Identification of Lethal Toxin with the Thermostable Direct Hemolysin Produced by *Vibrio parahaemolyticus*, and Some Physicochemical Properties of the Purified Toxin

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Received for publication 13 August 1975

Lethal toxin was purified extensively from the culture filtrate of a Kanagawa phenomenon-positive strain of *Vibrio parahaemolyticus*. The purified toxin was a protein, and its homogeneity was demonstrated by sodium dodecyl sulfate-polyacrylamide gel disc electrophoresis and analytical ultracentrifugation. It was demonstrated that the thermostable direct hemolysin was identical to the lethal toxin and that it was the main, if not only, lethal toxin in the culture filtrate. The purified toxin had a lethal effect when injected into mice either intravenously or intraperitoneally. Its lethal effect was very rapid, a dose of 5 μ g of toxin per mouse killing the animals within 1 min. The lethal activity was inhibited by a ganglioside mixture. Some physicochemical properties of the purified toxin are reported.

The lethal effect of *Vibrio parahaemolyticus* on small experimental animals was first described by Fujino and co-workers, who were also the first to isolate this organism from a victim of food poisoning (6, 7). Kuriyama (9) reported that the deoxyribonucleic acid fraction of the cell envelopes of this organism had a lethal effect on mice. Akehashi and Yoneda (1) also reported the existence of a toxic substance in the cells. On the other hand, Ueyama et al. (20) isolated a toxin lethal to mice from the culture filtrate of *V. parahaemolyticus*. They reported that the toxin was free from sugar and nucleic acids and had the properties of a protein.

Since a relation was found between the pathogenicity of *V. parahaemolyticus* to man and its hemolytic activity (12), several workers have isolated and purified the thermostable direct hemolysin from culture filtrates of this organism (11, 14-17, 21-23). In some of these studies the hemolysin was also found to be lethal to small experimental animals (15, 22, 23). However, it is uncertain whether the hemolysin reported by these workers is identical with the lethal toxin reported by others, especially Ueyama et al. (20). The present study was done to determine the identity of the thermostable direct hemolysin with the lethal toxin found in the culture filtrate of *V. parahaemolyticus*.

MATERIALS AND METHODS

Strains and cultivation of the organism. *V. parahaemolyticus* WP-1, a Kanagawa phenomenon (12)-

positive strain, was cultured in medium containing 30 g of sodium chloride, 10 g of peptone (Difco), 5 g of dibasic sodium phosphate, and 5 g of glucose per liter in distilled water. The pH of the medium was 7.6 to 7.8. After incubating the cells at 37 C for about 15 h with shaking, the culture filtrate was collected by centrifugation.

Isolation of crude toxin from the culture filtrate. Solid ammonium sulfate (35.1 g/100 ml) was added to the culture filtrate, and the resulting precipitate was dissolved in a small amount of 0.01 M phosphate buffer ($\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$, pH 7.0). Then it was dialyzed overnight against the same buffer and used as crude toxin.

Diethylaminoethylcellulose column chromatography. Crude toxin prepared from about 10 liters of culture filtrate was applied to a diethylaminoethylcellulose column (2.2 by 50 cm) equilibrated with 0.01 M phosphate buffer (pH 7.0). The column was eluted with about 1,000 ml of the same buffer containing 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 with toxic activity were collected and concentrated by adding solid ammonium sulfate (40.0 g/100 ml). The resulting precipitate was dissolved in a small volume of 0.01 M phosphate buffer (pH 7.0) and dialyzed against the same buffer.

Hydroxyapatite column chromatography. The toxin concentrated to a small volume was applied to a hydroxyapatite column (2.2 by 35 cm) equilibrated with 0.01 M phosphate buffer (pH 7.0). The column was eluted with about 500 ml of 0.1 M phosphate buffer (pH 7.0) and then with 800 ml of a linear gradient of 0.1 to 0.3 M phosphate buffer (pH 7.0). Fractions containing toxic activity were collected and concentrated to a small volume by using a colloidal bag.

Sephadex G-200 gel filtration of the toxin. The preparation from the hydroxyapatite column was applied to a Sephadex G-200 column (2.2 by 50 cm) equilibrated with 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.0). The column was eluted with the same buffer, and fractions with toxic activity were collected and concentrated to a small volume by using a collodion bag. This preparation was used as purified toxin in this study.

Assay of hemolytic activity. Hemolytic activity was assayed essentially as described previously (18). The standard reaction mixture (2.5 ml) contained 10 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.2), 5 mM CaCl_2 , 0.9% NaCl, the toxin, and 1.25 ml of a 1% suspension of human erythrocytes (about 8.3×10^7 cells/ml). The reaction mixture was incubated at 37°C for 30 min 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.

Assay of lethal toxicity. Lethal toxicity was assayed by injecting the toxin either intravenously or intraperitoneally into 4- to 6-week-old mice (ddO strain) and measuring the survival time of the animals.

SDS-polyacrylamide disc gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide disc gel electrophoresis on 0.1% SDS gel was carried out as described by Fairbanks et al. (4). Electrophoresis was performed at a constant current of 5 mA per tube for about 2 h. Then the gel was stained with Coomassie brilliant blue. After destaining, the gel was scanned at 540 nm with a Gfrod spectrophotometer equipped with a linear transport device.

Analytical ultracentrifugation. Centrifugation was performed by using a Hitachi analytical ultracentrifuge equipped with a Schlieren optical system. Before centrifugation, the purified toxin was dialyzed against 0.01 M phosphate buffer (pH 7.0) for 24 h. The toxin at a concentration of 4 mg/ml was centrifuged at a rotor speed of 60,000 rpm and an angle of 70° at a constant temperature of 20°C. Photographs were taken at 5-min intervals after the rotor had reached maximum speed.

Determination of molecular weight by Sephadex G-100 gel filtration. The molecular weight of the purified toxin was determined by Sephadex G-100 gel filtration as described by Andrews (2). The column (2.2 by 50 cm) was eluted with 0.01 M phosphate buffer (pH 7.0). The absorbance of standard proteins at 280 nm and the hemolytic activity of purified toxin in the eluate were measured.

Isoelectric focusing of the toxin. Electrofocusing of the purified toxin using ampholine carrier ampholytes was carried out with an LKB electrofocusing system 8100-10, using a pH gradient of 3 to 5. A sample of 1 mg of purified toxin was applied and electrophoresis was performed at a constant voltage of 450 V for 32 h. Then fractions were collected and their hemolytic activity was determined as described above.

Amino acid analysis of the toxin. The purified toxin in 0.01 M phosphate buffer (pH 7.0) was hydrolyzed with 6 N hydrochloric acid for either 36, 48, or 72 h at 105°C and then subjected to amino acid

analysis. Amino acid contents were calculated from the values obtained with these different hydrolysates. Cysteine was determined as cysteic acid after performic acid oxidation. Tryptophan was estimated spectrophotometrically.

RESULTS

Isolation and purification of the toxin. The culture filtrate of *V. parahaemolyticus* WP-1 was fractionated with ammonium sulfate, and the lethal toxicity of each fraction was examined. It was found that toxin was isolated by adding 35.1 g of solid ammonium sulfate per 100 ml of culture filtrate (55% saturation of ammonium sulfate). The crude toxin fractionated by ammonium sulfate was purified by successive column chromatographies on diethylaminoethylcellulose (Fig. 1A), hydroxyapatite (Fig. 1B), and Sephadex G-200 (Fig. 1C). On diethylaminoethylcellulose column chromatography, the lethal toxin was eluted with about 0.4 M NaCl and coincided with the hemolytic activity (Fig. 1A). On further chromatographies on hydroxyapatite and Sephadex G-200, the lethal toxin was consistently eluted with the hemolytic activity. Typical results on purification of the toxin (hemolytic activity) are summarized in Table 1.

Homogeneity of purified toxin. Homogeneity of the purified toxin was examined by SDS-polyacrylamide disc gel electrophoresis and analytical ultracentrifugation. The purified toxin gave a single band on SDS-polyacrylamide disc gel electrophoresis (Fig. 2). The purified toxin also formed a single symmetric peak on sedimentation analysis (Fig. 3).

Lethal toxicity of the purified toxin. The lethal toxicity of the purified toxin was studied in mice (Table 2). Intravenous injection of the toxin killed mice very rapidly, and 5 µg of the toxin per mouse (6 weeks old) killed the animals within 1 min. Even a dose of 1 µg of toxin per mouse killed the animals within 20 min. No death occurred within 3 days after injection of 0.5 µg of the toxin. Intraperitoneal injection of the toxin also killed mice, although this route was less effective than intravenous administration. When injected with toxin, the animals became motionless and sometimes showed signs of cramp.

It was reported previously that a ganglioside mixture (Sigma Chemical Co., type II) inhibited the hemolytic activity of the purified thermostable direct hemolysin (19). Thus, its effect on the lethal toxicity of the purified toxin was examined. The ganglioside mixture inhibited the lethal effect in parallel with the hemolytic activity of the toxin (Table 3).

Physicochemical properties of the purified

FIG. 1. Purification of the lethal toxin. Crude toxin was chromatographed on a diethylaminoethylcellulose column (A), a hydroxyapatite column (B), and a Sephadex G-200 column (C) as described in the text. The lethal activity and hemolytic activity of samples of each fraction were assayed as described in the text. Symbols: ●, Absorbance at 280 nm; ▲, lethal activity; ○, hemolytic activity; - - -, concentration of NaCl in (A) and of phosphate in (B). The bar (↔) indicates the fractions concentrated for further analyses.

TABLE 1. Purification of the thermostable direct hemolysin produced by *V. parahaemolyticus*

Fraction	Total vol (ml)	Total protein (mg) ^a	Total activity (HU) ^b	Specific activity (HU/mg)	Relative activity	Yield of hemolytic activity (%)
Culture filtrate	46,000	25,760	223,744	8.7	(1.0)	100
Ammonium sulfate fraction ...	400	14,036	201,142	14.3	1.64	89.9
Diethylaminoethylcellulose column eluate	10.8	59.4	68,314	1,150.1	132.2	30.6
Hydroxyapatite column eluate .	1.2	11.6	46,966	4,048.8	465.4	21.1
Sephadex G-200 column eluate .	1.76	7.4	34,520	4,664.9	536.2	15.5

^a Protein content was measured by the method of Lowry et al. (10).

^b Hemolytic activity was determined as described in the text. One hemolytic unit (HU) was defined as the amount with an absorbance of 0.5 at 540 nm.

FIG. 2. SDS-polyacrylamide disc gel electrophoresis of the purified toxin. SDS-polyacrylamide disc gel electrophoresis of a sample of the purified toxin was carried out as described in the text.

FIG. 3. Analytical ultracentrifugation of the purified toxin. Experimental conditions were as described in the text. The photograph was taken 30 min after the rotor had reached 60,000 rpm.

toxin. The purified toxin was found to be a pure protein. Five hundred micrograms of the purified toxin did not contain detectable amounts of carbohydrate and phospholipid, which were determined according to Morris (13) and Baginski et al. (3), respectively. The sedimentation coefficient was calculated to be 6.9S. The molecular weight of the purified toxin was determined to be about 42,000 by Sephadex G-100 gel filtration (Fig. 4). The isoelectric point of the purified toxin, examined as described above, was around pH 4.2 (Fig. 5). The amino acid composition of the purified toxin is shown in Table 4.

DISCUSSION

Thermostable direct hemolysin produced by *V. parahaemolyticus* has been purified by several workers (11, 14, 16, 21-23). Zen-Yoji and his co-workers (22, 23) extensively purified the thermostable direct hemolysin (which they named "enteropathogenic toxin") and demon-

strated its homogeneity. They found that it was lethal when injected into mice intravenously. Obara et al. (15) reported that oral administration of the purified hemolysin killed mice.

In this work, we demonstrated that the purified lethal toxin found in the culture filtrate of *V. parahaemolyticus* was identical to the thermostable direct hemolysin. Moreover, fractionation of the culture filtrate with ammonium sulfate and diethylaminoethylcellulose column chromatography of the crude toxin (Fig. 1A) showed that the hemolysin is the main, if not the only, lethal toxin produced by Kanagawa phenomenon-positive strains of *V. parahaemolyticus*. These results indicate that the

TABLE 2. Lethal activity of the purified thermostable direct hemolysin after intravenous and intraperitoneal injection into mice^a

Amt of hemolysin injected (μ g of protein/mouse)	Survival time after injection
Intravenous injection	
10.0	35.5 $\pm$ 4.8 ^b s
5.0	49.0 $\pm$ 8.4 s
2.5	561.2 $\pm$ 368.8 s
1.0	1,121.5 $\pm$ 291.0 s
0.5	No death
Intraperitoneal injection	
40.0	29.2 $\pm$ 13.5 min
20.0	47.4 $\pm$ 27.1 min
10.0	113.8 $\pm$ 38.0 min
5.0	115.5 $\pm$ 44.2 min
2.5	No death

^a Six-week-old mice (ddO strain) were used, and each dose was tested on 10 mice.

^b Mean $\pm$ standard deviation.

TABLE 3. Inhibition of lethal activity by ganglioside

Amt of ganglioside ^a (mg)	Lethal activity ^b (s)	Hemolytic activity ^c
0	49.0 $\pm$ 4.2 ^d	0.775
0.1	270.0 $\pm$ 35.5	0.304
0.4	444.0 $\pm$ 147.4	0.089
1.0	No death	0.005

^a The indicated amounts of ganglioside mixture (Sigma Chemical Co., type II) were incubated with 50 μ g of purified toxin in 1 ml of 0.01 M phosphate buffer (pH 7.0) at 37 C for 20 min.

^b Assayed by injecting the preincubated toxin (5 μ g) intravenously into five 6-week-old mice (ddO strains) and measuring the survival time of the animals.

^c The hemolytic activity of each sample of preincubated toxin (5 μ g) was assayed as described in the text. Each value is the average of duplicate determinations.

^d Mean $\pm$ standard deviation.

FIG. 4. Determination of the molecular weight of the purified toxin by Sephadex G-100 gel filtration. Sephadex G-100 gel filtration of (1) cytochrome c, (2) chymotrypsinogen, (3) ovalbumin, (4) bovine serum albumin, and (T) purified toxin was carried out as described in the text. The elution volumes of the standard proteins are plotted against the logarithms of their molecular weight, and the molecular weight of the toxin was calculated from its elution volume.

lethal toxin reported by Ueyama et al. (20) is identical with the thermostable direct hemolysin.

Previously, a method was developed in this laboratory for purification of the thermostable direct hemolysin (16). The procedure included ammonium sulfate fractionation, acid precipitation, diethylaminoethylcellulose column chromatographies (gradient elutions and stepwise elution), diethylaminoethyl-Sephadex A-50 column chromatography, and Sephadex G-200 gel filtration after urea treatment. The present paper describes a simpler purification method involving ammonium sulfate fractionation, diethylaminoethylcellulose column chromatography, hydroxyapatite column chromatography, and Sephadex G-200 gel filtration.

The physicochemical properties of the purified thermostable direct hemolysin (lethal toxin) reported in this paper are similar in general to those reported by Zen-Yoji et al. (22, 23). Study with gel diffusion tests also showed that our toxin and theirs were antigenically identical (unpublished observation).

There have been several reports on the biological activities of the thermostable direct hemolysin. Zen-Yoji et al. (22, 23) reported that 200 to 500 μ g of the purified hemolysin gave a positive ileal loop test in rabbits. Therefore,

FIG. 5. Isoelectric focusing of the purified toxin. Procedure was as described in the text. Symbols: O, pH; ●, hemolytic activity; Δ, absorbance at 280 nm.

TABLE 4. Amino acid composition of the purified toxin

Amino acid	Amino acid residues (%) ^a
Lys	6.1
His	2.2
Arg	1.9
Asp	12.1
Thr	6.2
Ser	8.9
Glu	9.4
Pro	4.6
Gly	5.5
Ala	4.7
Cys	3.1
Val	12.0
Met	1.9
Ile	3.1
Leu	3.9
Tyr	6.2
Phe	6.6
Trp	1.6

^a The total of the individual amino acid residues is taken as 100.0%.

they named the hemolysin "enteropathogenic toxin." However, the amount of toxin required for a positive reaction is much higher than the effective dose of cholera, which is reported to be 2.0 μ g (5), and the question still remains of whether the lethal toxin is really enteropathogenic.

Recently, we found that the purified thermostable direct hemolysin (lethal toxin) has cardiotoxic activity (8). We found that rhythmic

beating of the heart in rats was significantly disturbed by intravenous injection of the purified toxin. Beating of mouse heart cells cultured in vitro was also affected by the purified toxin. This explains the rapid death of animals after intravenous injection of the toxin.

ACKNOWLEDGMENTS

We would like to express our thanks to S. Sakakibara and M. Ogura of the Protein Research Foundation for analysis of amino acid composition and to A. Miyatake for performing the analytical ultracentrifugation.

LITERATURE CITED

1. Akehashi, H., and M. Yoneda. 1967. Toxins of *Vibrio parahaemolyticus* (in Japanese), p. 215-231. In T. Fujino and H. Fukumi (ed.), *Vibrio parahaemolyticus* II. Naya Shoten Co., Tokyo.
2. Andrews, P. 1964. Estimation of the molecular weight of proteins by Sephadex gel filtration. *Biochem. J.* 91:222-233.
3. Baginski, E. S., P. P. Foa, and B. Zek. 1967. Microdetermination of inorganic phosphate, phospholipids, and total phosphate in biological materials. *Clin. Chem.* 13:326-332.
4. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* 10:2606-2617.
5. Finkelstein, R. A. 1973. Cholera. *CRC Crit. Rev. Microbiol.* 2:553-623.
6. Fujino, T. 1974. Discovery of *Vibrio parahaemolyticus*, p. 1-4. In T. Fujino, G. Sakaguchi, R. Sakazaki, and Y. Takeda (ed.), *International symposium on Vibrio parahaemolyticus*. Saikon Publishing Co., Tokyo.
7. Fujino, T., Y. Okuno, D. Nakada, A. Aoyama, K. Fukai, T. Mukai, and T. Ueho. 1953. On the bacteriological examination of shirasu-food poisoning. *Med. J. Osaka Univ.* 4:299-304.
8. Honda, T., K. Goshima, Y. Takeda, Y. Sugino, and

- T. Miwatani. 1976. Demonstration of the cardiotoxicity of thermostable direct hemolysin (lethal toxin) produced by *Vibrio parahaemolyticus*. *Infect. Immun.* 13:163-171.
9. Kuriyama, K. 1964. Biological and immunological studies on the DNA derived from *Vibrio parahaemolyticus*. I. On the purification of the toxin fraction isolated from *Vibrio parahaemolyticus*. *Jpn. J. Bacteriol.* 19:418-424 (in Japanese with English summary).
10. Lowry, O. H., J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
11. Miwatani, T., J. Sakurai, Y. Takeda, and S. Shinoda. 1974. Studies on direct hemolysins of *Vibrio parahaemolyticus*, p. 245-251. *In* T. Fujino, G. Sakaguchi, R. Sakazaki, and Y. Takeda (ed.), *International symposium on Vibrio parahaemolyticus*. Saikon Publishing Co., Tokyo.
12. 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.
13. Morris, D. L. 1948. Quantitative determination of carbohydrates with Dreywood's anthrone reagent. *Science* 107:254-255.
14. Nikkawa, T., Y. Obara, S. Yamai, and Y. Miyamoto. 1972. Purification of a hemolysin from *Vibrio parahaemolyticus*. *Jpn. J. Med. Sci. Biol.* 25:197-200.
15. Obarba, Y., S. Yamai, T. Nikkawa, Y. Miyamoto, M. Ohashi, and T. Shimada. 1974. Histopathological change in the small intestine of suckling mice challenged orally with purified hemolysin from *Vibrio parahaemolyticus*, p. 253-257. *In* T. Fujino, G. Sakaguchi, R. Sakazaki, and Y. Takeda (ed.), *International symposium on Vibrio parahaemolyticus*. Saikon Publishing Co., Tokyo.
16. Sakurai, J., A. Matsuzaki, and T. Miwatani. 1973. Purification and characterization of thermostable direct hemolysin of *Vibrio parahaemolyticus*. *Infect. Immun.* 8:755-780.
17. Sakurai, J., A. Matsuzaki, Y. Takeda, and T. Miwatani. 1974. Existence of two distinct hemolysins in *Vibrio parahaemolyticus*. *Infect. Immun.* 9:777-780.
18. Takeda, Y., Y. Hori, and T. Miwatani. 1974. Demonstration of a temperature-dependent inactivating factor of the thermostable direct hemolysin in *Vibrio parahaemolyticus*. *Infect. Immun.* 10:6-10.
19. Takeda, Y., T. Takeda, T. Honda, J. Sakurai, N. Ohtomo, and T. Miwatani. 1975. Inhibition of hemolytic activity of the thermostable direct hemolysin of *Vibrio parahaemolyticus* by ganglioside. *Infect. Immun.* 12:931-933.
20. Ueyama, T., T. Baba, and Y. Bito. 1964. Studies on the toxic substance of *Vibrio parahaemolyticus*. *Jpn. J. Bacteriol.* 19:480-482 (in Japanese with English summary).
21. Zen-Yoji, H., H. Hitokoto, S. Morozumi, and R. A. LeClair. 1971. Purification and characterization of a hemolysin produced by *Vibrio parahaemolyticus*. *J. Infect. Dis.* 123:665-667.
22. Zen-Yoji, H., Y. Kudoh, H. Igarashi, K. Ohta, and K. Fukai. 1974. Purification and identification on enteropathogenic toxins "a" and "a'" produced by *Vibrio parahaemolyticus* and their biological and pathological activities, p. 237-243. *In* T. Fujino, G. Sakaguchi, R. Sakazaki, and Y. Takeda (ed.), *International symposium on Vibrio parahaemolyticus*. Saikon Publishing Co., Tokyo.
23. Zen-Yoji, H., Y. Kodoh, H. Igarashi, K. Ohta, K. Fukai, and T. Hoehino. 1974. Further studies on characterization and biological activities of an enteropathogenic toxin of *Vibrio parahaemolyticus*. *Proc. 4th Int. Symp. Animal Plant Microbiol. Toxins.*