

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 carboxy-methylated enterotoxin and also a combination of fast atom bombardment mass spectrometry and carboxy-peptidase 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% CH₃CN in 0.05% trifluoroacetic acid (pH 2.35) and developed with a linear gradient of 10–40% CH₃CN. 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

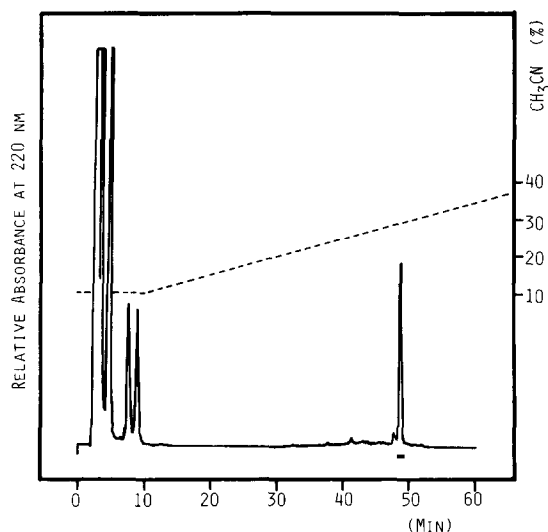


Fig.1. Reversed-phase HPLC of reductively carboxymethylated enterotoxin. Chromatographic conditions were as described in section 2.

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

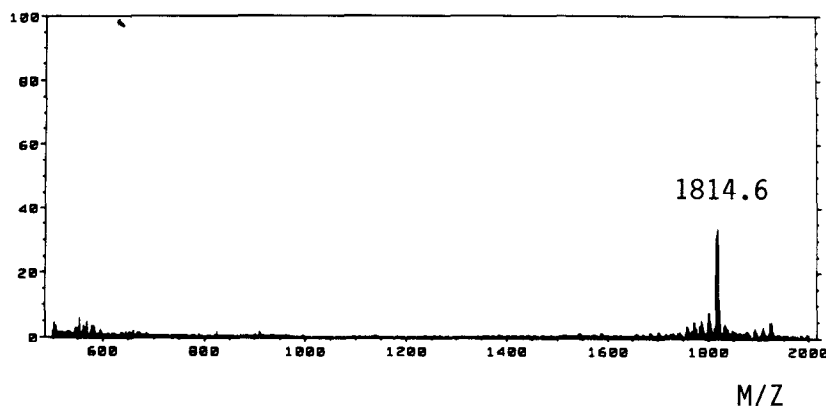


Fig.2. FAB mass spectrum of native enterotoxin.

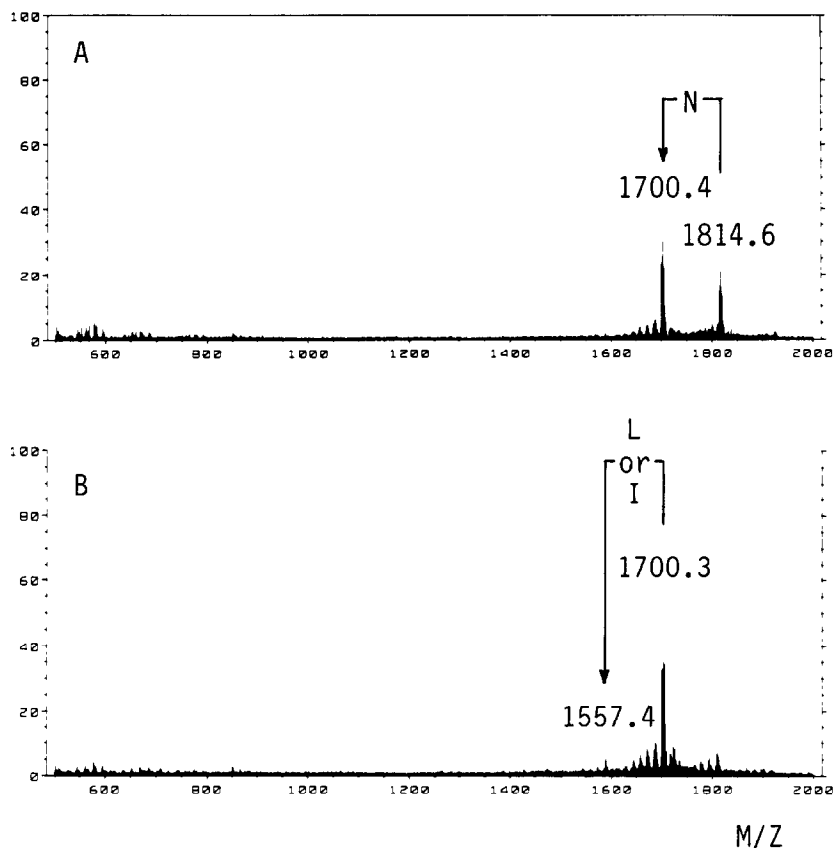


Fig.3. FAB mass spectra of: (A) a digest of native enterotoxin with carboxypeptidase Y at 37°C for 90 min and (B) a digest of (A) with carboxypeptidase B at 37°C for 20 h.



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 im-

munologically 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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