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Purification and sequence determination of heat-stable enterotoxin elaborated by a cholera toxin-producing strain of *Vibrio cholerae* O1

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Four molecular species of heat-stable enterotoxins elaborated by a cholera toxin-producing strain of *Vibrio cholerae* O1 were isolated from its culture supernatant. The amino acid sequence of one of the enterotoxins was determined to be Phe-Ile-Lys-Gln-Val-Asp-Glu-Asn-Gly-Asn-Leu-Ile-Asp-Cys-Cys-Glu-Ile-Cys-Cys-Asn-Pro-Ala-Cys-Phe-Gly-Cys-Leu-Asn with three intramolecular disulfide linkages. The other enterotoxins had shorter amino acid sequences in the N-terminal regions, but possessed the same sequence in their C-terminal regions including the three disulfide linkages. The enterotoxins with the shorter N-terminal sequences showed more potent toxicities, and the minimum effective dose of the longest one with 28 amino acid residues was 10-folds of that of the shortest one.

Heat-stable enterotoxin; Primary structure; Cholera; *Vibrio cholerae* O1

1. INTRODUCTION

Vibrio cholerae belonging to the serogroup O1, the etiologic agent responsible for epidemics and pandemics of cholera, produce a heat-labile enterotoxin known as cholera toxin (CT) which is responsible for the drastic intestinal electrolyte secretion and fluid loss leading to the clinical state of cholera. In contrast, *V. cholerae* non-O1 do not have the epidemic potential but are associated with sporadic cases of gastroenteritis. CT production is rarely associated with *V. cholerae* non-O1 but strains of *V. cholerae* non-O1 are known to produce a heat-stable enterotoxin (ST) named NAG-ST [1] or Vc-H-ST [2]. Attempts to develop an effective live oral vaccine against the disease cholera have not been completely successful because one-third of the human volunteers fed with genetically engineered candidate live oral cholera vaccine strains incapable of producing biologically active CT suffered from mild to moderate diarrhoea [3]. This led investigators to search for ancillary secretogenic factors which might be responsible for the residual diarrhoea caused by the attenuated live oral cholera vaccine strains. It was recently found that an ST-like enterotoxin is produced by an environmental strain of CT-producing *V. cholerae* O1 [4]. The fact that *V. cholerae* O1 produces not only CT but also an ST-like toxin analogous to that of enterotoxigenic *Escherichia coli* implied that a cholera vaccine should be produced against both toxins for being completely effective. This formed the impetus for the present study in which we report for the first time the purification and sequence determination of the ST-like toxin (named O1-ST) obtained from the culture supernatant of a CT-producing strain of *V. cholerae* O1.

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2. EXPERIMENTAL

2.1. *Bacterial strain and culture conditions*

A CT-producing strain (GP156) of *V. cholerae* O1 (biotype eltor and serotype Inaba) originally isolated from Australia was the source of O1-ST [4]. The strain was cultured as previously described [4].

2.2. *Isolation of O1-ST*

The culture supernatant of *V. cholerae* O1 GP156 was fractionated by ammonium sulfate precipitation (60% saturation). After centrifugation at 15,000 × *g* for 30 min at 4°C, the resulting pellet was dialyzed against deionized and distilled water (DDW), and then applied to a DEAE-Sephadex A-25 column (2 × 40 cm; acetate form) equilibrated with DDW. The column was washed with 1000 ml of DDW and eluted with a linear gradient from 0 to 1.0 M acetic acid (1000 ml of total volume), at a flow rate of 100 ml/h. The eluate was collected in fractions of 12 ml. An aliquot of each fraction was tested for toxicity in suckling mice and the active fractions were pooled. The toxic fractions were concentrated, dialyzed against DDW and then subjected to immunoaffinity chromatography on anti-NAG-ST monoclonal antibody (mAb2F)-conjugated CNBr-Sepharose 4B column (1.6 × 4.5 cm) equilibrated with 100 mM sodium phosphate buffer (pH 7.0) [5]. The column was washed with 30 ml of the equilibration buffer and eluted with 15 ml of 100 mM Gly buffer (pH 2.5). The eluate was collected in fractions of 1.5 ml. The fractions containing toxic materials were concentrated and then purified further by reverse-phase high-performance liquid chromatography (RP-HPLC).

2.3. *High-performance liquid chromatography*

The HPLC instrument used for peptide separation was a high-pressure gradient system which consisted of two Waters Model 510 HPLC pumps. The column effluent absorbance was monitored at 214 and 280 nm with use of Waters Absorbance Detectors Model 441 and 440, respectively. The following elution programs were used in this study. Program I: a YMC R-ODS-5 column (4.6 × 250 mm) equilibrated with 10% CH_3CN in 0.1% trifluoroacetic acid (TFA) was eluted with a linear gradient of CH_3CN (10–50% in 40 min), at a flow rate of 1.0 ml/min. Program II: a Cosmosil 5C18-AR column (4.6 × 250 mm) equilibrated with 10 mM ammonium acetate ($AcONH_4$) (pH 5.7) was eluted with a linear gradient of CH_3CN (10–50% in 40 min), at a flow rate of 1.0 ml/min. Program III: a Cosmosil 5C18 column (4.6 × 150 mm) equilibrated with 10% CH_3CN in 0.1% TFA was eluted with a linear gradient of CH_3CN (10–50% in 40 min), at a flow rate of 1.0 ml/min. Program IV: a Cosmosil 5C18-AR column (4.6 × 150 mm) equilibrated with 10% CH_3CN in 10 mM $AcONH_4$ (pH 5.7) was eluted with a linear gradient of CH_3CN (10–50% in 40 min), at a flow rate of 1.0 ml/min. Program V: a Cosmosil 5C18 column (4.6 × 150 mm) equilibrated with 5% CH_3CN in 0.1% TFA was eluted with a linear gradient of CH_3CN (5–50% in 45 min), at a flow rate of 1.0 ml/min.

2.4. *Amino acid analysis, automated Edman degradation, and fast atom bombardment (FAB) mass spectrometry*

Amino acid analysis was performed using a Hitachi L-8500 amino acid analyzer as documented previously [6]. Automated Edman degradation was carried out with a Shimadzu PSQ-1 gas-phase protein sequencer. FAB mass spectra were obtained with a JEOL JMS-HX100 double-focusing mass spectrometer as previously described [6].

2.5. *Peptide synthesis*

NAG-ST (IDCCEICCNPACFGCLN) was synthesized as documented previously [7]. *N-t*-Butyloxycarbonyl (Boc)-Leu-NAG-ST and Boc-Asn-Leu-NAG-ST were synthesized by condensation of the synthetic NAG-ST and Leu-NAG-ST with Boc-L-Leu *N*-hydroxysuccinimide ester (–ONSu) and Boc-L-Asn-ONSu, respectively. Boc group was removed by the treatment with 90% TFA. Amino acid compositions (mol/mol of Ala) and observed mass values ($[M+H]^+$) of the synthetic peptides were as follows (the value in parentheses is shown as the theoretical value): Found for Leu-NAG-ST: Asp, 3.18 (3); Glu, 0.98 (1); Pro, 1.03 (1); Gly, 1.04 (1); Ala, 1.00 (1); 1/2Cys, 5.55 (6); Ile, 1.88 (2); Leu, 2.06 (2); Phe, 1.12 (1). Observed m/z value: 1927.7 (1927.7). Found for Asn-Leu-NAG-ST: Asp, 4.21 (4); Glu, 0.97 (1); Pro, 1.03 (1); Gly, 1.02 (1); Ala, 1.00 (1); 1/2 Cys, 5.36 (6); Ile, 1.83 (2); Leu, 2.02 (2); Phe, 1.09 (1). Observed m/z value: 2041.6 (2041.7).

2.6. *Biological assay*

Toxic activity was assayed in suckling mice as previously described [8]. The fluid accumulation (FA) ratio was calculated as the ratio of the weight of entire intestine to that of the rest of body. An FA ratio of over 0.09 was considered to indicate a positive response. The minimum effective dose (MED) was evaluated as the minimum amount generating the toxicity.

3. RESULTS

Crude toxin obtained from 15 L of the culture supernatant of *V. cholerae* O1 GP156 was partially purified by DEAE-Sephadex A-25 ion-exchange chromatography and mAb2F-conjugated CNBr-Sepharose 4B immunoaffinity chromatography, as described in section 2. The toxic fractions which eluted with 100 mM Gly buffer in affinity chromatography, were separated by RP-HPLC using Program I to four active peak fractions, as shown in Fig. 1. Each of these fractions was subjected to rechromatography using Program II to give a pure peptide. Four different active peptides (O1-ST-1, -2, -3, and -4) were purified in final amounts of 22.91, 40.10, 2.41, and 0.85 nmol, respectively. The amino acid compositions, mass values, and MED values of the purified toxins are summarized in Table I. Differences in mass by 6 units between native and reduced toxins in their FAB mass spectra indicate the presence of three intramolecular disulfide linkages.

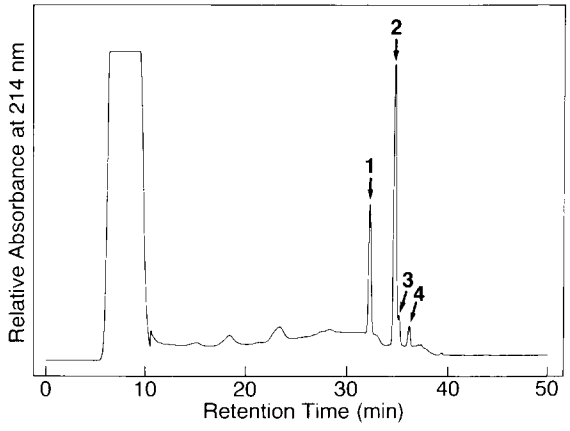

Fig. 1. Reverse-phase HPLC of the toxic fractions obtained by the immunoaffinity chromatography. Four active peak fractions indicated by arrows (1, 2, 3 and 4) were further subjected to rechromatography with the other solvent system (Program II, see section 2) to yield four pure peptides, O1-ST-1, -2, -3 and -4, respectively.

The amino acid composition and observed mass value of O1-ST-1 suggested that it has the same amino acid sequence (IDCCEICCNPACFGCLN) [1] and disulfide pairing mode (between Cys^3 and Cys^8, Cys^4 and Cys^{12}, and Cys^7 and Cys^{15}) [9] as those of NAG-ST. In fact, O1-ST-1 showed not only the same retention time as the synthetic NAG-ST on RP-HPLC using Programs III and IV but coeluted with synthetic NAG-ST as a

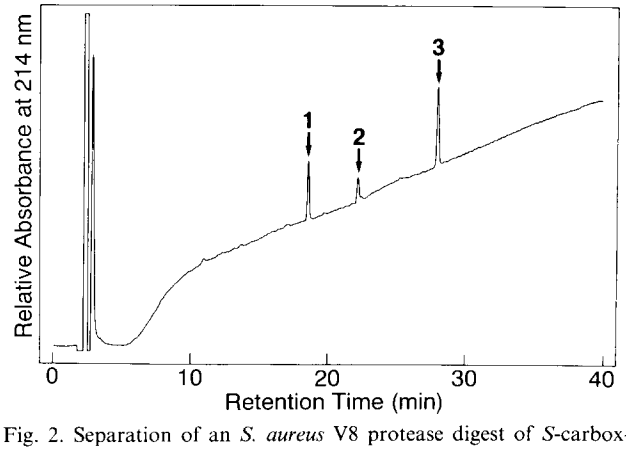

Fig. 2. Separation of an *S. aureus* V8 protease digest of *S*-carboxymethylated O1-ST-4 by RP-HPLC.

Table I

Amino acid compositions, mass values, and MED values of heat-stable enterotoxins produced by *V. cholerae* O1 GP156

	O1-ST-1	O1-ST-2	O1-ST-3	O1-ST-4
Asp	3.13 (3)	3.13 (3)	4.08 (4)	5.92 (6)
Glu	0.99 (1)	0.99 (1)	0.98 (1)	2.69 (3)
Pro	1.00 (1)	1.02 (1)	0.98 (1)	1.04 (1)
Gly	1.05 (1)	1.02 (1)	0.98 (1)	2.12 (2)
Ala	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
1/2 Cys	5.49 (6)	5.73 (6)	5.32 (6)	4.03 (6)
Val	–	–	–	0.85 (1)
Ile	1.84 (2)	1.89 (2)	1.78 (2)	2.54 (3)
Leu	1.00 (1)	2.06 (2)	1.96 (2)	2.06 (2)
Phe	1.06 (1)	1.08 (1)	1.05 (1)	1.76 (2)
Lys	–	–	–	0.80 (1)
Total	(17)	(18)	(19)	(28)
$[M + H]^+$				
Intact	1814.7	1927.7	2041.7	3072.5
	(1814.6)	(1927.7)	(2041.7)	(3072.2)
Reduced	1820.7	1933.6	2047.8	3078.4
	(1820.7)	(1933.7)	(2047.8)	(3078.3)
MED (ng)	5.0	6.3	12	87
(pmol)	2.8	3.3	5.9	28

The amino acid composition is shown as a normalized value with alanine to have one residue, and the number in parentheses refers to the number of residues in the peptide found by sequencing. The mass value in parentheses denotes the theoretical mass value of the peptide.

single peak under the both conditions. In addition, the MED value of O1-ST-1 in the suckling mice assay was nearly identical to the value (5.4 ng, 3.0 pmol) of the synthetic NAG-ST.

Next, O1-ST-4 was subjected to a gas-phase protein sequencer. The N-terminal sequence of the peptide was determined to be Phe-Ile-Lys-Gln-Val-Asp-Glu-Asn-Gly-Asn-Leu-Ile-Asp-. Then, O1-ST-4 was treated with iodoacetic acid in the presence of dithiothreitol, and the resulting *S*-carboxymethylated peptide was digested with *Staphylococcus aureus* V8 protease in 50 mM NH_4HCO_3 (pH 7.8) at 37°C for 7 h. Fractionation of the digest by RP-HPLC with use of Program V yielded three peptide fragments (Fig. 2). The amino acid sequences of fragments 1 and 2 were determined by automated Edman degradation to be Phe-Ile-Lys-Gln-Asp-Glu and Asn-Gly-Asn-Leu-Ile-Asp-CmCys-CmCys-Glu, respectively. The retention time of fragment 3 on RP-HPLC using Program V was identical to that of a peptide, Ile-CmCys-CmCys-Asn-Pro-Ala-CmCys-Phe-Gly-CmCys-Leu-Asn, which was obtained from an *S. aureus* V8 protease digest of *S*-carboxymethylated synthetic NAG-ST. Taken together all the evidence described above, the amino acid sequence of O1-ST-4 was determined as shown in Table I.

Taking the amino acid sequences of O1-ST-1 and -4 and the data of O1-ST-2 shown in Table II into consideration, O1-ST-2 was suggested to be a peptide that one more Leu linked to the N-terminus of O1-ST-1, as shown in Table II. Chromatographic analyses and biological assay supported that O1-ST-2 was identical to the synthetic Leu-NAG-ST (MED value: 6.9 ng, 3.6 pmol). According to the same procedure, the amino acid sequence of O1-ST-3 was determined as shown in Table II. (The MED value of the synthetic Asn-Leu-NAG-ST was 12 ng, 5.9 nmol.)

4. DISCUSSION

In the present study, four molecular species of STs elaborated by a CT-producing strain of *V. cholerae* O1 were isolated and their primary structures determined. They had the same amino acid sequence but varied in their length at the N-terminal portions. Although the amino acid sequence (CCEICCNPACFGC) essential for expression of the biological activity was completely identical among the peptides [9,10], their toxicities became more potent by the loss of the N-terminal amino acids. One of the enterotoxins produced by *V. cholerae* O1, O1-ST-1, was the same toxin as that elaborated by *V. cholerae* non-O1 (named NAG-ST) [1] and *V. mimicus* (named VM-ST) [11]. Furthermore, the primary structure of O1-ST-2 was identical to that of Vc-H-ST produced by *V. cholerae* non-O1 serogroup Hakata [2]. Very recently, the nucleotide sequence of the structural gene encoding O1-ST has been determined [12]. All of the determined amino acid sequences were consistent

Table II

Amino acid sequences of O1-STs and their comparisons with those of STs produced by the other bacteria belonging to the genus *Vibrio*

O1-ST-1:	Ile-Asp-Cys-Cys-Glu-Ile-Cys-Cys-Asn-Pro-Ala-Cys-Phe-Gly-Cys-Leu-Asn
O1-ST-2:	Leu-Ile-Asp-Cys-Cys-Glu-Ile-Cys-Cys-Asn-Pro-Ala-Cys-Phe-Gly-Cys-Leu-Asn
O1-ST-3:	Asn-Leu-Ile-Asp-Cys-Cys-Glu-Ile-Cys-Cys-Asn-Pro-Ala-Cys-Phe-Gly-Cys-Leu-Asn
O1-ST-4:	Phe-Ile-Lys-Gln-Val-Asp-Glu-Asn-Gly-Asn-Leu-Ile-Asp-Cys-Cys-Glu-Ile-Cys-Cys-Asn-Pro-Ala-Cys-Phe-Gly-Cys-Leu-Asn
NAG-ST:	Ile-Asp-Cys-Cys-Glu-Ile-Cys-Cys-Asn-Pro-Ala-Cys-Phe-Gly-Cys-Leu-Asn
Vc-H-ST:	Leu-Ile-Asp-Cys-Cys-Glu-Ile-Cys-Cys-Asn-Pro-Ala-Cys-Phe-Gly-Cys-Leu-Asn
VM-ST:	Ile-Asp-Cys-Cys-Glu-Ile-Cys-Cys-Asn-Pro-Ala-Cys-Phe-Gly-Cys-Leu-Asn

NAG-ST, Vc-H-ST and VM-ST are produced by *V. cholerae* non-O1 [1], *V. cholerae* non-O1 serogroup Hakata [2], and *V. mimicus* [11], respectively.

with those deduced from the nucleotide sequence. Thus, the four O1-STs should have originated from a single structural gene. From several lines of evidence, it was suggested that an immature form of toxin is excreted into the extracellular media by crossing through the outer membrane without complete proteolytic processing. The nucleotide sequence of the O1-ST gene is highly homologous to that of the NAG-ST gene of *V. cholerae* non-O1 [12,13]. However, only one species of toxin (NAG-ST) could be recovered from the culture supernatant of *V. cholerae* non-O1 [1]. The difference in gene products between *V. cholerae* O1 and non-O1 may be ascribed to the specificity of proteases secreted into the periplasmic space and extracellular media of these bacteria [14].

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