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Demonstration of a Temperature-Dependent Inactivating Factor of the Thermostable Direct Hemolysin in *Vibrio parahaemolyticus*

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A factor was found in *Vibrio parahaemolyticus* which inactivated the hemolytic activity of a purified, thermostable direct hemolysin. The inactivating factor was associated with the hemolysin but could be separated from it by diethylaminoethyl-cellulose column chromatography. The inactivating factor was activated by heating at 50 to 60 C, but was itself thermolabile and lost activity on heating to 70 to 100 C. The mechanism of the Arrhenius effect, observed with crude hemolysin of *V. parahaemolyticus*, is as follows. At 50 to 60 C, the temperature-dependent inactivating factor associated with hemolysin inactivates the hemolysin, whereas at 80 to 100 C the crude hemolysin retains activity because at this temperature the factor is inactivated.

It has been reported that the thermostable direct hemolysin of *Vibrio parahaemolyticus* is closely correlated with human pathogenicity of this organism (11). During the course of our study to purify the thermostable direct hemolysin (12), we found that a crude hemolysin of *V. parahaemolyticus* was inactivated by heating at 50 to 60 C, but not by heating at 90 to 100 C (8-10). Since the purified thermostable hemolysin was found to be a protein and its activity was not inactivated by heating at either 50 to 60 or 90 to 100 C (12), the above finding is peculiar and should be explained by further experimentation.

This peculiar characteristic of the hemolysin has been reported for staphylococcal alpha-toxin and named the Arrhenius effect (1, 3). Although the mechanism of the Arrhenius effect shown by staphylococcal alpha-toxin has been studied by several workers (1, 2, 4, 6, 7, 13), it is not fully understood. The mechanism is difficult to study mainly because purified staphylococcal alpha-toxin itself is thermolabile. In the present study, the mechanism of the Arrhenius effect was studied with the thermostable hemolysin of *V. parahaemolyticus*.

MATERIALS AND METHODS

Isolation and purification of the thermostable direct hemolysin. *V. parahaemolyticus* OV-3, originally isolated from a case of gastroenteritis and maintained in the culture collection of this institute, was used throughout. Cultivation of bacteria and fractionation of the culture filtrate with ammonium sulfate were as described previously (5). Purification of the hemolysin by column chromatographies on diethylaminoethyl (DEAE)-cellulose, DEAE-Sephadex A-50, and Sephadex G-200 was carried out as described previously (9, 12).

Isolation of a temperature-dependent inactivating factor by DEAE-cellulose column chromatography. Material precipitated from the culture filtrate of *V. parahaemolyticus* OV-3 with ammonium sulfate was dissolved in 0.01 M phosphate buffer (pH 7.0) containing 6 mM 2-mercaptoethanol. An appropriate amount of the solution was applied to a DEAE-cellulose column (2.2 by 80 cm) previously equilibrated with 0.01 M phosphate buffer (pH 7.0). The column was eluted with about 800 ml of 0.01 M phosphate buffer (pH 7.0) containing 6 mM 2-mercaptoethanol and 0.2 M NaCl, and then with 1,000 ml of a linear gradient of 0.2 to 1.0 M NaCl in the same buffer. Fractions of 12 ml were collected.

Fractions which showed inactivating activity were combined and concentrated by adding 40 g of ammonium sulfate per ml. The resulting precipitate was dissolved in 0.01 M phosphate buffer (pH 7.0) containing 6 mM 2-mercaptoethanol. This preparation was used as the inactivating factor after extensive dialysis against 0.01 M phosphate buffer (pH 7.0) containing 6 mM 2-mercaptoethanol.

Assay of hemolytic activity. In most experiments, the following assay procedure was adopted; however, the assay was carried out as described previously (10) in some experiments. The standard reaction mixture (2.5 ml) for assay of hemolytic activity contained 0.01 M phosphate buffer (pH 7.0), 1.25 ml of a 1% suspension of human erythrocytes, 0.9% NaCl, and the hemolysin. The reaction mixture was incubated at 37 C for 2 h and then centrifuged at 3,000 rpm for 5 min. The hemolytic activity was determined by

measuring the absorbance of the resulting supernatant fluid at 540 nm. The hemolytic activity determined in this way was porportional to the amount of hemolysin added up to the amount causing complete hemolysis (Fig. 1).

Assay of inactivating factor. The reaction mixture, containing the inactivating factor and the hemolysin in 0.25 ml of 0.01 M phosphate buffer (pH 7.0), was heated at 60 C for 10 min. Then 1.25 ml of a 1% suspension of erythrocytes in 0.01 M phosphate buffer (pH 7.0) containing 1.8% NaCl and 1 ml of 0.01 M phosphate buffer (pH 7.0) was added, and hemolysis was measured as described above. The inactivating activity of the factor was determined from the reduction in absorbance at 540 nm over that of the control.

RESULTS

Effect of heat on hemolysin. Fractions of hemolysin at various purification steps were heated, and their hemolytic activity was assayed. Crude hemolysin was inactivated by heating at 60 to 70 C, whereas the most purified hemolysin was not (Table 1). The degree of inactivation of the hemolysin on heating at 60 to 70 C decreased with increase in purity of the preparation. This indicates that some factor(s) inactivating hemolysin was removed from the hemolysin during purification.

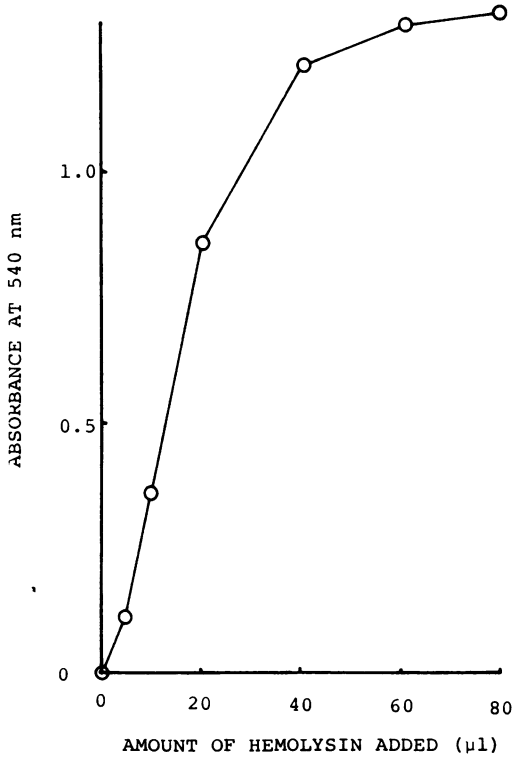

FIG. 1. *Dependence of hemolysis on the amount of the hemolysin. The procedure for assay of hemolytic activity is described in the text. Hemolysin (26.3 µg of protein per ml) from DEAE-Sephadex A-50 was added as indicated. Under these conditions, complete hemolysis occurred with about 40 µliters of hemolysin.*

Isolation of a factor inactivating the thermostable direct hemolysin. A factor inactivating the thermostable direct hemolysin could be separated from the hemolysin by DEAE-cellulose column chromatography (Fig. 2). In this experiment, curde hemolysin precipitated with ammonium sulfate was applied to a DEAE-cellulose column, and chromatography was carried out as described above. The hemolysin was eluted with about 0.5 M NaCl, and a factor which inactivated the hemolysin was eluted with 0.2 M NaCl. The hemolysin fraction from the first DEAE-cellulose column still showed the Arrhenius effect, indicating that the fraction still contained some inactivating factor(s) (Table 1). This was confirmed by the fact that further inactivating factor was eluted with 0.2 M NaCl from the second DEAE-cellulose column.

Demonstration of the Arrhenius effect with the isolated inactivating factor and purified hemolysin. For the experiment shown in Fig. 3, the inactivating factor was mixed with the purified thermostable direct hemolysin, and hemolysis was assayed after heating at various temperatures. Heating the mixture at 50 to 60 C for 10 min resulted in significant loss of hemolytic activity, whereas heating at 80 to 90 C did not inactivate the hemolysin. The effect of heat (Arrhenius effect) with a mixture of the inactivating factor and the purified hemolysin (Fig. 3) was essentially similar to that observed with crude hemolysin (Table 1). This indicates that the Arrhenius effect of the crude hemolysin is due to the presence of the inactivating factor with the hemolysin.

Characteristics of the inactivating factor. Inactivating activity was dependent on the amount of the inactivating factor added to the purified hemolysin (Fig. 4). This was also demonstrated by using inactivating factor which had been extensively dialyzed against 0.01 M phosphate buffer containing 6 mM 2-mercaptoethanol.

Results on the kinetics of the inactivation are shown in Fig. 5. For these results, the inactivating factor was mixed with purified hemolysin and heated at 60 C for the indicated times. Heating for 30 min resulted in almost complete inactivation of the hemolysin, although in the absence of the inactivating factor, the hemolysin was stable on heating at 60 C for 30 min.

TABLE 1. *Effect of heat treatment on the hemolytic activities of various preparations of hemolysin*

Heat treatment	Thermostability[a]									
	1:4[b]	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1,024	1:2,048
Ammonium sulfate-treated fraction										
37 C, 10 min	3+	3+	3+	3+	3+	3+	2+	2+	+	+
50 C, 10 min	3+	3+	3+	3+	3+	2+	+	±	−	−
60 C, 10 min	3+	2+	+	±	−	−	−	−	−	−
70 C, 10 min	3+	3+	2+	+	±	−	−	−	−	−
90 C, 10 min	3+	3+	3+	3+	2+	2+	+	±	−	−
1st DEAE-cellulose fraction										
37 C, 10 min	3+	3+	3+	2+	2+	+	+	±	−	−
50 C, 10 min	3+	3+	3+	2+	2+	+	−	−	−	−
60 C, 10 min	3+	3+	2+	2+	+	±	−	−	−	−
70 C, 10 min	3+	3+	2+	2+	2+	+	±	−	−	−
90 C, 10 min	3+	3+	3+	2+	2+	+	±	−	−	−
Sephadex G-200 fraction										
37 C, 10 min	3+	3+	3+	2+	+	±	−	−	−	−
50 C, 10 min	3+	3+	2+	2+	+	±	−	−	−	−
60 C, 10 min	3+	3+	2+	2+	+	±	−	−	−	−
70 C, 10 min	3+	3+	2+	2+	+	±	−	−	−	−
90 C, 10 min	3+	3+	2+	2+	±	±	−	−	−	−

[a] Symbols: 3+, complete hemolysis; 2+, +, ±, degree of partial hemolysis; −, no hemolysis.
[b] Dilution. Protein content of original fraction was as follows: ammonium sulfate-treated fraction, 16.3 mg/ml; first DEAE-cellulose fraction, 0.27 mg/ml; Sephadex G-200 fraction, 40.9 μg/ml.

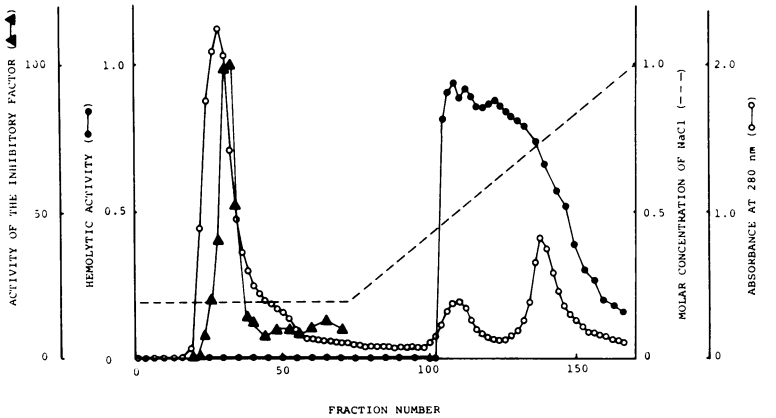

FIG. 2. *DEAE-cellulose column chromatography of the inactivating factor and hemolysin. Material precipitated from culture filtrates with ammonium sulfate was chromatographed as described in the text. Inactivating activity in 0.5-ml samples of each fraction was assayed as described in the text. Complete inhibition of the hemolysis with 40 μliters of the DEAE-Sephadex A-50 fraction (26.3 μg of protein per ml) is expressed as 100. Hemolysis was assayed as described in the text, with 0.5 ml of each fraction.*

The inactivating factor was active only when heated.

The inactivating factor itself was thermolabile (Fig. 6). For this determination, the inactivating factor alone was heated at various temperatures for 10 min and then mixed with purified hemolysin and heated at 60 C to measure its inactivating activity. The inactivating activity was almost completely lost when the factor was heated at or above 60 C for 10 min (Fig. 6).

DISCUSSION

As early as 1907, staphylococcal alpha-toxin was found to show the Arrhenius effect (3), and the mechanism of this effect has been studied

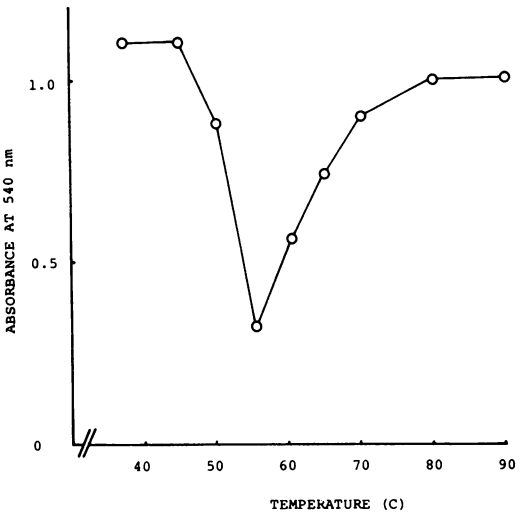

FIG. 3. *Effect of heat on a mixture of the hemolysin and isolated inactivating factor. A 40-μliter amount of the inactivating factor (0.58 mg of protein per ml) and a 40-μliter amount of the DEAE-Sephadex A-50 fraction of hemolysin (26.3 μg of protein per ml) in 0.25 ml of 0.01 M phosphate buffer (pH 7.0) were heated at the temperatures indicated for 10 min. Other experimental conditions were as described in the text.*

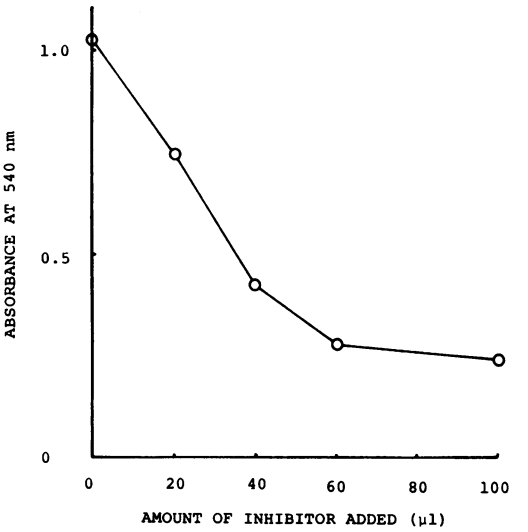

FIG. 4. *Dependence of the inactivating activity on the amount of the inactivating factor added. Various amounts of inactivating factor (0.58 mg of protein per ml) and the DEAE-Sephadex A-50 fraction of hemolysin (26.3 μg of protein per ml) in 0.25 ml of 0.01 M phosphate buffer were heated at 60 C for 10 min. Other experimental conditions were as described in the text.*

by several workers. Landsteiner and von Rauchenbichler (6), Tager (13), and Manohar et al. (7) proposed the existence of some substance which interacts with alpha-toxin at 60 C. On

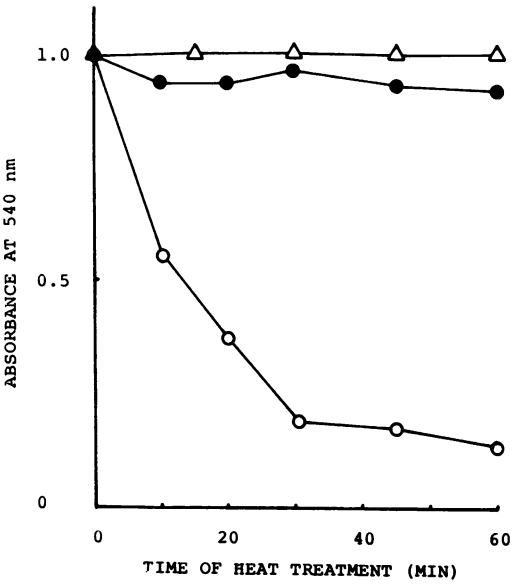

FIG. 5. *Kinetics of the activity of the inactivating factor. A 40-μliter amount of the DEAE-Sephadex A-50 fraction of the hemolysin (26.3 μg of protein per ml) was incubated with or without 40 μliters of the inactivating factor (0.58 mg of protein per ml) at 60 or 37 C for the times indicated. Other experimental conditions were as described in the text. Symbols: ○, heated at 60 C with the inactivating factor; △, incubated at 37 C with the inactivating factor; ●, heated at 60 C without the inactivating factor.*

the other hand, Cooper et al. (4) and Arbuthnott et al. (2) reported that active alpha-toxin aggregates at 60 C to an insoluble, nontoxic form, whereas at higher temperatures soluble active toxin is released.

Crude hemolysin of *V. parahaemolyticus* shows an Arrhenius effect similar to that of staphylococcal alpha-toxin (8–10). However, purified hemolysin from *V. parahaemolyticus* is thermostable (12), whereas staphylococcal alpha-toxin is thermolabile (7). In the present study on the mechanism of the Arrhenius effect of the hemolysin of *V. parahaemolyticus*, a factor inactivating the hemolysin was isolated from *V. parahaemolyticus*. The inactivating factor was associated with the hemolysin but could be separated from it by DEAE-cellulose column chromatography (Fig. 2). The factor was nondialyzable and was inactive without heat treatment. However, this factor was itself thermolabile and lost activity on heating at or above 60 C. These results suggest that the factor has the nature of a protein.

Crude hemolysin is probably inactivated by heating at 50 to 60 C, because the inactivating factor associated with it is activated at this temperature. The finding that the inactivating factor itself is thermolabile and is inactivated

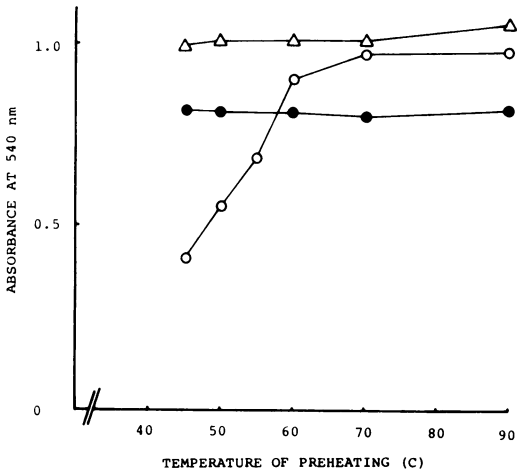

FIG. 6. *Effect of heat on the inactivating factor. A 40-μliter amount of the inactivating factor (0.58 mg of protein per ml) in 0.15 ml of 0.01 M phosphate buffer (pH 7.0) was heated at the temperatures indicated for 10 min. Then 40 μliters of the DEAE-Sephadex A-50 fraction of the hemolysin (26.3 μg of protein per ml) and 60 μliters of 0.01 M phosphate buffer (pH 7.0) were added and mixtures were again incubated at either 37, 60, or 100 C for 10 min. Other experimental conditions were as described in the text. Temperatures of second heat treatment: 60 C (○); 37 C (△); 100 C (●).*

by heating at 80 to 100 C explains why crude hemolysin is not inactivated by heating at 80 to 100 C.

The inactivating factor might destroy the hemolysin when heated together at 60 C. Manohar et al. (7) reported the presence of a thermolabile inhibitor in preparations of staphylococcal alpha-toxin and postulated that this inhibitor combines with the alpha-toxin. It is still uncertain whether the factor inactivating the hemolysin of *V. parahaemolyticus* simply combines with the hemolysin or whether it has some other function in relation to the hemolysis.

LITERATURE CITED

1. Arbuthnott, J. P. 1970. Staphylococcal α-toxin, p. 189-236. *In* T. C. Montie, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. 3. Academic Press Inc., New York.
2. Arbuthnott, J. P., J. H. Freer, and A. W. Bernheimer. 1967. Physical states of staphylococcal α-toxin. J. Bacteriol. **94**:1170-1177.
3. Arrhenius, S. 1907. Immunochemie. Anwendungen der physikalischen Chemie auf die Lehre von den physiologischen Antikoerpern. Akademische Verlagsgesellschaft, Leipzig.
4. Cooper, L. Z., M. A. Madoff, and L. Weinstein. 1966. Heat stability and species range of purified staphylococcal α-toxin. J. Bacteriol. **91**:1686-1692.
5. Fujino, T., T. Miwatani, Y. Takeda, and A. Tomaru. 1969. A thermolabile direct hemolysin of *Vibrio parahaemolyticus*. Biken J. **12**:145-148.
6. Landsteiner, K., and R. von Rauchenbichler. 1909. Ueber das Verhalten des Staphylolysins bein Erwarmen. Z. Immunitaetsforsch. **1**:439-448.
7. Manohar, M., S. Kumar, and R. K. Lindorfer. 1966. Heat reactivation of the α-hemolytic, dermonecrotic, and lethal activities of crude and purified staphylococcal α-toxin. J. Bacteriol. **91**:1681-1685.
8. Miwatani, T., J. Sakurai, Y. Takeda, and S. Shinoda. 1974. Studies on direct hemolysins of *Vibrio parahaemolyticus*, p. 245-252. *In* T. Fujino, G. Sakaguchi, R. Sakazaki, and Y. Takeda (ed.), International Symposium on *Vibrio parahaemolyticus*. Saikon Publishing Co., Tokyo.
9. Miwatani, T., J. Sakurai, A. Yoshihara, and Y. Takeda. 1972. Isolation and partial purification of thermolabile direct hemolysin of *Vibrio parahaemolyticus*. Biken J. **15**:61-66.
10. Miwatani, T., Y. Takeda, J. Sakurai, A. Yoshihara, and S. Taga. 1972. Effect of heat (Arrhenius effect) on crude hemolysin of *Vibrio parahaemolyticus*. Infect. Immunity **6**:1031-1033.
11. Miyamoto, Y., T. Kato, Y. Obara, S. Akiyama, K. Takizawa, and S. Yamai. 1969. In vitro hemolytic characteristic of *Vibrio parahaemolyticus*: its close correlation with human pathogenicity. J. Bacteriol. **100**:1147-1149.
12. Sakurai, J., A. Matsuzaki, and T. Miwatani. 1973. Purification and characterization of thermostable direct hemolysin of *Vibrio parahaemolyticus*. Infect. Immunity **8**:775-780.
13. Tager, M. 1941. Factors modifying the reaction of alpha-staphylolysin to heat. Yale J. Biol. Med. **14**:69-88.