbuthnott, 1970). Recently, we isolated a factor from the culture filtrate of *V. parahaemolyticus* which inhibited the hemolytic activity of the hemolysin. This factor can be separated from hemolysin by DEAE-cellulose column chromatography and its inhibitory activity is activated by heating at 60 – 70 C for 10 min (Miwatani et al., unpublished observation). These findings may explain the above mentioned phenomenon that the crude hemolysin was inactivated by heating at 60 – 70 C for 10 min, but not by heating at 100 C for 10 min.

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