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Purification and Characterization of *Vibrio metschnikovii* Cytolysin

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An extracellular cytolysin produced by *Vibrio metschnikovii* was purified by acid precipitation, phenyl-Sepharose CL-4B chromatography, and rechromatography on a phenyl-Sepharose CL-4B column and high-performance liquid chromatography on a Mono Q (anion-exchange) column. The purified cytolysin had a molecular weight of 50,000 and an isoelectric point of 5.1. It was inactivated by heating at 60°C for 5 min and was inhibited by Zn^{2+}, Cu^{2+}, and high concentrations of cholesterol. Lysis of calf erythrocytes by cytolysin was temperature dependent and occurred only above 18°C. Moreover, no lysis was observed at high concentrations of erythrocytes, suggesting that the cytolysin lyses erythrocytes by a multihit mechanism. This cytolysin had no immunological cross-reactivities with hemolysins from other *Vibrio* species tested, indicating that it is a new cytolysin. *V. metschnikovii* cytolysin lysed erythrocytes from several animal species (calf, rabbit, guinea pig, mouse, human, sheep, chicken, and horse) and cultured cells (Vero and Chinese hamster ovary), caused fluid accumulation in the intestines of infant mice, and increased vascular permeability in rabbit skin.

At least 10 *Vibrio* species have been reported to be associated with human diseases (8, 21, 22, 29, 30, 35, 39). One of these, *Vibrio metschnikovii*, was first described in 1888 (7) and redefined as a new *Vibrio* species in 1978 (24). Lee and colleagues (24) reported that this organism is widely distributed; and they isolated it from rivers, estuaries, sewage, cockles, oysters, lobsters, and a bird that had died of a choleralike disease. They also isolated *V. metschnikovii* from human feces.

In 1981, Jean-Jacques et al. (21) described a strain isolated from the blood of an elderly woman with peritonitis and an inflamed gallbladder. It is uncertain whether the organism was the primary pathogen in that case. There have been no other reports suggesting that the organism causes diseases in humans or other animals.

We recently isolated this organism from the feces of a patient with diarrhea. The patient was a 60-year old woman who was admitted to the hospital in June 1985 because of general languor and who was found to have diabetes mellitus and a hepatoma. During treatment with insulin, she suffered from diarrhea with fever. *V. metschnikovii* was isolated from the diarrheal feces in pure culture; no other enteropathogens were found. When the patient was given minocycline hydrochloride, she recovered from diarrhea and fever and *V. metschnikovii* disappeared from her stool. Thus, this organism may have been a causative agent of diarrhea in this case (M. Tanaka, S. Funahashi, M. Miyake, T. Honda, and T. Miwatani, manuscript in preparation).

To investigate the pathogenicity of this organism, we examined the toxins that it produces. A sandwich enzyme-linked immunosorbent assay (ELISA), with anti-cholera toxin antibody used as a coating antibody, and the infant mouse assay were used to screen for the presence of cholera toxin or *Escherichia coli* heat-labile enterotoxin (18) and *E. coli* heat-stable enterotoxin (12), respectively. The culture supernatant of the bacteria induced fluid accumulation in infant mice, but did not give a positive reaction on ELISA. Results of preliminary studies suggested that the factor causing fluid accumulation in infant mice was a cytolysin or a hemolysin. Thus, in this study we purified and characterized this extracellular cytolysin.

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MATERIALS AND METHODS

Bacterial strains and cultivation. *V. metschnikovii* MIT-1, MIY-920 (NCTC 8443, type strain), and MIY-1323 (ATCC 7708) were used for cytolysin purification.

V. vulnificus Braumann 329, *V. fluvialis* AQ0067, *V. furnissii* 542 (ATCC 35016), *V. mimicus* 546 (ATCC 33653), *V. cholerae* TQ17, and *V. cholerae* non-O1 AQ1121 were grown in brain heart infusion broth supplemented with 0.5% NaCl at 37°C for 15 h with rotation. The supernatants of cultures were collected by centrifugation at 1,000 × *g* for 15 min and subjected to ELISA and a cross-neutralization test as crude hemolysin preparations.

V. metschnikovii MIT-1, which was isolated from the patient described above, was plated onto tryptic soy agar (Difco Laboratories, Detroit, Mich.) containing 10% (vol/vol) defibrinated sheep blood. A highly hemolytic colony was picked, inoculated into brain heart infusion broth, and cultured at 37°C for 3 h with rotation. Then, 2 ml of this seed culture was inoculated into 500 ml of brain heart infusion broth in a 5-liter flask and cultivated at 37°C for 7 h with shaking (80 cycles per min).

Purification of cytolysin. (i) Acid precipitation (step 1). The culture supernatant was collected by centrifugation at 16,000 × *g* for 20 min, and the pooled culture supernatant was adjusted to pH 4.0 with concentrated (ca. 10 N) hydrochloric acid. The mixture was held at 4°C for 10 to 12 h, and then insoluble material was recovered by centrifugation (16,000 × *g*, 15 min), dissolved in about 50 ml of 10 mM sodium phosphate buffer (PB) containing 1 M urea (pH 7.0), and dialyzed against the same buffer for 48 h.

(ii) First phenyl-Sepharose CL-4B chromatography (step 2). The crude cytolysin preparation obtained in step 1 was dialyzed against PB containing 2 M NaCl and 1 M urea (pH 6.1) for 3 h and then applied to a column (2.2 by 23 cm) of phenyl-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) equilibrated with PB containing 2 M NaCl and 1 M urea (pH

6.1). The column was washed (flow rate, 50 ml/h) with 2,000 ml of equilibrating buffer and 2,000 ml of PB containing 0.6 M NaCl and 1 M urea (pH 6.8). Then, the cytolysin was eluted with PB containing 1 M urea (pH 7.0). The hemolytic fractions were pooled, concentrated to 5 ml by ultrafiltration by using a membrane (PM-10 Diaflo; Amicon Corp., Danvers, Mass.), and dialyzed against PB containing 1.5 M urea (pH 7.3).

(iii) Second phenyl-Sepharose CL-4B chromatography (step 3). The concentrated cytolysin preparation was applied to a phenyl-Sepharose CL-4B column (1.5 by 25 cm) equilibrated with PB containing 1.5 M urea (pH 7.3) and was eluted with the same buffer at a flow rate of 40 ml/h. Fractions of 7 ml were collected, and their A_{280} and hemolytic activity were measured.

(iv) High-performance liquid chromatography (step 4). The fractions with hemolytic activity obtained in step 3 were pooled and subjected to high-performance liquid chromatography (HPLC) on a Mono Q column (Pharmacia) equilibrated with 50 mM Tris hydrochloride buffer containing 1 M urea (pH 8.0). The column was washed, and then cytolysin was eluted with a linear gradient of 0 to 0.3 M NaCl in the equilibrating buffer. Material with hemolytic activity was stored at 4°C.

Hemolytic activity. Calf erythrocytes were used to determine hemolytic activity unless otherwise mentioned. Samples of 0.2 ml were mixed with an equal volume of a 2% erythrocyte suspension that had been washed 2 or 3 times with PB containing 0.85% NaCl (PBS; pH 7.0). This mixture was incubated at 37°C for 30 min and then centrifuged at 1,000 × *g* for 2 min, and the A_{540} of the supernatant was determined automatically in a colorimeter (Titertek Multiskan MC; Flow Laboratories, Inc., McLean, Va.). The A_{540} value after 100% lysis of a control erythrocyte suspension with 1% Triton X-100 was also determined. One hemolytic unit (HU) was defined as the amount of cytolysin causing 50% hemolysis.

Cytolytic activity. Vero and CHO cells were grown in Eagle minimal essential medium supplemented with 10% calf serum. Cytolytic activity was assayed in 96-well flat-bottom microtiter plate (Corning Glass Works, Corning, N.Y.). Samples of 100 μl of suspensions containing 2×10^4 cells were dispensed into the wells and incubated overnight at 37°C in a humidified atmosphere of 5% CO_2 in air. A 50-μl volume of the cytolysin preparation diluted with serum-free minimal essential medium was added to each well, and the microtiter plate was incubated at 37°C. Cytotoxicity was assessed 10 h after the addition of cytolysin by the dye exclusion test with eosin Y (33).

Electrophoresis. Conventional disc polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulfate (SDS)-slab PAGE, and polyacrylamide disc gel isoelectric focusing were carried out by the methods of Davis (11), Laemmli (23), and Wrigley (44), respectively.

Antiserum and antibody. Antiserum was raised in rabbits. Doses of 20 μg of the purified cytolysin with adjuvants were injected subcutaneously into the footpads of rabbits 3 times at 3-week intervals. The purified cytolysin solution was emulsified with an equal volume of Freund complete adjuvant (Difco) for the first injection and incomplete adjuvant for the other injections. Blood was taken 7 days after the third injection, and the serum was separated.

Antibody was purified from the antiserum by immunoaffinity chromatography on a column of purified cytolysin coupled to CNBr-activated Sepharose 4B (Pharmacia) by the method of Cuatracasas (10). Antibody was eluted with 0.2 M glycine hydrochloride buffer (pH 2.7) containing 0.5 M NaCl. A minimal 100-ng dose of antibody was required to neutralize the activity of 10 ng of cytolysin.

Antisera against *V. parahaemolyticus* thermostable direct hemolysin and *V. mimicus* hemolysin (17) were prepared by the same method by injecting the purified antigens.

Inactivation study. For measurement of heat stability, 250 ng of purified cytolysin per ml diluted with PBS containing 0.1% bovine serum albumin (BSA) was incubated in a water bath at 37, 60, or 100°C for various times; and then, 0.2-ml portions were assayed for hemolytic activity as described above.

The effects of trypan blue (Wako Pure Chemical Industries, Osaka, Japan) and divalent cations (Cu^{2+} and Zn^{2+}) on cytolysin activity were examined by assaying the hemolytic activity in the presence of these reagents. For this, a mixture of the reagent in 10 mM Tris hydrochloride containing 0.85% NaCl (Tris-buffered saline [TBS]; pH 7.0) and 0.1 ml of 100 ng of purified cytolysin (3.3 HU) per ml was incubated with 0.2 ml of 2% calf erythrocyte suspension in TBS at 37°C for 30 min, and the hemolytic activity was determined.

The effect of cholesterol on cytolysin activity was examined as described below. Portions of a solution of 5 mg of cholesterol (Wako) per ml in ethanol were rapidly injected through a microtip (Gilson) into a solution of 50 ng of cytolysin per ml in TBS to give final concentrations of 1, 10, and 100 μg/ml, according to the single-bilayer liposome preparation method of Batzri and Korn (6). The mixtures were incubated at 23°C for 10 min, and then 0.2-ml portions of each mixture were tested for hemolytic activity as described above.

Infant mouse assay. A volume of 0.01 ml of 2% Evans blue solution (Sigma Chemical Co., St. Louis, Mo.) was added to 0.5 ml of each specimen, and 0.1 ml of the mixture was given intragastrically to mice (weights, 1.9 to 2.3 g). The mice were kept at room temperature (20 to 25°C) and sacrificed with chloroform, and the degree of fluid accumulation was determined from the ratio of the intestinal weight to the weight of the rest of the body (FA ratio). By the criteria proposed by Dean et al. (12), the response was considered positive if the ratio was higher than 0.09.

Vascular permeability factor activity assay. The vascular permeability factor activity was assayed essentially as described previously (41). A volume of 0.1 ml of serial fivefold dilutions of samples was injected intradermally into shaved areas of rabbit skin 3 h before intravenous injection of 5% Evans blue solution (Sigma) dissolved in 0.85% NaCl (1 ml/kg of body weight). One hour after injection of the dye, the diameter of the blue lesion in the skin was measured.

Ouchterlony immunodiffusion test. The double gel immunodiffusion test was carried out by the method of Ouchterlony (32) with 1% Noble agar gel (Difco) in PBS.

ELISA. ELISA was carried out by a previously described method (18) by using purified antibody against *V. metschnikovii* cytolysin instead of that against *E. coli* heat-labile enterotoxin.

Determination of protein. Protein content was determined by the method of Lowry et al. (25). BSA was used as a standard.

RESULTS

Production of cytolysin. Hemolytic activity in the culture supernatant of strain MIT-1 cells reached a maximum in the exponential phase of growth and then gradually decreased. No hemolytic activity was detected in cultures that were more than 16 h old.

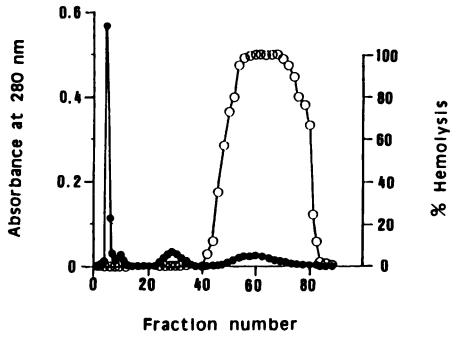

FIG. 1. Phenyl-Sepharose CL-4B (second) chromatography of *V. metschnikovii* cytolysin. Symbols: ●, A_{280}; ○, hemolytic activity of 10-fold-diluted samples of each fraction.

Purification of cytolysin. The elution profiles of the *V. metschnikovii* cytolysin on the second phenyl-Sepharose CL-4B column and HPLC are shown in Fig. 1 and 2, respectively. On the first phenyl-Sepharose CL-4B column chromatography, the bound cytolysin was eluted with buffer without NaCl. Fractions with hemolytic activity were pooled, concentrated by ultrafiltration, and applied to the second phenyl-Sepharose CL-4B column. Under these conditions, cytolysin was eluted after about six bed volumes of the column (fraction 40; Fig. 1), indicating that the gel interacted with this cytolysin. It was therefore well separated from most contaminating proteins. The final step of purification was HPLC on an anion-exchange column (Fig. 2). The cytolysin was eluted at 0.18 M NaCl.

The cytolysin preparation obtained by HPLC gave one protein band on conventional disc PAGE (Fig. 3), and the hemolytic activity was found in the same position as the protein band (Fig. 3, lane 2), indicating that the cytolysin was highly purified. The final yield of cytolysin activity from the culture was 18.5% of that initially present; about 3 mg of cytolysin was obtained from 6 liters of culture (Table 1).

Three cytolysin preparations were purified from different bacterial strains, namely, MIT-1 (clinical isolate), MIY-920 (type strain), and MIY-1323. No difference was found in these cytolysins by conventional PAGE and SDS-PAGE (Fig. 4, lanes 5, 6, and 7) or dose-dependent lysis of calf

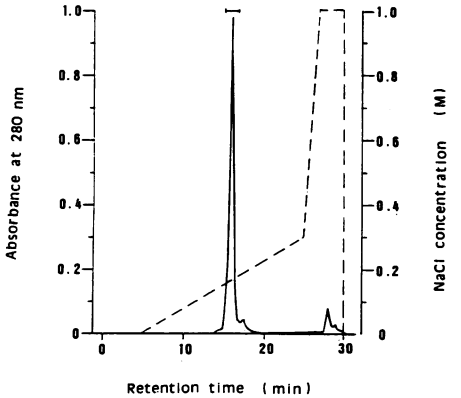

FIG. 2. Elution profile of *V. metschnikovii* cytolysin on HPLC on a Mono Q column. A sample of 1.0 ml of cytolysin (protein concentration, about 1 mg/ml) from the second hydrophobic column was applied. Symbols: ——, A_{540}; – – –, NaCl concentration. The hemolytic activity was observed in a major peak eluted with ca. 0.18 M NaCl (indicated by the bar).

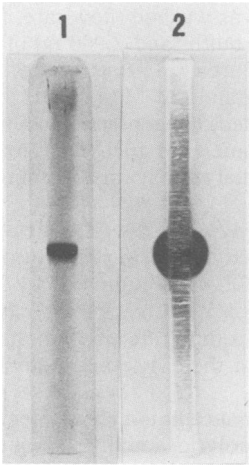

FIG. 3. Conventional (undenatured) disc PAGE of the purified cytolysin. Lanes: 1, purified cytolysin (40 μg); 2, zone of hemolysis by gel dipped in blood agar (2% calf erythrocytes in 1% Noble agar-PBS).

erythrocytes. Furthermore, they appeared immunologically identical in an Ouchterlony double immunodiffusion test (Fig. 5).

Estimation of molecular weight and isoelectric point. The SDS-PAGE pattern of the cytolysin preparations obtained at each purification step is shown in Fig. 4. The purified cytolysin migrated as a single band even under denatured conditions. The molecular weight of cytolysin estimated by SDS-PAGE was 50,000. On gel filtration on Sephadex G-100 or Sephacryl S-200, the cytolysin eluted after the bed volume of the column, and so its molecular weight could not be determined from its elution volume on gel filtration. A probable explanation for this observation is that the purified cytolysin interacted with the dextran-based gels, and so was retarded on the gel columns. Similar results have been obtained in studies on the molecular weights of *V. vulnificus* cytolysin (15) and *Clostridium perfringens* delta toxin (1).

On polyacrylamide gel electrofocusing, the purified cytolysin revealed a single band in the gel. No other active species of cytolysin was found in the purified preparation. The isoelectric point of the purified cytolysin was estimated as 5.1 from the standard curve obtained with marker proteins

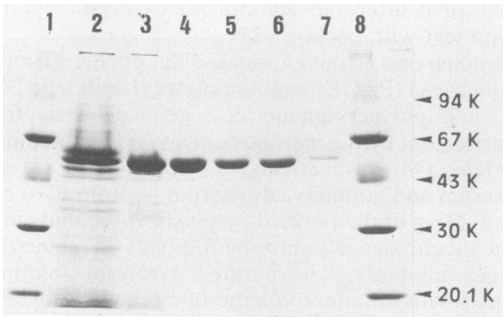

FIG. 4. SDS-PAGE of cytolysin preparations. Lanes: 1 and 8, molecular weight markers (K indicates molecular weight, in thousands) (Pharmacia); 2, acid-precipitated fraction; 3, eluate from first phenyl-Sepharose CL-4B column; 4, eluate from second phenyl-Sepharose CL-4B column; 5, purified cytolysin from MIT-1; 6, purified cytolysin from MIY-920; 7, purified cytolysin from MIY-1323.

TABLE 1. Purification of *V. metschnikovii* cytolysin from MIT-1

Purification step	Vol (ml)	Total protein (mg)	Total activity (HU)	Sp act (HU/μg)	Relative activity	Yield (%)
Culture supernatant	5,430	8.4×10^4	4.1×10^6	0.049	1	100
Acid precipitation (crude cytolysin)	87	6.0×10^2	3.0×10^6	5.0	102	73.2
Phenyl-Sepharose CL-4B (first)	7.1	20.8	1.7×10^6	82	1,700	41.5
Phenyl-Sepharose CL-4B (second)	14.2	4.6	7.1×10^5	150	3,100	17.3
HPLC	10.9	3.2	7.6×10^5	240	4,900	18.5

(cytochrome *c* and its acetylated derivatives; Oriental Yeast Co., Tokyo, Japan).

Inactivation study. The cytolysin was heat labile. It was inactivated by heating at 60°C for 5 min, even in the presence of 0.1% BSA as a stabilizer (Table 2). Divalent cations, such as Zn^{2+} and Cu^{2+}, at millimolar concentrations, inhibited the cytolytic activity. Takeda et al. (40) have reported that the inhibitory effects of zinc ion on the hemolytic activity of thermostable direct hemolysin from *V. parahaemolyticus* and Triton X-100 were false inhibitions (pseudoinhibitions) due to precipitation of hemoglobin (4). We examined whether this was also the case with cytolysin by doing the following experiments. First, a suspension of 2% erythrocytes was treated with an equal volume of 400 ng of cytolysin per ml in the presence of 1.0 mM $CuCl_2$ in TBS at 37°C for 30 min. During this treatment, no hemolysis was observed, as described above. Then, we washed erythrocytes with TBS without Cu^{2+}, suspended them in TBS, and incubated them further. When this incubation was carried out at 37°C, complete hemolysis was observed within 15 min, whereas no hemolysis was observed when they were incubated at 4°C (Table 3). The amount of hemoglobin released from erythrocytes in this experiment was almost the same as that released from erythrocytes that had not been treated with Cu^{2+}. These findings and the fact that the hemoglobin precipitated by this cation could not be recovered in a soluble form by cation-free buffer showed that the inhibitory effect of Cu^{2+} was not due to pseudoinhibition by precipitation of hemoglobin. Similar results were obtained with Zn^{2+}.

Cholesterol is known to inactivate thiol-activated (oxygen-labile) cytolysin (37). The cytolysin isolated in this study was also inhibited by cholesterol (Table 2). The MIC of cholesterol for this cytolysin was, however, about 100-fold more than that commonly used to inactivate thiol-activated cytolysins (9, 27, 34, 36, 43). Thiol-activated cytolysins are also inactivated by oxidation of their thiol groups and are activated by reduction of their thiol groups by reducing reagents, such as dithiothreitol and cysteine hydrochloride (37). The hemolytic activity of the *V. metschnikovii* cytolysin was not inhibited by air oxidation or activated by dithiothreitol (data not shown). Thus, this cytolysin is not thiol activated.

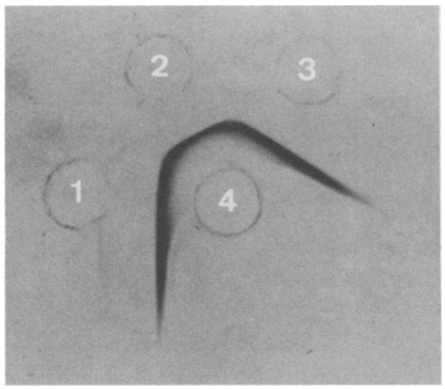

FIG. 5. Ouchterlony double gel immunodiffusion test of purified cytolysin from various strains. Wells contained cytolysins from MIY-920 (well 1), MIT-1 (well 2), MIY-1323 (well 3), and anti-purified cytolysin of MIT-1 (well 4).

Biological activities. The cytolysin was active against erythrocytes from all eight animal species examined (Table 4). It was most active against calf erythrocytes; the specific activity of the purified cytolysin for lysis of calf erythrocytes was about 333 HU/μg of protein.

The cytolysin caused fluid accumulation in the intestines of infant mice. After injection of 5 μg of cytolysin, the FA ratio rapidly increased within 30 min, reaching a maximum after 2 h that persisted for 18 h, which is different from the time course reported for *E. coli* heat-stable enterotoxin (13). The dose-response curve of the FA ratio 3 h after the inoculation is shown in Fig. 6. The minimum amount of cytolysin that induced an FA ratio of more than 0.09 was about 2 to 3 μg.

The purified cytolysin increased the vascular permeability of rabbit skin. A blue response appeared immediately after injection of the cytolysin and reached a maximum after 2 to 3 h. This early response (followed by a quick decrease) was

TABLE 2. Inactivation of *V. metschnikovii* cytolysin by heat, cholesterol, trypan blue, and divalent cations

Treatment	Residual activity (%)
None[a]	100
37°C, 30 min[b]	100
60°C, 5 min	0
100°C, 1 min	0
Cholesterol (μg/ml)[c]	
100	14.7
10	57.0
1	90.8
TBS[d]	100
Trypan blue[d] (40 μg/ml[e])	95.0
Cu^{2+}[d] (mM)	
1.0	0
0.2	55.5
Zn^{2+}[d] (mM)	
1.2	6.7
0.6	92.5

[a] Not subjected to heat treatment.
[b] These heat treatments are described in detail in the text.
[c] Final concentration of cholesterol in cytolysin solution.
[d] A concentration of 100 ng of cytolysin per ml and an equal volume of the indicated concentrations of the reagents were mixed and immediately subjected to the hemolytic activity assay.
[e] Dose commonly used to inactivate streptolysin S (14).

TABLE 3. Restoration of hemolytic activity of *V. metschnikovii* cytolysin on removal of copper ion[a]

Suspended in:	A_{540} at[b]:	
	37°C	4°C
TBS	1.008 ± 0.011	0.021 ± 0.006
TBS-Cu^{2+}[c]	0.013 ± 0.016	0.018 ± 0.015

[a] A suspension of 2% calf erythrocytes was incubated with 100 ng of cytolysin per ml in the presence of 1 mM $CuCl_2$. Then, the erythrocytes were washed and suspended in TBS with or without $CuCl_2$ and incubated at 37 or 4°C for 30 min.
[b] Values are means ± standard deviations.
[c] TBS containing 1 mM $CuCl_2$.

quite different from that to cholera enterotoxin (and to the heat-labile enterotoxin of *E. coli*), which did not appear so rapidly; reached a maximum in 8 to 10 h; and persisted for 20 to 30 h. Administration of 200 ng of the cytolysin induced an obvious blue response, and at more than 1 μg the toxin induced necrosis in the center of the blue region. These phenomena are also different from the reaction caused by cholera toxin.

The cytolysin also lysed cultured mammalian cells, such as Vero and CHO. The amounts of cytolysin required for 50% lysis of Vero and CHO cells were 8 and 1 ng, respectively.

All the biological activities of cytolysin were neutralized by anti-cytolysin antibody and inactivated by heating the cytolysin at 60°C for 5 min (data not shown).

Mechanism of the lysis of erythrocytes. The mechanism of lysis of calf erythrocytes by *V. metschnikovii* cytolysin was examined. Lysis of the erythrocytes was temperature dependent (Fig. 7), and the optimal temperature for the lysis was 37 to 43°C. At 37°C, lysis of the erythrocytes by 3.3 HU of cytolysin began after 5 min and reached 100% within 30 min. At 18°C, however, no hemolysis was observed during this time period.

The percentage of hemolysis decreased with an increase in the target cell concentration (Fig. 8). Moreover, the absolute amount of lysis (measured as the A_{540}) was highest with 0.5 to 1.5% erythrocytes. These findings suggest that more than one molecule of the cytolysin is required to lyse a single erythrocyte (20, 31).

ELISA and cross-neutralization test. It has been reported that many vibrios produce various hemolysins or cytolysins and that some hemolysins or cytolysins from certain vibrio species are immunologically related to those from the other species (17, 41, 42; S. Yamada, S. Matsushita, Y. Kudoh, and M. Ohashi, Proc. 20th Joint Conf. U.S.-Japan Cooperative Med. Sci. Prog. Cholera Panel, p. 25, 1984). We examined whether this cytolysin is immunologically related

TABLE 4. Specific activities of the purified cytolysin for lysis of erythrocytes from various animal species

Erythrocyte source	Sp act (HU/μg of cytolysin)
Calf	333.3
Rabbit	138.9
Guinea pig	131.6
Mouse	125.0
Human	14.3
Sheep	12.5
Chicken	6.3
Horse	4.9

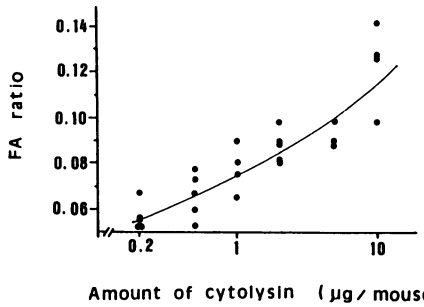

FIG. 6. Fluid accumulation in infant mice induced by purified cytolysin. Doses of 10, 5, 2, 1, 0.5, and 0.2 μg were administered to groups of at least three mice. Each point represents the value for one mouse.

to hemolysins or cytolysins from other vibrios by the ELISA by using anti-*V. metschnikovii* cytolysin antibody as a coating antibody and by the neutralization test (Table 5). The antibody against the purified *V. metschnikovii* cytolysin neither neutralized the hemolytic activities of nor reacted in the ELISA with crude hemolysin preparations. Furthermore, the hemolytic activity of *V. metschnikovii* cytolysin was not neutralized by antibodies against thermostable direct hemolysin or *V. mimicus* hemolysin, whereas these antibodies completely neutralized the activities of the homologous antigens (data not shown).

DISCUSSION

As far as we know, this is the first report of purification and characterization of the cytolysin produced by *V. metschnikovii*.

During purification of the cytolysin, we found that acid precipitation as an initial step was simpler and more economical with a large volume of culture supernatant than other methods, such as ammonium sulfate precipitation or ultrafiltration. Furthermore, we found that this step is very effective, increasing the specific activity of the cytolysin 100-fold (Table 1). Similar results were obtained by Asao et al. (2) during purification of *Aeromonas hydrophila* hemolysin.

The cytolysin tended to aggregate and form various complexes with other coexisting proteins when concentrated by acid precipitation, which made it difficult to purify the toxin. The most effective method to overcome this difficulty was the addition of 1 M urea to the buffer during purification.

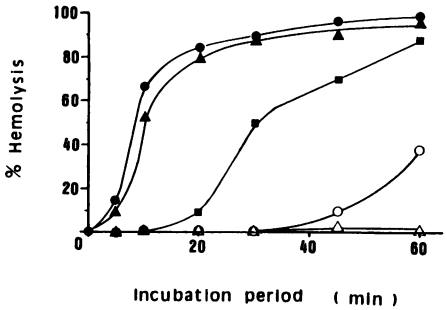

FIG. 7. Effect of temperature on hemolysis by purified cytolysin. Mixtures of 0.2 ml of 2% washed calf erythrocytes and 0.2 ml of 50 ng of purified cytolysin per ml (3.3 HU) were incubated at 4 to 18°C (△), 23°C (○), 30°C (■), 37°C (●), and 43°C (▲) for the indicated periods of time. Values are for three samples.

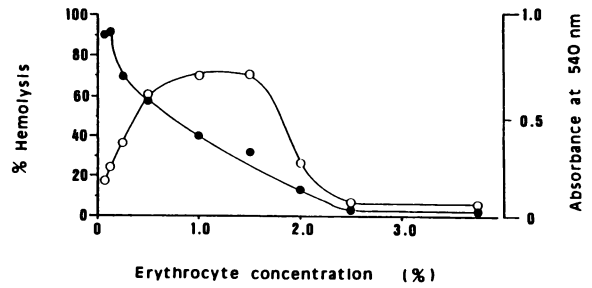

FIG. 8. Effect of erythrocyte concentration on hemolysis by purified cytolysin. Samples of 0.2-ml suspensions of washed calf erythrocytes were incubated with 0.2 ml of 20 ng of purified cytolysin per ml (1.3 HU) at 37°C for 30 min. Then, the mixtures were centrifuged and the A_{540} of the supernatant (○) was determined as described in the text. The percent hemolysis (●) was also calculated. Lysis of 100% was taken as that of the supernatant of each concentration of erythrocytes treated with 1% Triton X-100.

Neither hemolytic activity nor heat stability of the cytolysin was affected by the addition of urea to the buffer.

The purified cytolysin was a heat-labile protein with a molecular weight of 50,000 and an isoelectric point of 5.1. It was inactivated by heat treatment at 60°C for 5 min in the presence of 0.1% BSA as a stabilizer. The lability of the cytolysin was different under different experimental conditions. At a concentration of less than 1 μg/ml, it was inactivated at 23°C within 5 h without BSA, but in the presence of 0.1% BSA it was not inactivated appreciably under the same conditions (data not shown). Under these heat-labile properties, this cytolysin differed from the *V. parahaemolyticus* thermostable direct hemolysin, which is not inactivated even when it is heated at 100°C for 10 min (22).

The hemolytic activity of this cytolysin was inhibited by divalent cations (Table 2). This inhibitory effect was not a pseudoinhibition due to precipitation of hemoglobin by these cations after its release from the erythrocytes (4, 40), because the inhibition in the presence of cations was reversed by removing the cations from the extracellular medium, and because the hemoglobin content of the erythrocytes was not changed by treatment with cations and cytolysin. These observations also indicate that inhibition of binding of this cytolysin to erythrocytes is not the primary action of divalent cations; i.e., divalent cations block hemolysis at a stage of lesion formation subsequent to its binding. An interesting finding was that after treatment with the cytolysin in the presence of cations, erythrocytes were lysed in cation-free buffer at 37°C but not at 4°C (Table 3). We postulate that after binding of the cytolysin to the cell membrane, membrane fluidity is needed for the formation of lesions. If this hypothesis is correct, the inhibitory effect of divalent cations might be due to fixation or disturbance of membrane fluidity as the result of an alteration in the state of the lipid bilayer (3–5, 16) by reversible binding of the cations.

TABLE 5. Neutralization of hemolytic activity of crude hemolysin preparations and results of sandwich ELISA by using anti-*V. metschnikovii* cytolysin antibody

Culture supernatant of:	Hemolytic activity with[a]:		ELISA (A_{405})
	TBS	Antibody	
V. metschnikovii	100	0	0.833
V. vulnificus	41.7	41.7	0.065
V. fluvialis	43.7	40.2	0.051
V. furnissi	83.0	87.6	0.047
V. mimicus	142.5	151.4	0.051
V. cholerae	125.7	135.6	0.053
V. cholerae non-O1	111.6	111.6	0.044
Brain heart infusion broth	NT[b]	NT	0.055

[a] Samples were mixed with equal volumes of 50 μg of anti-*V. metschnikovii* cytolysin antibody or TBS per ml, followed by incubation at 37°C for 30 min, and were subjected to the hemolytic activity assay by using calf erythrocytes. The values are the mean percentage of HU of hemolysin preparations compared with the activity of *V. metschnikovii* culture supernatant (TBS).

[b] NT, Not tested.

Thiol-activated cytolysins are known to be inhibited by cholesterol. It is interesting that the hemolytic activity of the cytolysin examined in this study was inhibited by cholesterol (Fig. 2), although it had no thiol-activated properties. The amount of cholesterol needed to inhibit the *V. metchnikovii* cytolysin was about 100 times that generally used to inhibit thiol-activated cytolysin (9, 27, 34, 36, 43). Similarly, Gray and Kreger (15) have found that *V. vulnificus* cytolysin is inhibited by a large amount of cholesterol, but Miyoshi et al. (28) have reported that *V. vulnificus* cytolysin is inhibited by cholesterol at a concentration similar to that which inhibits thiol-activated cytolysin. This discrepancy might be due to differences in the preparations of cholesterol micelles or in the reaction conditions. It is possible that a much lower concentration of cholesterol would inhibit *V. metschnikovii* cytolysin under different conditions.

V. metschnikovii did not seem to produce other hemolysins than the cytolysin we reported here, and no immunological similarities of this cytolysin with other hemolysins of vibrios were found (Table 5). The possibility, however, is left that another hemolysin which has an immunological cross-reactivity with other hemolysins of vibrios could be produced under different culture conditions of *V. metschnikovii*.

Of the biological activities of the purified cytolysin demonstrated in this study, those that caused fluid accumulation in mice and increased vascular permeability in rabbit skin seemed important, although the time courses of fluid accumulation in suckling mice and the increase in vascular permeability were quite different from those of typical enterotoxins such as *E. coli* heat-stable enterotoxin and cholera enterotoxin. These activities were also observed in the culture supernatant of *V. metschnikovii* and were completely neutralized by anti-cytolysin antibody against the purified cytolysin. Thus, this cytolysin may be the only toxin in the culture supernatant of *V. metschnikovii* that causes fluid accumulation and an increase in vascular permeability. In several reports (19, 26, 38), it has been suggested that hemolysins are the causative agents of diarrhea. These results and data such as those from the positive suckling mouse assay with the cytolysin (Fig. 6) purified from a *V. metschnikovii* strain derived from stools of a patient with diarrhea led us to consider the possibility that this cytolysin acts as an enterotoxin. The data presented here, however, are not enough to make this conclusion. Further studies on the etiological, clinical, and immunological aspects of *V. metschnikovii* infection are needed for the establishment of the pathogenicity of the cytolysin described here.

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