

Non-O1 *Vibrio cholerae* Hemolysin: Purification, Partial Characterization, and Immunological Relatedness to El Tor Hemolysin

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Hemolysin of a non-O1 *Vibrio cholerae* strain was purified and characterized. The purified hemolysin gave a single protein band on conventional and sodium dodecyl sulfate-gel electrophoresis. Its molecular weight was estimated as 60,000 by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. It had a pI of 5.7. The purified hemolysin caused increased vascular permeability of rabbit skin and rapid death of mice on intravenous injection and also lysed erythrocytes of various animal species. An Ouchterlony double gel diffusion test using antiserum against the purified hemolysin indicated that the hemolysin from non-O1 *V. cholerae* was immunologically related, but not identical, to the hemolysin from El Tor *V. cholerae*. Antiserum against the purified hemolysin neutralized the hemolytic activity of the hemolysins from not only non-O1 but also El Tor *V. cholerae*.

Vibrio cholerae strains other than serovar O1 (non-O1 *V. cholerae*) have long been known to cause diarrhea. Some non-O1 *V. cholerae* strains have been found to produce an enterotoxin that is similar to cholera enterotoxin (2, 8, 13, 15-18). We purified enterotoxins from non-O1 *V. cholerae* and found that one was identical to cholera enterotoxin (15) and another was similar but not identical to it (16). The production of cholera-like enterotoxin in non-O1 *V. cholerae* suggested that this enterotoxin might cause diarrhea in some non-O1 *V. cholerae* infections. However, non-O1 *V. cholerae*, which does not produce cholera-like enterotoxin, has been repeatedly isolated from patients (13, 17), whereas no diarrheagenic factor(s) has yet been identified.

It is also known that a hemolysin is produced by most clinical isolates of non-O1 *V. cholerae* (7, 10), although its pathogenic significance is unclear. In this paper we report the purification of a hemolysin from non-O1 *V. cholerae* and studies on its biological, physicochemical, and immunological properties.

MATERIALS AND METHODS

Bacterial strains. The strain used for purification of hemolysin was non-O1 *V. cholerae* S7, which was obtained from Y. Zinnaka of the Defence Medical College, Tokorozawa, Saitama, Japan. Strain S7 produces cholera-like enterotoxin that is biologically similar but not identical to cholera enterotoxin (16). Other non-O1 and El Tor *V. cholerae* strains were all isolated from diarrheal patients.

Cultivation. A loopful of bacterial cells from a nutrient agar slant was inoculated into 5 ml of brain heart infusion broth (Difco) and incubated for 6 h at 37°C. Then 5 ml of the culture was transferred to a Roux bottle containing 150 ml of

syncase broth (4) and incubated for 18 h at 37°C. Volumes of 1.5 ml of the culture were then inoculated into 150-ml volumes of syncase broth supplemented with 3% glycerol in Roux bottles (surface/volume ratio: 2 cm²/ml) and incubated for 48 h at 30°C without shaking.

Preparation of crude hemolysin. After addition of 0.02% NaN₃, the culture (4,000 ml) was centrifuged at 25,000 × *g* for 20 min and the supernatant fluid (3,728 ml) was collected. It was mixed with 1/20 volume of 1 M Tris-hydrochloride buffer (pH 8.0) and then with 1,526 g of solid ammonium sulfate (60% saturation) at 4°C. The mixture was centrifuged at 25,000 × *g* for 30 min, and the precipitate was suspended in 25 ml of Tris-hydrochloride buffer (50 mM, pH 8.0) containing 1 mM EDTA and 3 mM NaN₃ (TEA buffer) and dialyzed against the same buffer. The dialyzed sample was centrifuged, and the supernatant (28 ml) was used as crude hemolysin.

Determination of hemolytic activity. Samples (1 ml) of a 2% sheep erythrocytes suspension (standardized to give an optical density of 0.6 at 540 nm when lysed and diluted 10-fold with distilled water) were incubated for 30 min at 37°C with samples (1 ml) of diluted hemolysin preparations. After centrifugation for 2 min at 3,000 rpm, hemoglobin released into the supernatant was measured as the absorbance at 540 nm. One hemolytic unit (HU) was defined as the amount of hemolysin causing 50% hemolysis of 1 ml of 1% sheep erythrocytes solution in 30 min at 37°C.

Antiserum. Rabbit antihemolysin antiserum was obtained by subcutaneous injection of 50 µg of the purified hemolysin into the footpads in three doses, one with an equal volume of Freund complete adjuvant (Difco) and the others with an equal volume of incomplete adjuvant (Difco), at 4-week intervals. Serum was taken 7 days after the third injection.

Polyacrylamide gel electrophoresis. Conventional polyacrylamide gel disk electrophoresis, sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis, and polyacrylamide gel isoelectrofocusing were carried out as described previously (16) by the methods of Davis (3), Laemmli (6), and Wringley (14), respectively. A molecular-weight marker kit was purchased from Boehringer-Mannheim GmbH (Mannheim, W. Germany). Cytochrome *c* and its acetylated deriv-

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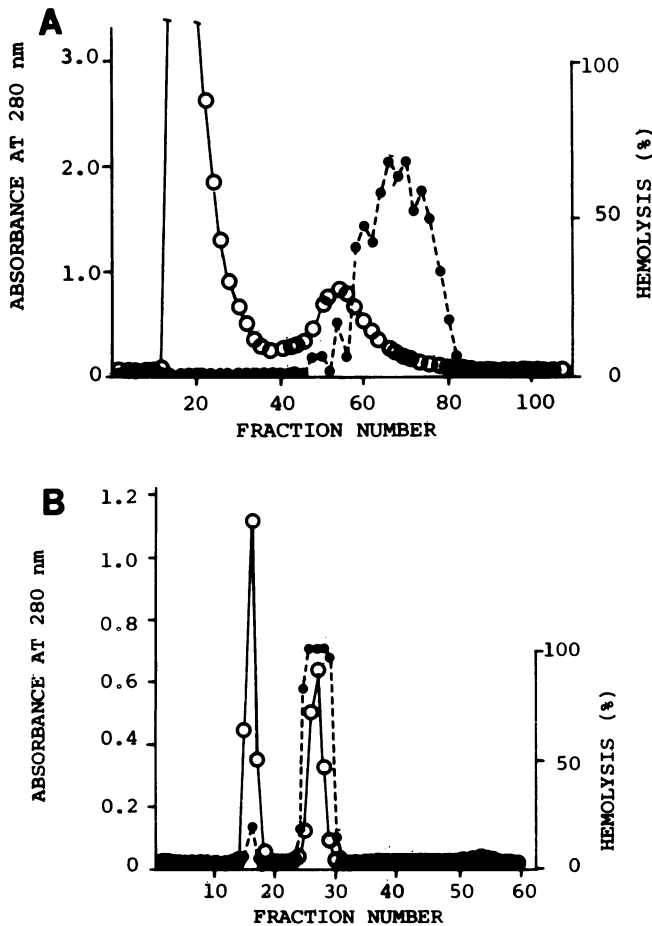


FIG. 1. Sephadex G-100 gel filtration of the hemolysin. (A) The crude hemolysin was applied to a Sephadex G-100 regular column previously equilibrated with TEA buffer. Material was eluted with TEA buffer. Flow rate was 90 ml/h, and 15 ml was collected in each fraction. (○) Absorbance at 280 nm; (●) hemolytic activity of 500-times-diluted samples of each fraction (100% hemolysis gave an optical density of 3.0 at 540 nm). (B) The Sephadex G-100 regular gel eluate was applied to a Sephadex G-100 superfine column previously equilibrated with TEA buffer containing 0.25 M glucose. The elution was made with TEA buffer containing 0.25 M glucose with the flow rate of 10 ml/h; 5 ml was collected in each fraction. (○) Absorbance at 280 nm; (●) hemolytic activity of 5,000-times-diluted samples of each fraction.

atives (Oriental Yeast Co., Tokyo) were used as pI marker proteins.

Vascular permeability factor assay. Permeability factor assay was carried out essentially as described previously (15, 16). Volumes of 0.1 ml of serial fourfold dilutions of samples were injected intradermally into shaved areas of

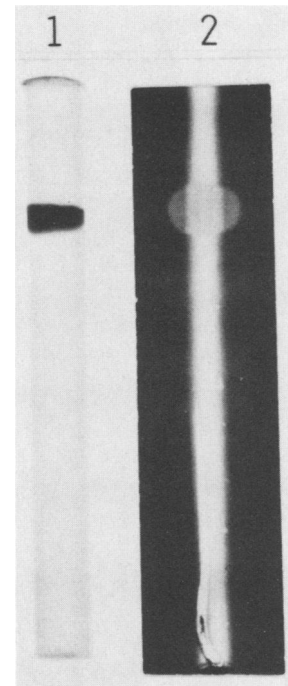


FIG. 2. Polyacrylamide gel disk electrophoresis of the purified hemolysin. Lane 1, Purified hemolysin (100 µg); lane 2, zone of hemolysis obtained when the purified hemolysin (10 µg) was subjected to electrophoresis and then the gel was dipped in melted blood agar, solidified, and incubated at 37°C for 4 h.

rabbit skin at 23, 4, 2, 1, and 0 h before intravenous injection of 5% Evans blue solution (Sigma Chemical Co., St. Louis, Mo.) in 0.75% 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 to the nearest 0.5 mm.

Ouchterlony immunodiffusion test. The double gel diffusion test was carried out by the method of Ouchterlony (9) with 1.2% Noble agar gel (Difco) in 20 mM phosphate buffer (pH 7.0) containing 0.02% NaN₃. Samples of 50 µl were put into each well. The gel plates were stained with Coomassie brilliant blue (Wako Pure Chemicals, Osaka, Japan) and destained with a solution of 10% acetic acid and 50% methyl alcohol.

Determination of protein. Protein content was determined as described by Bradford (1) with a Bio-Rad Protein Assay System (Bio-Rad Laboratories, Richmond, Calif.), using bovine serum albumin as a standard.

RESULTS

Conditions for hemolysin production. The production of hemolysin by several strains of non-O1 *V. cholerae* was examined on syncase blood agar plates. Of 53 non-O1 *V.*

TABLE 1. Purification of non-O1 hemolysin

Fraction	Vol (ml)	Total protein (mg)	Total hemolytic activity (HU)	Sp act (HU/µg)	Relative activity	Yield (%)
Culture supernatant	3,728	ND ^a	5.5×10^6	NA ^b	NA	100
Ammonium sulfate precipitate (crude hemolysin)	28	2,250	2.4×10^6	1.1	1	44
Sephadex G-100 regular column eluate	2	46	8.3×10^5	18	17	15
Sephadex G-100 superfine column eluate	2	18.2	4.6×10^5	25	23	8.2

^a ND, Not done.

^b NA, Not applicable.

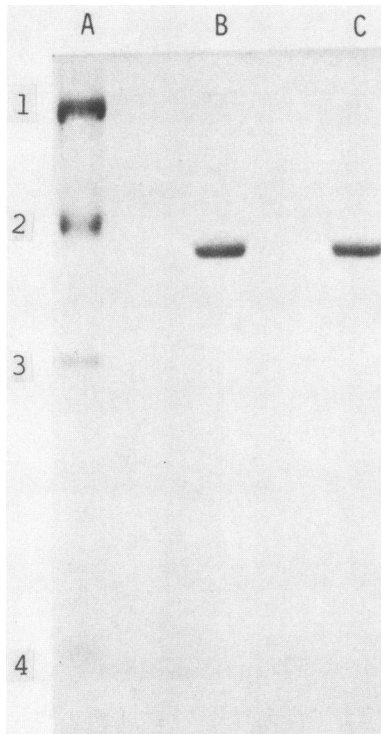


FIG. 3. Sodium dodecyl sulfate slab gel electrophoresis of the purified hemolysin. Lane A, Molecular weight marker proteins: 1, β and β' subunits of RNA polymerase of *Escherichia coli* ($M_r = 155,000$ and $165,000$, respectively); 2, bovine serum albumin ($M_r = 68,000$); 3, α subunit of RNA polymerase of *E. coli* ($M_r = 39,000$); 4, soybean trypsin inhibitor ($M_r = 21,500$). Lane B, Purified hemolysin without dithiothreitol. Lane C, Purified hemolysin with dithiothreitol.

cholerae strains examined, 52 produced the hemolysin, resulting in transparent hemolytic zones around the colonies. Strain S7 was found to produce relatively high amounts of the hemolysin and so was used for further studies on production and purification of the hemolysin.

Among various media studied, such as syncase broth, brain heart infusion broth, and heart infusion broth, syncase broth enhanced the production of the hemolysin more than other media; stationary culture in syncase broth at 30°C produced the most hemolysin. Production of the hemolysin was significantly enhanced by the addition of 3% glycerol to the medium, like the production of hemolysin by *V. cholerae* biotype El Tor (5). Addition of 3% glycerol increased the production of the hemolysin in 48 h more than 10 times.

Purification of the hemolysin. Crude hemolysin prepared as described above was applied to a column (4 by 70 cm) of Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden). A typical pattern of elution is shown in Fig. 1A. Hemolytic activity was eluted after the bed volume (fraction no. 58) of the column. This delayed elution suggests that the hemolysin interacts with the Sephadex gel. Active fractions were pooled and concentrated on an Amicon PM10 membrane (Amicon Co., Lexington, Mass.), and the concentrated material was applied to a Sephadex G-100 column (superfine, 2.5 by 95 cm) equilibrated with TEA buffer containing 0.25 M glucose, which interferes with the interaction of the hemolysin with Sephadex gel. Two protein peaks were eluted, the second of which coincided with the hemolytic activity (Fig. 1B). Active fractions were pooled, concentrated with an Amicon PM10 membrane, and used as purified

hemolysin. Typical data of the purification steps are summarized in Table 1.

Polyacrylamide gel electrophoresis of the purified hemolysin. The result of conventional polyacrylamide gel disk electrophoresis of the purified hemolysin is shown in Fig. 2. The purified hemolysin gave one protein band (lane 1) with the same mobility as the hemolytic activity (hemolytic zone in lane 2). The purified hemolysin also gave one protein band on sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis in the presence and absence of dithiothreitol (Fig. 3). These data indicate that the purified hemolysin is homogeneous. The result of polyacrylamide gel isoelectrofocusing also showed the homogeneity of the purified hemolysin (Fig. 4, insert lane A).

Determination of the molecular weight of the purified hemolysin. The molecular weight of the purified hemolysin was estimated to be 60,000 by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis with marker proteins (Fig. 3).

pI of purified hemolysin. Polyacrylamide gel isoelectrofocusing was carried out to determine the pI of the purified hemolysin. From the standard curve obtained with marker proteins (Fig. 4), the pI of the purified hemolysin was determined to be 5.7.

Biological activities. The specific activity of the purified hemolysin for lysis of sheep erythrocytes was about 25 HU/ μg of protein. The activity was destroyed by heating for 10 min at 70 or 100°C . The purified hemolysin lysed erythrocytes from various animal species, namely, rabbit, guinea pig, pig, sheep, goat, dog, horse, calf, mouse, goose, and chicken. The specific activities for lysis of each blood cell type are shown in Table 2.

The purified hemolysin increased the vascular permeability of rabbit skin (Fig. 5). A blueing response appeared immediately after injection of the hemolysin and reached a maximum after 3 to 5 h. Administration of more than $1 \mu\text{g}$ of the hemolysin induced necrosis in the center of the blueing region.

The purified hemolysin was lethal to mice when injected

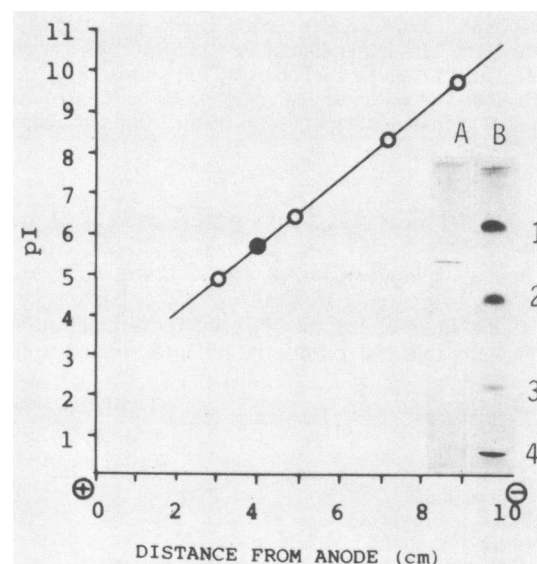


FIG. 4. Determination of the isoelectric point of the purified hemolysin by polyacrylamide gel isoelectrofocusing. (○) Purified hemolysin. (●) Standard markers: 1, pI 4.9; 2, pI 6.4; 3, pI 8.4; 4, pI 9.7.

TABLE 2. Specific activity of the purified hemolysin for lysis of various blood cells

Source of blood cells	Sp act (HU ^a /μg of hemolysin)
Rabbit.....	125
Guinea pig.....	83
Pig.....	36
Sheep.....	25
Goat.....	25
Dog.....	25
Horse.....	25
Calf.....	11
Mouse.....	9.6
Goose.....	2.3
Chicken.....	0.6

^a One HU is defined as the amount of hemolysin causing 50% hemolysis of 1 ml of 1% blood cells in 30 min at 37°C.

intravenously. The 50% lethal dose was 0.3 μg in mice of about 20 g. Administration of more than 1 μg of the hemolysin killed the mice within 1 min.

Immunological relatedness of the hemolysin of non-O1 *V. cholerae* to El Tor hemolysin. An Ouchterlony double gel diffusion test was carried out with the purified hemolysin and crude hemolysins from various strains of non-O1 and El Tor *V. cholerae* against antiserum raised against the purified hemolysin from *V. cholerae* S7. The precipitin lines that formed between the antiserum and the purified hemolysin or crude hemolysins from various strains of non-O1 *V. cholerae* were observed to fuse (Fig. 6). Spur formations were seen between the precipitin line with the purified hemolysin and those with crude hemolysins from various strains of El Tor *V. cholerae*. These results indicate that the hemolysin from non-O1 *V. cholerae* shares an antigenic determinant(s) with hemolysin from El Tor *V. cholerae*. This notion was further supported by the results (Fig. 7) on the ability of the antiserum to purified hemolysin to neutralize the hemolytic activities of hemolysins from various strains of non-O1 and El Tor *V. cholerae*. The hemolytic activities of the hemolysins from all non-O1 *V. cholerae* were neutralized with the antiserum diluted about 10³ to 10⁴ times, whereas about 10 to 100 times more antiserum was needed to neutralize the hemolytic activity of the hemolysin from El Tor *V. cholerae*.

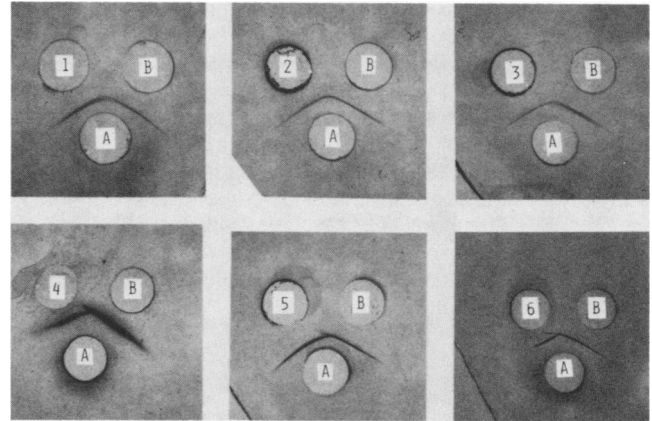


FIG. 6. Ouchterlony double gel diffusion test of hemolysins from various strains of non-O1 and El Tor *V. cholerae*. (A) Anti-purified hemolysin from *V. cholerae* S7. (B) Purified hemolysin (5 μg) from *V. cholerae* S7. The wells contained undiluted crude hemolysins from non-O1 *V. cholerae* strains 5047 (1), 1112 (2), and 1122 (3) and from *V. cholerae* El Tor strains Marraca (4), Q3735 (5), and K4746 (6), all of which were prepared as described in the text.

DISCUSSION

It is known that non-O1 *V. cholerae* produces a hemolysin (7, 11), but little is known about the characteristics of this hemolysin. In this work we purified the hemolysin from a strain of non-O1 *V. cholerae* and examined its biological, physicochemical, and immunological properties. The purified hemolysin had a molecular weight of about 60,000 (Fig. 3) and a pI of about 5.7 (Fig. 4). It had a lethal effect on mice, permeability factor activity in rabbits (Fig. 5), and hemolytic activity on erythrocytes from various animal species (Table 2). These biological activities were similar to those of the hemolysin purified previously from a strain of El Tor *V. cholerae* (5). Immunological relatedness of the purified hemolysin from non-O1 *V. cholerae* S7 to that from El Tor *V. cholerae* was also demonstrated (Fig. 6 and 7).

The production by non-O1 *V. cholerae* of an enterotoxin that is similar to cholerae enterotoxin has been reported (2, 8, 13, 15-18), and the biological, physicochemical, and immunological properties of this enterotoxin have recently been clarified (15, 16). However, little is known about the

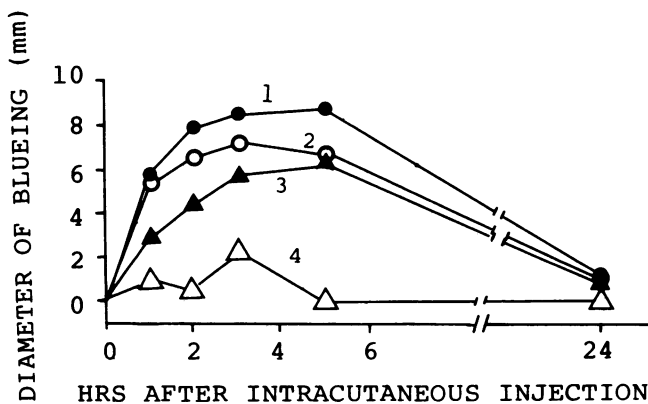


FIG. 5. Time course of permeability factor activity of the purified hemolysin in rabbit skin. Values are means of four determinations. The amounts of the hemolysin administered were (1) 1,000, (2) 250, (3) 63, and (4) 16 ng.

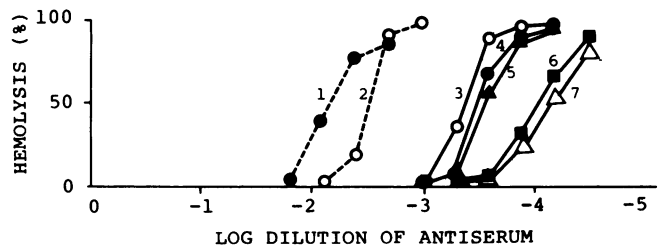


FIG. 7. Neutralization of hemolytic activity of hemolysins from various strains of non-O1 and El Tor *V. cholerae* by antiserum against the purified hemolysin from *V. cholerae* S7. Crude hemolysin containing 4 HU/ml was mixed with equal volumes (0.5 ml) of serially diluted anti-purified hemolysin and incubated for 30 min at 37°C. An equal volume of 2% sheep erythrocytes was added to the mixtures, and incubation was continued for 30 min at 37°C. After incubation, the mixtures were centrifuged and the absorbance of the supernatants at 540 nm was measured. (—) Non-O1 hemolysins; (---) El Tor hemolysins. Strains: (1) Marraca, (2) A40, (3) 5644, (4) 1113, (5) 1122, (6) 1112, (7) S7.

possible pathogenic role of the hemolysin produced by non-O1 *V. cholerae*. It is interesting that the hemolysin from non-O1 *V. cholerae* is immunologically related to that from El Tor *V. cholerae* (Fig. 6 and 7). Although the role of the hemolysin from El Tor *V. cholerae* in infection is not well understood, Sanyal et al. (12) recently reported production of a diarrheagenic factor other than cholera enterotoxin by *V. cholerae* O1, suggesting that diarrhea due to non-O1 *V. cholerae* is caused not only by cholera-like enterotoxin, but also by another diarrheagenic factor(s). Further studies on the purified hemolysin may reveal its role in diarrhea due to non-O1 *V. cholerae*.

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