Purification and Characterization of *Vibrio cholerae* Non-O1 Heat-Stable Enterotoxin

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A toxin which causes rapid fluid accumulation in a suckling mouse assay and which was produced by *Vibrio cholerae* non-O1 was investigated. The toxin was purified from the culture supernatant of *V. cholerae* non-O1 (strain A-5) by ammonium sulfate fractionation, hydroxyapatite treatment, ethanol extraction, column chromatographies on SP-Sephadex C-50 and DEAE-Sephadex A-25, and high-pressure liquid chromatography on a Lichrosorb RP-8 column. About 1.4×10^5-fold purification was achieved, with a recovery of about 12%. Although the crude preparation was heat labile, the purified toxin was heat stable. The minimum effective dose of purified toxin was about 5 ng in the suckling mouse assay. The amino acid composition of the purified toxin was determined to be Asp(3), Glu(1), Gly(1), Ala(1), half-Cys(6), Ile(2), Leu(1), Phe(1), and Pro(1). These data show the production of a new type of heat-stable enterotoxin (NAG-ST) by *V. cholerae* non-O1.

Vibrio cholerae non-O1 strains, also referred to as nonagglutinable (NAG) or noncholera vibrios, have been recognized as the causative agents of outbreaks and sporadic cases of gastroenteritis (2, 3, 8, 13), although large epidemics and pandemics like those caused by *V. cholerae* O1 have not been reported. More than one type of gastroenteritis syndrome may be associated with *V. cholerae* non-O1; that is, some strains of *V. cholerae* non-O1 cause an illness clinically indistinguishable from cholera, but others cause fever and bloody diarrhea (3, 8).

The pathogenic effects of *V. cholerae* non-O1 are not well defined. A number of investigators have shown that some *V. cholerae* non-O1 strains produce a toxin similar to cholera enterotoxin. Craig et al. (4) reported that certain *V. cholerae* non-O1 strains produce a toxin(s) that is virtually identical to cholera enterotoxin, while Yamamoto et al. (19) reported that such strains produce a toxin that is similar but not identical to cholera enterotoxin. Moreover, some investigators reported evidence suggesting that certain *V. cholerae* non-O1 strains produce an enterotoxin(s) that is not related to cholera enterotoxin. Spira et al. (13) suggested the production of a toxin similar to *Escherichia coli* heat-stable enterotoxin (EC-ST) by two strains of closely related *V. mimicus*, a group that was previously included taxonomically in the non-O1 *V. cholerae* group, and Nishibuchi et al. (9, 10) reported production of a heat-labile enterotoxin (vibrio factor) that is active in the infant mouse assay. As these workers studied only crude materials, however, they could not determine the nature of the toxin or its role in diarrhea caused by *V. cholerae* non-O1.

This paper reports the isolation and purification of a new type of heat-stable enterotoxin produced by *V. cholerae* non-O1 and a demonstration of its similarity to EC-ST.

MATERIALS AND METHODS

Strains of *V. cholerae* non-O1. One hundred strains of *V. cholerae* non-O1 used in this study were isolated at Osaka Airport Quarantine Station from 100 patients with travellers

diarrhea who had just returned from cholera-infected areas of Southeast Asia. Eight other strains were isolated from frozen seafoods imported from Southeast Asia.

Cultures. In preliminary experiments, we tested several media including CAYE (Casamino Acids-yeast extract), tryptic soy broth, and brain heart infusion broth (BHI) with or without 0.5% NaCl. As Nishibuchi and Seidler reported (9), BHI with 0.5% NaCl (BHI') was found to be the best medium for production of toxin(s) causing intestinal fluid accumulation in suckling mice. For screening toxin production, 2-ml volumes of BHI' in test tubes (13 by 100 mm) were inoculated with the test *V. cholerae* non-O1 strain and cultured at 37°C for 18 h on a roller drum (40 rpm). Culture supernatants prepared by centrifugation ($3,000 \times g$ for 20 min) of the broth cultures and filtration of the supernatants through a 0.2-μm membrane filter were tested in suckling mice. For large-scale fermentation, a culture (at 37°C for 5 h in 5 ml of BHI') of the best toxin-producing strain (A-5) isolated from frozen seafood was transferred to 1,000 ml of BHI' in a 5-liter Erlenmeyer flask and cultured at 37°C for 14 h with vigorous shaking.

Isolation of toxin. *V. cholerae* non-O1 enterotoxin was isolated and purified essentially by the method used for purification of EC-ST (1, 14, 16, 17).

Ammonium sulfate fractionation. Solid ammonium sulfate (39 g/100 ml) was added to the culture supernatant obtained by centrifugation ($15,000 \times g$ for 20 min). The resulting precipitate was collected by centrifugation ($15,000 \times g$ for 20 min), dissolved in a small amount of distilled water, and dialyzed in Spectrapor-6 dialyzing tubing (Spectrum Medical Industries, Inc.) against distilled water, as described previously (16).

Treatment with hydroxyapatite. Hydroxyapatite (Nihon Chemical Co.) (7 g/1,000 ml of original culture supernatant) was added to the ammonium sulfate-treated preparation. The unadsorbed supernatant was collected and concentrated in a rotary evaporator as described before (16).

Ethanol extraction. Absolute ethanol (−20°C) was added to the hydroxyapatite-treated preparation to a final concentration of 90%. The supernatant obtained by centrifugation
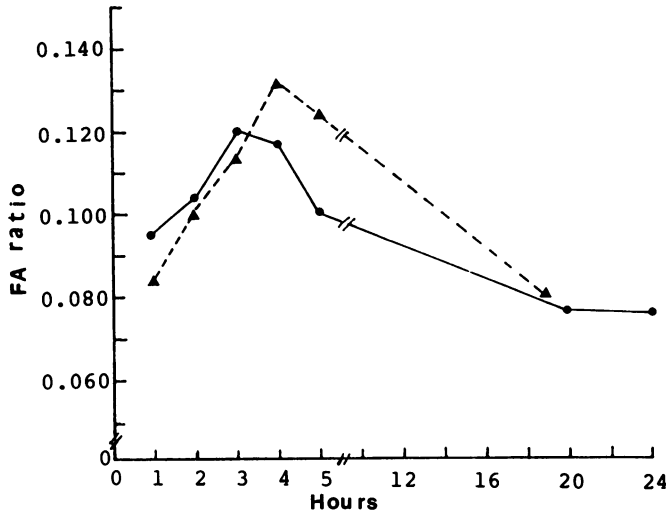
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FIG. 1. Time course of change in FA ratio induced by crude and purified NAG-ST in the suckling mouse assay. Symbols: ●, with 2 mouse units of purified NAG-ST; ▲, with 2 mouse units of crude toxin.

(15,000 × g for 20 min) was dried in a rotary evaporator and the residue was suspended in a small amount of distilled water.

SP-Sephadex C-50 column chromatography. The preparation was applied to a column (2 by 40 cm) of SP-Sephadex C-50 (H^+ cycle) equilibrated with distilled water. Material was eluted with about 500 ml of distilled water and then with 0.05 M ammonium acetate (pH 6.6). Fractions containing toxin activity were collected.

DEAE-Sephadex A-25 column chromatography. Fractions obtained from SP-Sephadex C-50 chromatography were applied to a column (2 by 50 cm) of DEAE-Sephadex A-25 (acetate form) equilibrated with distilled water. Materials were eluted with 1,000 ml of a linear gradient of 0 to 1 M acetic acid.

HPLC. The active fractions obtained from the DEAE-Sephadex A-25 column were further purified by high-pressure liquid chromatography (HPLC) on a Lichrosorb RP-8 column. Materials were applied to a column equilibrated with 10% acetonitrile in 0.01 M ammonium acetate (pH 5.7). The column was washed with 2 ml of 10% acetonitrile in 0.01 M ammonium acetate and then developed with a linear gradient of 10 to 35% acetonitrile in 0.01 M ammonium acetate (pH 5.7) at a flow rate of 2 ml/min. Fractions containing toxic activity were further subjected to HPLC (Lichrosorb RP-8 column) in 0.05% trifluoroacetic acid; materials were eluted with 2 ml of 30% acetonitrile in 0.05% trifluoroacetic acid and then with 60 ml of a gradient of 30 to 70% acetonitrile in 0.05% trifluoroacetic acid.

Suckling mouse assay. Toxin activity was assayed in suckling mice as reported previously (17). Briefly, samples of 0.1 ml with Evans blue dye as a marker were introduced by gastric tube into 2- to 3-day-old mice. At the indicated times after administration of the samples, animals were killed, and the fluid accumulation (FA) ratio of each animal was calculated as the ratio of the weight of the entire intestine to that of the rest of the body.

Heat stability. Samples of crude and purified toxin were heated at 60°C for 10 and 30 min, at 100°C for 10 and 30 min, and at 120°C for 30 min. The remaining activity was assayed in suckling mice.

Amino acid composition. The amino acid composition was

examined in a Hitachi type 835 analyzer, using samples hydrolyzed in 4 M methanesulfonic acid for 24 h at 110°C in vacuum-sealed tubes (1).

RESULTS

Toxin production. Of 100 strains of *V. cholerae* non-O1 isolated from humans and 8 strains isolated from seafoods, 55 and 5 strains, respectively, were enterotoxigenic in the suckling mouse assay. All positive strains gave an FA ratio of ≥0.090, while all negative strains gave an FA ratio of ≤0.065. Of the positive strains, strain A-5, which showed the highest toxin active in the suckling mouse assay, was used in further experiments.

Kinetics of the secretory response. Figure 1 shows the changes with time in the FA ratios induced by crude and purified toxins. Fluid accumulation was observed 1 to 8 h after toxin inoculation, but thereafter the FA ratio decreased to a negative value. As the maximal activity was observed 2 to 5 h after toxin challenge, we used a 3-h assay period.

Purification. The toxic activity in culture supernatants was precipitated with ammonium sulfate. In further purification steps, most of the activity was recovered in the supernatants on treatments with hydroxyapatite and ethanol. The crude toxin thus obtained was chromatographed on SP-Sephadex C-50 (Fig. 2). Most of the activity was recovered late (fractions 15 to 20) in the elution with distilled water, whereas many contaminants were eluted in the first 10 fractions with water and in those with ammonium acetate.

The fractions containing toxic activity were then applied to a DEAE-Sephadex A-25 column. The toxic activity was recovered in fractions eluted with 0.5 M acetic acid (Fig. 3).

The material was finally purified by reversed-phase HPLC. Most of the toxic activity was eluted as a sharp single peak with about 28% acetonitrile (Fig. 4). The fractions containing toxic activity were pooled, lyophilized, and suspended in a small volume of distilled water. Then the preparation was rechromatographed on the same column but eluted with about 60% acetonitrile in 0.05% trifluoroacetic
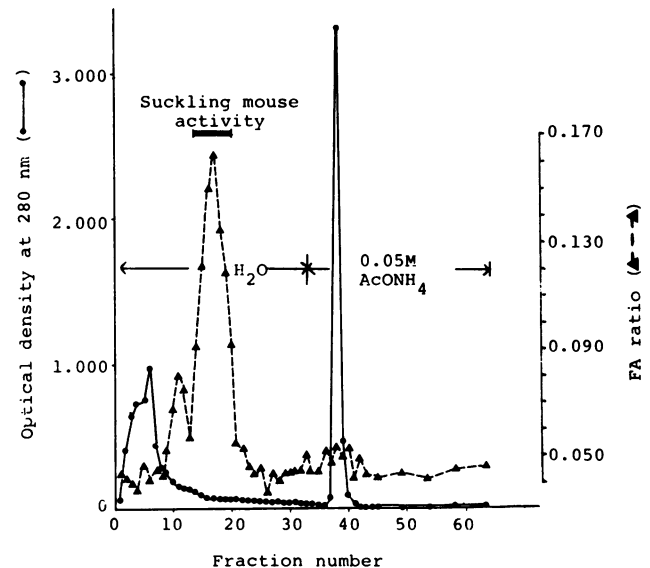

FIG. 2. Chromatographic profile of crude toxin on SP-Sephadex C-50. An aqueous solution of crude toxin was applied to the column. The column was developed with distilled water and then 0.05 M ammonium acetate.

acid (Fig. 5), whereas *E. coli* heat-stable enterotoxin (STh) was eluted with about 50% acetonitrile (Fig. 5).

Recovery of toxin during purification. Table 1 summarizes typical data on the purification of NAG-ST. The specific activity of the toxin increased about 1.4×10^5-fold, and the final recovery rate was about 11.8%. About 100 μg (dry weight) of purified toxin protein was obtained from 10 liters of culture supernatant. The minimum effective dose of purified toxin in the suckling mouse assay was about 5 ng.

Physicochemical properties of the purified toxin. The purified toxin was stable on heating at 100°C for 30 min and at 60°C for 30 min. The crude material was heat labile with the same treatments (Table 2), although some activities of crude materials remained upon heating at 60 or 100°C for 10 min.

After reducing the purified toxin (40 mouse units/ml) in the presence of 0.5 mM (or more) dithiothreitol for 30 min at room temperature, its activity was measured directly without dialysis. This reduction of the toxin resulted in a loss of activity, but the toxic activity was not inhibited by treating animals with the same concentrations of dithiothreitol 5 min before challenging the toxin, suggesting that dithiothreitol does not alter directly the suckling mouse response to native toxin and that disulfide bridges in the toxin molecule were necessary for biological activity.

The isoelectric point of the purified toxin was determined by polyacrylamide gel electrofocusing (17) to be 3.9, as its activity was recovered in the same position as *E. coli* STh (17).

Amino acid composition. The amino acid composition of the purified toxin is given in Table 3. The NAG-ST was similar to *E. coli* STh and STp and *Yersinia enterocolitica* ST in biological activity and amino acid composition (for example, it contained six half-Cys). It consisted of 17 amino acid residues, one less than *E. coli* STp (14) and two less than *E. coli* STh (1). The molecular weight of the purified toxin was estimated to be 3,000 by gel filtration on Sephadex G-75 and 1,000 to 5,000 by ultrafiltration (data not shown). From its amino acid composition, the molecular weight of the purified toxin was estimated to be 1,811 to 1,815, because Asp and Asn and Glu and Gln were analyzed together as Asp and Glu, respectively, in the acid hydrolysate.

DISCUSSION

Fluid accumulation in the suckling mouse assay was originally used to detect EC-ST (5), and this assay has
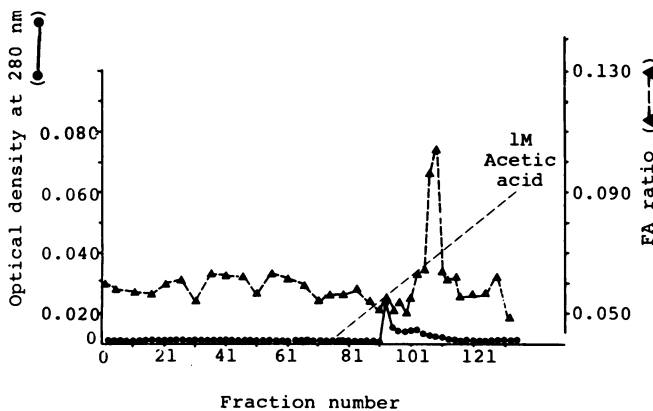

FIG. 3. Chromatographic profile of NAG-ST on DEAE-Sephadex A-25. The column was developed with distilled water and then a linear gradient of 0 to 1 M acetic acid.
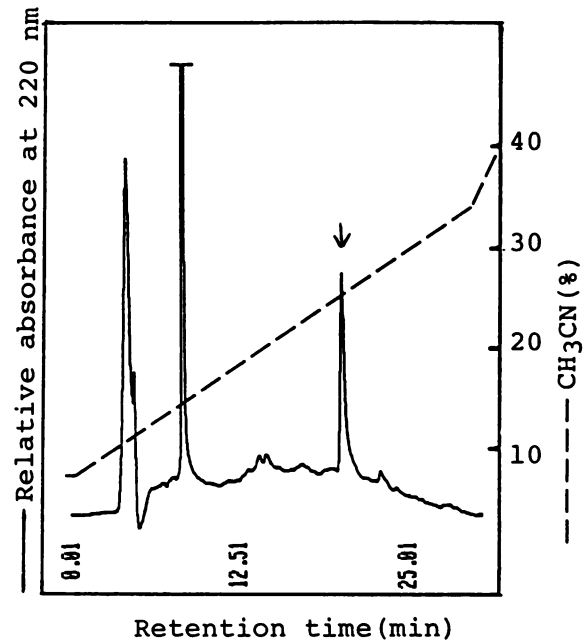

FIG. 4. Reversed-phase HPLC profile of NAG-ST. A linear gradient (-----) of 10 to 35% acetonitrile in 0.01 M ammonium acetate was used.

subsequently been used to demonstrate the enterotoxic activities of *Y. enterocolitica* (11), *Aeromonas hydrophila* (6), and *V. cholerae* non-O1 (9, 10, 13).

By monitoring enterotoxic activity with the suckling mouse assay, we purified a heat-stable enterotoxin (NAG-ST) from *V. cholerae* non-O1 cultures. NAG-ST could be purified by the method developed for purification of EC-ST (1, 14, 16), with minor differences. (i) On SP-Sephadex C-50 column chromatography, NAG-ST and EC-ST eluted with distilled water and 0.05 M ammonium acetate, respectively. (ii) On DEAE-Sephadex A-25 column chromatography, NAG-ST and EC-ST eluted with 0.5 and 0.2 M acetic acid, respectively. (iii) The difference between the two could be demonstrated directly by the HPLC profile of a mixture of NAG-ST and EC-ST (Fig. 5).

Purified NAG-ST was heat stable like EC-ST even at 100°C for 30 min, but was inactivated at 120°C for 10 min. On the other hand, in the crude preparation it was heat labile (Table 2) at 60°C for 30 min. Analysis of the interesting discrepancy between pure and crude preparations is now in progress in our laboratory, and according to our preliminary data there are two possibilities: (i) crude preparations contain a second and heat-labile factor, such as hemolysin, which is active in the suckling mouse assay; and (ii) the NAG-ST molecule is coprecipitated and masked by heat-denaturing products in crude preparations.

The amino acid composition of purified NAG-ST showed similarities to and differences from that of EC-ST. (i) NAG-ST is a peptide enterotoxin composed of 17 amino acids, while *E. coli* STp (14) and STh (1) are peptides consisting of 18 and 19 residues, respectively. (ii) NAG-ST has the same numbers of Glu, Gly, Pro, and half-Cys residues as EC-ST, but Thr and Tyr are found only in EC-ST and Ile is found only in NAG-ST. (iii) NAG-ST contains six half-Cys residues per molecule, like EC-ST (1, 14). Recently (15) *Y. enterocolitica* ST was also found to contain six half-Cys residues per molecule.
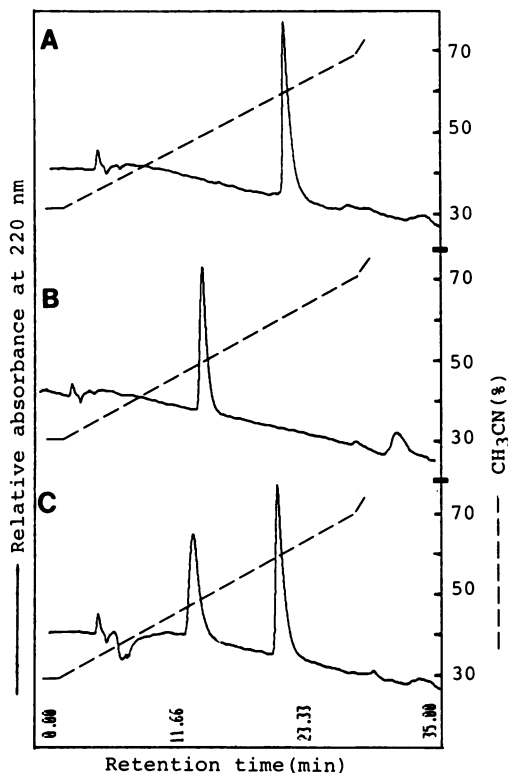
FIG. 5. Reversed-phase HPLC profiles of NAG-ST and *E. coli* STh. Toxin(s) was eluted with a linear gradient (-----) of 30 to 70% acetonitrile in 0.05% trifluoroacetic acid. A, NAG-ST; B, *E. coli* STh; C, NAG-ST plus *E. coli* STh.

As expected, NAG-ST did not react with anticholera toxin, but we observed immunological cross-reactivity between NAG-ST and EC-ST (manuscript in preparation). Thus the present study showed that *V. cholerae* non-O1 strains produce an enterotoxin that is similar, but not identical, to EC-ST and is distinct from cholera (-like) toxin produced by *V. cholerae* non-O1 (4, 19).

Madden et al. (7) reported that *V. cholerae* non-O1 strains did not produce an enterotoxin with activity in the suckling mouse assay. However, Nishibuchi and Seidler (9) reported production by *V. cholerae* non-O1 strains of a possible new heat-labile enterotoxin active in the suckling mouse assay. The characteristics of their toxin are similar to those of our crude preparation: (i) toxin production was highest in BHI', (ii) the toxin was heat labile with culture supernatants, and

TABLE 1. Summary of purification of NAG-ST produced by *V. cholerae* non-O1 A-5

Step	Total protein (mg)	Sp act (μg/mouse unit)[a]	Relative activity	Recovery (%)
Culture supernatant (9.4 liters)	117,056	688.9	1	100
Ethanol extraction	1,800	10.9	63.2	64.7
SP-Sephadex C-50	59.9	1.19	578.9	29.7
Second HPLC	0.1	0.005	140,000	11.8

[a] The minimal amount of toxin giving an FA ratio of >0.09 after 3 h was designated as 1 mouse unit.

TABLE 2. Heat stabilities of crude and purified NAG-ST

Amt of toxin (mouse unit)[b]	FA ratio[a]					
	Unheated control	Heated				
		60°C, 10 min	60°C, 30 min	100°C, 10 min	100°C, 30 min	120°C, 10 min
1 (crude)	0.101	0.063	0.064	0.067	0.058	NT
2						
Crude	0.105	0.093	0.075	0.092	0.074	NT
Pure	0.115	0.132	0.103	0.131	0.117	0.054
4						
Crude	0.108	0.102	0.087	0.092	0.085	NT
Pure	0.136	0.142	0.150	0.131	0.124	0.053

[a] Mean of three assays. NT, Not tested.
[b] See footnote a, Table 1, for definition of mouse unit.

(iii) its activity was detectable 3 to 5 h after its introduction into suckling mice. There were, however, marked differences between their findings and ours. For example, the estimated molecular weight of their toxin was 40,000, whereas that of ours was 1,800. Spira et al. (13) also reported that two isolates, both from the Chesapeake Bay, produced a toxin very similar to EC-ST that was heat stable, dialyzable, had a more rapid effect than cholera enterotoxin in suckling mice, and was not neutralized by anticholera toxin. They did not, however, give details of the nature of this toxin because they used only crude materials and the strains they investigated were actually sucrose-negative *V. mimicus*, not *V. cholerae* non-O1 (9).

Thus, we believe that this paper clearly shows for the first time the existence and the nature of a new heat-stable enterotoxin produced by *V. cholerae* non-O1 strains.

ACKNOWLEDGMENTS

TABLE 3. Amino acid composition of NAG-ST compared with those of *E. coli* STh and STp and *Y. enterocolitica* ST (Y-ST) determined previously

Amino acid	Molar ratio (nearest integer)	STh[a]	STp[b]	Y-ST[c]
Asp, Asn	3.20 (3)[d]	3	2	5
Thr	—[e]	1	1	—
Ser	0.12 —	2	—	3
Glu, Gln	1.20 (1)	1	1	2
Pro	0.83 (1)	1	1	5
Gly	1.11 (1)	1	1	1
Ala	1.00 (1)	1	2	4
Half-Cys	5.10 (6)[f]	6	6	7
Val	0.04 —	—	—	2
Ile	1.82 (2)	—	—	—
Leu	1.11 (1)	1	1	—
Tyr	0.05 —	2	2	—
Phe	1.17 (1)	—	1	—
Trp	—	—	—	1

[a] From reference 1.
[b] From reference 13.
[c] From reference 14.
[d] Values were calculated as moles per mole of Ala.
[e] Not detectable.
[f] The recovery of half-Cys was about 80% in this assay.

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