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NOTES

Purification and Characterization of a New Heat-Stable Enterotoxin Produced by *Vibrio cholerae* Non-O1 Serogroup Hakata

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The possible production of a heat-stable enterotoxin (Vc-H-ST) by *Vibrio cholerae* non-O1 serogroup Hakata was investigated, and the purified Vc-H-ST was characterized. It has a unique amino acid sequence, LIDCCEICCNPACFGCLN. This sequence is quite similar to that of the heat-stable enterotoxin (NAG-ST) produced by *V. cholerae* non-O1 except for one amino acid (leucine) residue excess at the N terminus. Other characteristics, including biological activity, are compatible with those of NAG-ST.

Vibrio cholerae non-O1 serogroup Hakata, often isolated from the environment and from imported seafood in Japan, is a new serotype of *V. cholerae* non-O1 proposed by Shimada et al. (7). *V. cholerae* non-O1 serogroup Hakata is agglutinated with polyvalent anti-*V. cholerae* O1 antisera but not with antisera against the A antigen (factor) that is a common antigen among *V. cholerae* O1 (7). This sometimes causes misidentification of *V. cholerae* non-O1 serogroup Hakata as *V. cholerae* O1.

In this study, the possible production of a heat-stable enterotoxin (ST) by these organisms was examined by using the suckling mouse assay (11), and it was found that some strains produce a new type (Vc-H-ST) of ST similar to the STs previously described in *V. cholerae* non-O1 (NAG-ST) (2, 9) and *Vibrio mimicus* ST (VM-ST) (3), both of which are also similar to well-known STs produced by enterotoxigenic *Escherichia coli* (1, 8), *Yersinia enterocolitica* (10), and *Citrobacter freundii* (5, 6).

A total of 28 strains (20 strains isolated from the environment [five sites] and 8 strains from imported seafood [7 cases]) were screened, by the suckling mouse assay (11) using at least three mice per sample, for the production of ST by culturing test strains in either tryptic soy broth or brain heart infusion broth supplemented with 0.5% NaCl. As a result of the screening, four strains produced ST and heat-labile toxin(s) active in causing fluid accumulation in the suckling mouse assay, giving a fluid accumulation ratio of 0.09 or more (11). One strain (TQ-C3) was positive in the suckling mouse assay of samples cultured in tryptic soy broth, and four strains (TQ-C3, NQ-13, NQ-16, and NAHA-1) were positive in the suckling mouse assay of samples cultured in brain heart infusion broth supplemented with 0.5% NaCl. Upon heating of these supernatants at 100°C for 10 min, the toxin from strain TQ-C3 proved to be heat stable. Therefore, this ST (Vc-H-ST) was investigated in more detail.

Vc-H-ST was purified essentially as described previously (2). In brief, culture supernatant of strain TQ-C3 cultured in brain heart infusion broth supplemented with 0.5% NaCl was fractionated by ammonium sulfate precipitation (60% saturation). After dialysis against distilled water, samples were treated with hydroxyapatite, the nonadsorbed supernatants were concentrated in an evaporator, and ethanol extraction was performed. The samples thus treated were applied to columns of SP-Sephadex C-25, DEAE-Sephadex A-25, and finally Nucleosil 5C8 (reversed-phase high-performance liquid chromatography [HPLC]).

During purification, suckling mouse Vc-H-ST activity was recovered in essentially the same position as that of NAG-ST (2). That is, Vc-H-ST was precipitated by 60% saturated ammonium sulfate and recovered in the unadsorbed fraction of hydroxyapatite and was 90% ethanol soluble. Active portions of these treatments were applied on columns. Vc-H-ST was eluted with 0.05 M ammonium acetate on SP-Sephadex C-25 column chromatography and then recovered in fractions eluted with 0.4 M acetic acid on DEAE-Sephadex A-25 column chromatography. After reversed-phase HPLC was performed twice with a 10 to 70% acetonitrile gradient in 0.05% trifluoroacetic acid at 2 ml/min for 60 min, most of the Vc-H-ST activity was eluted as a sharp, single peak with about 40% acetonitrile in 0.05% trifluoroacetic acid. When the mixture of purified NAG-ST (2) and Vc-H-ST was applied, these two toxins were eluted in the same position, which suggests that Vc-H-ST is identical or very similar to NAG-ST. However, when the elution was done by using an acetonitrile gradient in 10 mM ammonium acetate instead of trifluoroacetic acid, these two toxins were eluted separately; Vc-H-ST and NAG-ST were eluted at acetonitrile concentrations of 27 and 25%, respectively, and their retention times were 22 and 20 min, respectively (Fig. 1). This indicates that Vc-H-ST and NAG-ST are not identical.

The amino acid composition of Vc-H-ST was determined by analysis of native and carboxymethylated ST and was found to be composed of Asp or Asn (three residues), Glu

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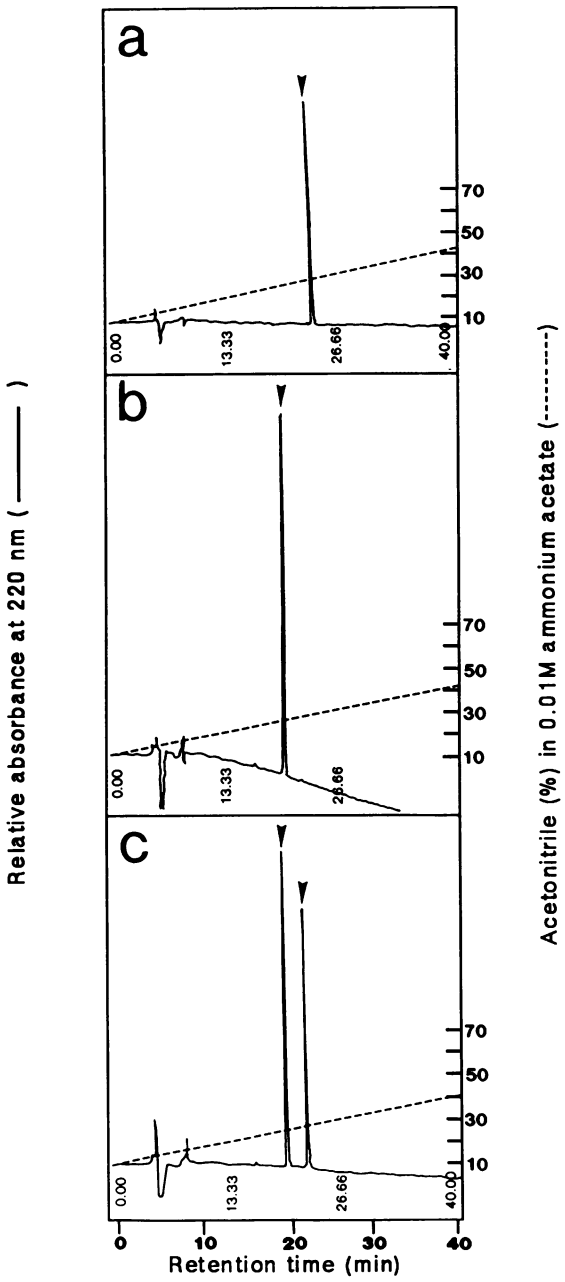

FIG. 1. Comparison of elution profiles of purified Vc-H-ST and NAG-ST from HPLC using a Nucleosil 5C8 column. A linear gradient (-----) of 10 to 40% acetonitrile in 0.01 M ammonium acetate was used ▼, positive in the suckling mouse assay, giving a fluid accumulation ratio of more than 0.09. (a) Vc-H-ST. (b) NAG-ST. (c) Mixture of Vc-H-ST and NAG-ST.

(one), Pro (one), Gly (one), Ala (one), Cys (six), Ile (two), Leu (two), and Phe (one), consisting of 18 amino acid residues, which is one amino acid residue (leucine) more than NAG-ST (9).

The amino acid sequence, as determined by Edman degradation (4) of native and reductively carboxymethylated Vc-H-ST and also by a combination of fast-atom bombardment mass spectrometry and carboxypeptidase Y digestion of native Vc-H-ST (12), was LIDCCEICCNPACFGCLN (Fig. 2). The composition and sequence were similar but not identical to those of NAG-ST. From 4 liters of culture supernatants, purified Vc-H-ST was obtained with a recovery rate of 17%. The minimum effective dose of Vc-H-ST in the suckling mouse assay was 12.5 ng, which is comparable to that of NAG-ST (5.0 ng) (2), STh (2.5 ng) (11), STp (2.5 ng) (8), and Yersinia-ST (20.0 ng) (10). The molecular weight of Vc-H-ST was 1,926.6 by fast-atom bombardment mass spectrometry. Purified Vc-H-ST (2 mouse units) was heat treated at 60°C for 10 and 30 min, at 100°C for 10 and 30 min, and at 120°C for 10 min, and the residual Vc-H-ST activity assayed in suckling mice was almost 100%, except for a sample treated at 120°C for 10 min, which lost its activity.

In conclusion, it was demonstrated that the *V. cholerae* non-O1 serogroup Hakata strain TQ-3C produces an ST-like enterotoxin similar to NAG-ST. It cannot yet be concluded that Vc-H-ST is a virulence factor in human gastroenteritis, since the *V. cholerae* non-O1 serogroup Hakata has been isolated only from seafoods and the environment (7), not from patients. However, because it is well established that STh and STp of enterotoxigenic *E. coli* are virulence factors of diarrhea of human and animals, the Vc-H-ST of *V. cholerae* non-O1 serogroup Hakata may play a role in gastroenteritis. Further studies or possible isolation of *V. cholerae* non-O1 serogroup Hakata from clinical specimens is in progress.

Vc-H-ST	(This Study)				1 L	I	D	C	5 C	E	I	C	C	10 N	P	A	C	F	15 G	C	L	N			
NAG-ST	(Ref. 2)					1 I	D	C	C	5 E	I	C	C	N	10 P	A	C	F	G	15 C	L	N			
EC-STh	(Ref. 1)	1 N	S	S	N	5 Y	C	C	E	L	10 C	C	N	P	A	15 C	T	G	C	Y					
EC-STp	(Ref. 8)		1 N	T	F	Y	5 C	C	E	L	C	10 C	N	P	A	C	15 A	G	C	Y					
Y-ST	(Ref. 10)	-----S	S	15 D	W	D	C	C	20 D	V	C	C	N	25 P	A	C	A	G	30 C					

FIG. 2. Amino acid sequence of Vc-H-ST. Other sequences were taken from other published works for comparison. Boxes enclose identical sequences among these ST-like toxins.

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