Amino acid sequence of heat-stable enterotoxin produced by *Vibrio cholerae* non-01

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The amino acid sequence of heat-stable enterotoxin, produced by *Vibrio cholerae* non-01 and isolated from its culture supernatant, was determined by both Edman degradation of native and reductively carboxymethylated enterotoxin and also a combination of fast atom bombardment mass spectrometry and carboxypeptidase Y digestion of native enterotoxin to be as follows: Ile-Asp-Cys-Cys-Glu-Ile-Cys-Cys-Asn-Pro-Ala-Cys-Phe-Gly-Cys-Leu-Asn. This sequence is very similar, but not identical, to those of heat-stable enterotoxins produced by enterotoxigenic *Escherichia coli* and *Yersinia enterocolitica*.

(Vibrio cholerae *non-01*) *Heat-stability* *Enterotoxin* *Primary structure*

1. INTRODUCTION

Vibrio cholerae non-01, also named non-agglutinating (NAG) or non-cholera vibrios (NCV), sometimes causes severe gastroenteritis in humans, which cannot be distinguished clinically from cholera, but the mechanism of its pathogenesis is still unknown [1]. Recently, many investigators have tried to isolate a virulence factor(s) related to this pathogenesis. Results have suggested that a cholera-like enterotoxin [2–5] and a heat-labile enterotoxin distinct from cholera enterotoxin [6,7] are produced by some strains of *V. cholerae* non-01 isolated from human patients and the environment. Moreover, a heat-stable enterotoxin (ST) similar to that of enterotoxigenic *Escherichia coli* was found to be produced by 2 strains of *V. cholerae* non-01 isolated from patients with diarrhea [8]. Very recently, some of us [9] succeeded in isolating a pure toxin from a strain (no.A-5) of *V. cholerae* non-01 that showed similar, but not identical, physicochemical and immunological properties to those of an ST of enterotoxigenic *E. coli*. These findings indicate that the toxin (named NAG-ST) has a similar structure to the STs of enterotoxigenic *E. coli* [10,11] and *Yersinia enterocolitica* [12,13]. Thus, it seemed interesting to determine the molecular structure of NAG-ST and to elucidate the structural, biological and immunological relation of NAG-ST and other STs.

This paper reports the amino acid sequence of NAG-ST produced by a strain (no.A-5) of *V. cholerae* non-01. The sequence is very similar to those of enterotoxigenic *E. coli* and *Y. enterocolitica*, suggesting that these STs are derived from a common ancestral transposon gene and form an ST-family like the cholera enterotoxin family [14].

2. MATERIALS AND METHODS

2.1. *Isolation of toxin*

The toxin was isolated from the culture superna-

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tant of a strain (no.A-5) of *V. cholerae* non-01, as described [9]. The purity of the isolated toxin was checked by reversed-phase HPLC under the conditions in [12].

2.2. *Amino acid analysis*

The purified toxin and its reduced and carboxymethylated derivative were hydrolyzed in 4 M methanesulfonic acid for 24 h at 110°C in vacuo in sealed tubes and the hydrolysates analyzed in a Hitachi type 835 amino acid analyzer.

2.3. *Reductive carboxymethylation*

A sample of native toxin (~100 μg) was reduced and carboxymethylated as in [10]. Carboxymethylated toxin was recovered in a yield of about 40% by reversed-phase HPLC, as described below.

2.4. *Edman degradation*

Native toxin (~12 nmol) was manually degraded by the Edman method [15]. The carboxymethylated toxin (2.45 nmol) was degraded sequentially with an Applied Biosystems model 470A gas-phase sequenator (California), as described [16]. The resulting Pth-amino acids were analyzed by HPLC as described below.

2.5. *Carboxypeptidase Y digestion*

Two samples of native toxin of 1.07 and 2.24 nmol, respectively, were digested with carboxypeptidase Y (0.1 and 1.2 μg, Oriental, Osaka) at 37°C for 90 min and 15 h in 0.1 M pyridinium acetate (10 μl) at pH 7.0. Part of the digest of the 1.07 nmol sample was subjected to FAB mass spectrometry and the remainder digested further with carboxypeptidase B (Boehringer), as described [17], and subjected to FAB mass spectrometry. The digest of the 2.24 nmol sample was subjected to amino acid analysis, as described above.

2.6. *Fast atom bombardment (FAB) mass spectrometry*

FAB mass spectra were recorded with a Jeol double-focusing mass spectrometer, JMS-HX100, fitted with a 2.33 T magnet, an FAB ion source and a post-accelerating system. A Jeol DA-5000 mass data analysis system was used for acquisition of mass spectra. Sample peptides (0.1–1.0 nmol) were applied to a stainless-steel sample holder and xenon gas was used as a neutral atom beam. Typical experimental conditions were as described [18].

2.7. *High-performance liquid chromatography (HPLC)*

For purification of native and carboxymethylated toxins, a Hitachi 655 liquid chromatograph (Tokyo) and a column (4 × 250 mm) of YMC-ODS (S-5, Yamamura, Kyoto) were used. The column was equilibrated with 10% CH_3CN in 0.05% trifluoroacetic acid (pH 2.35) and developed with a linear gradient of 10–40% CH_3CN. Pth-amino acids were identified using a Varian HPLC apparatus (Vista 5500) and a Varian micropack SP-ODS column (4.6 × 150 mm).

3. RESULTS AND DISCUSSION

The amino acid compositions of native and reductively carboxymethylated (CM) NAG-ST (fig.1) are shown in table 1. The M_r of NAG-ST was determined to be 1813.6 by measurements of the native and CM NAG-STs by FAB mass spectrometry, which gave intense signals at m/z = 1814.6 (fig.2) and 2168.6, respectively. The results clearly indicated that NAG-ST contains 6 half-cystine residues, which are intramolecularly linked

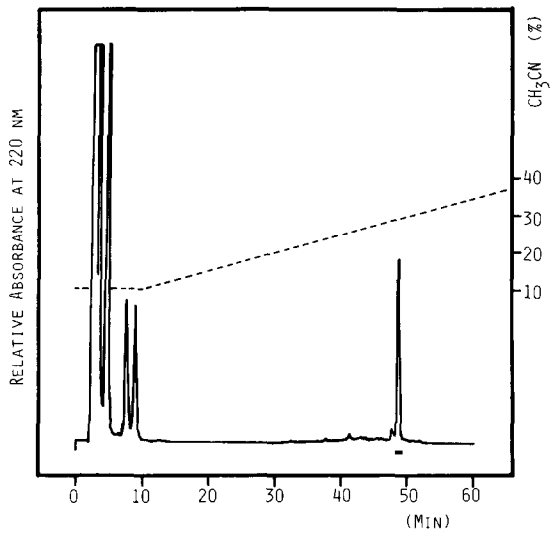

Fig.1. Reversed-phase HPLC of reductively carboxymethylated enterotoxin. Chromatographic conditions were as described in section 2.

Table 1

Amino acid composition of the purified enterotoxin

	Native	Carboxy-methylated	Nearest integer
CM-Cys	–	6.28	
Asp	3.20	3.10	3
Ser	0.12	0.05	
Glu	1.20	1.08	1
Gly	1.11	1.14	1
Ala	1.00	1.00	1
½Cys	5.10	0.07	6
Val	0.04	–	
Ile	1.82	1.84	2
Leu	1.11	1.02	1
Tyr	0.05	–	
Phe	1.17	1.03	1
Arg	–	0.10	
Pro	0.83	1.05	1
Total			17

Values were calculated as mol/mol Ala

by 3 disulfide bonds, and that 2 of 5 carboxyl groups of 3 Asp, 1 Glu and the C-terminus are present as amides.

For determination of the amino acid sequence of NAG-ST, the ST was subjected to direct Edman degradation and Ile and Asp were found in positions 1 and 2, respectively, from the N-terminus. The C-terminal amino acid residue of NAG-ST was examined by measurement [19] of the FAB mass spectra of the carboxypeptidase Y and B digests of native NAG-ST, as shown in fig.3. The differences between the mass values of native NAG-ST and the digests suggested that the C-terminal sequence was Leu (or Ile)-Asn. Moreover, native NAG-ST (2.24 nmol) released Asn (2.14 nmol) on amino acid analysis after digestion with carboxypeptidase Y. Thus, the C-terminal amino acid residue was concluded to be Asn.

Thereafter, CM-NAG-ST was analyzed in a gas-phase sequenator. As shown in fig.4, the CM toxin had a sequence of 17 amino acid residues from the N-terminus. This finding was supported not only by the amino acid composition and M_r values of the native and CM NAG-STs, but also by the mass values of fragment ion signals of CM-NAG-ST on FAB mass spectra (not shown). The sequence of NAG-ST thus determined was compared with those of enterotoxigenic *E. coli* and *Y. enterocolitica*. Except for Ile at position 6 and Phe at position 13, the sequence from position 3 to position 15 (core structure) was the same as those of the enterotoxins of *E. coli* and *Y. enterocolitica*, which is known to be the essential structure for expression of full enterotoxigenic activity [17]. This difference in the core structures and N- and C-terminal sequences may be responsible for the differences in degrees of immunological and biological activities: the minimum effective dose of NAG-ST is 5 ng [9], which is higher than that of enterotoxigenic *E. coli*. The presence of 6 half-cystine residues in the same positions as in the STs of *E. coli* and *Y. enterocolitica* strongly indicates that NAG-ST has the same secondary structure as these STs, although the positions of disulfide

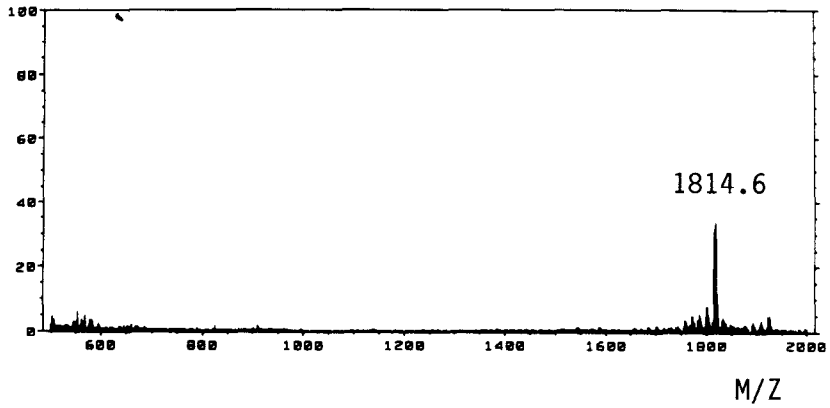

Fig.2. FAB mass spectrum of native enterotoxin.

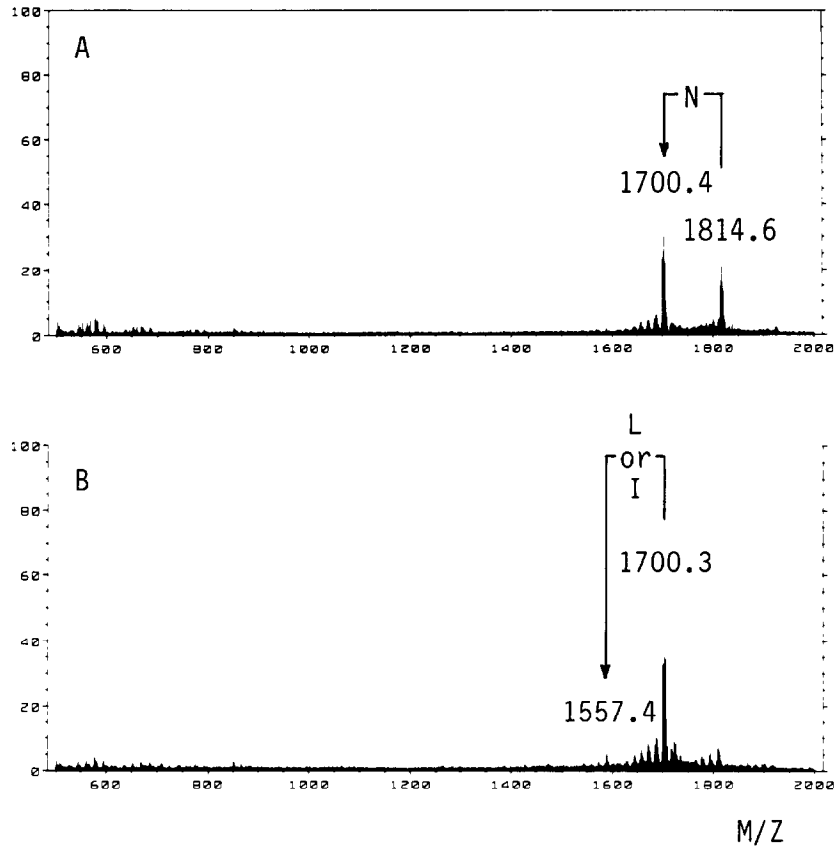

Fig.3. FAB mass spectra of: (A) a digest of native enterotoxin with carboxypeptidase Y at 37°C for 90 min and (B) a digest of (A) with carboxypeptidase B at 37°C for 20 h.

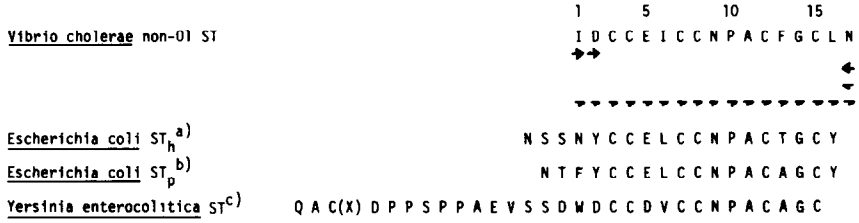

Fig.4. Comparison of amino acid sequence of the heat-stable enterotoxin (NAG-ST) of *Vibrio cholerae* non-01 with those of enterotoxigenic *E. coli* and *Y. enterocolitica* heat-stable enterotoxins: (⟶) residues determined by Edman degradation of native NAG-ST; (⟵ and ⟵) residues determined by FAB mass measurement and amino acid analysis of a CPase digest of native NAG-ST, respectively; (⟶) residues determined by Edman degradation of CM-NAG-ST; [a] see [10]; [b] see [11]; [c] see [12,13].

linkages are still unknown, and that the tertiary structure formed by these 6 half-cystine residues is responsible for heat stability. The present results suggest that structurally, functionally, and immunologically related heat-stable enterotoxins are present widely in enteric bacteria like the heat-labile enterotoxins produced by *V. cholerae* and enterotoxigenic *E. coli*.

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