Amino acid sequence of a heat-stable enterotoxin isolated from enterotoxigenic *Escherichia coli* strain 18D

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A heat-stable enterotoxin produced by a strain of enterotoxigenic *Escherichia coli* 18D was purified by ion-exchange and reversed-phase high-pressure liquid chromatography. The amino acid sequence of the purified toxin was determined by Edman-degradation and a combination of fast atom bombardment mass spectrometry and carboxypeptidase digestion to be Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr.

Enterotoxin *Isolation* *Amino acid sequence* *FAB mass spectrometry*

1. INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) produces at least two types of enterotoxins that cause diarrheal disease in man and various domestic animals: low-M_r, heat-stable enterotoxin (ST), and high-M_r, heat-labile enterotoxin (LT) [1]. It was recently confirmed [2,3] that there are two genetically distinct ST's, which were named STIa and STIb. The DNA sequences encoding STIa and STIb were determined in [3] and [4], respectively. Since STIa and STIb originated from porcine and human strains of ETEC, we here designate them as ST_p and ST_h, respectively.

Recently, we isolated and purified ST_h from ETEC of human patients [5,6] and determined its amino acid sequence of 19 amino acid residues [6]. The sequence was different from that deduced from the DNA sequence encoding ST_p but identical to that deduced from the DNA sequence of ST_h, except for the C-terminal amino acid residue. In [7] the amino acid sequence of an ST isolated from an ETEC strain 18D, was not similar to that of ST_h but was similar to that deduced from the DNA sequence of ST_p, although the strain was derived from human patients. However, strain 18D was found by the DNA hybridization technique to be a strain producing ST_p (P. Echeverria, personal communication).

Here, we describe the isolation of an ST ($= ST_p$) from ETEC strain 18D and its sequence determination by Edman-degradation and a combination of fast atom bombardment (FAB) mass spectrometry and carboxypeptidase digestion [8,9].

2. MATERIALS AND METHODS

2.1. *Bacterial strain and culture conditions*

E. coli 18D, kindly given by Dr Giannella (University of Cincinnati, OH), was used throughout. The cells were grown in CAYE medium [10] at pH 8.6 without glucose at 37°C for 24 h with vigorous shaking, as in [5].

2.2. *Isolation of the toxin*

Crude toxin was extracted from the culture filtrate (20 L), as in [5]. The crude toxin was chromatographed successively on SP-Sephadex C-50 (H^+ form) and DEAE-Sephadex A-25 (acetate

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form) as in [6]. In each case, the main fraction with biological activity was collected for further purification. Final purification was achieved by reversed-phase HPLC on a LiChrosorb RP-8 column under the conditions described in the legend to fig.3.

2.3. *Biological assay*

ST activity was assayed in suckling mice of 2–4 days old, as in [5]. The fluid accumulation ratio of each animal was calculated as the ratio of the weight of the entire intestine to that of the rest of the body. The minimal amount of ST giving a fluid accumulation ratio of over 0.09 was designated as 1 mouse unit (MU), as in [5].

2.4. *Amino acid analysis*

Purified toxin and its derivatives were hydrolyzed in 4 M methane-sulfonic acid for 24 h at 105°C in vacuum-sealed tubes and the hydrolysates were analyzed in a Hitachi type 835 analyzer.

2.5. *Aminoethylation and carboxymethylation*

Samples of 685 μg (~348 nmol) and 550 μg (~280 nmol) of native ST_p were reduced and then aminoethylated and carboxymethylated, respectively, as in [6]. The recoveries of aminoethylated ST_p and carboxymethylated ST_p were 540 μg and 310 μg, respectively.

2.6. *Enzymatic digestion*

Aminoethylated ST_p (540 μg, ~242 nmol) was dissolved in 0.5% NH_4HCO_3 (640 μl) with protease V8 (EC 3.4.21.19) (16 μg in 8 μl). The mixture was deaerated in a stream of nitrogen and kept at 37°C for 18 h. The digest was lyophilized repeatedly, and then the lyophilizate (405 μg) was dissolved in 0.1 M pyridine acetate (pH 8.0; 45 μl) and treated with carboxypeptidase B (EC 3.4.17.2) (8 μg) at room temperature. Aliquots were withdrawn from the digest at appropriate times for mass measurement.

2.7. *Edman degradation*

Carboxymethylated ST_p (155 μg, ~66.7 nmol) was degraded manually [11].

2.8. *FAB mass spectrometry*

Mass spectra were recorded with a Jeol double-focusing mass spectrometer JMS-HX100 equipped with a high-magnetic field and an FAB ion source. The sample (40–50 μg) was dissolved in 1 M HCl (1 μl), mixed with glycerol (3 μl), and applied to a stainless steel sample holder (3 × 3 mm). Typical experimental conditions were as follows: xenon atom beam source, 3 KeV accelerating potential and 200 μA emission current. Ion source, 5 KV accelerating potential.

3. RESULTS AND DISCUSSION

3.1. *Purification*

Crude toxin extracted from the culture supernatant of *E. coli* 18D was chromatographed on SP-Sephadex C-50 (fig.1). The main toxic activity was recovered in a peak eluted with 0.1 M $AcONH_4$ (pH 6.6). Since the peak fraction still contained a large amount of pigmented material, the area shown by a horizontal bar was then subjected to ion-exchange chromatography on DEAE-Sephadex A-25. The toxic activity emerged in the area shown by a horizontal bar in fig.2. Final purification was performed by HPLC on a reversed-phase column, as shown in fig.3. The main toxic fraction shown by a horizontal bar was confirmed to be pure by repeated HPLC (not shown). The purification of ST_p is summarized in table 1. The amino acid composition was determined to be $Asp_{2.01}$ (2), $Thr_{0.99}$ (1), $Glu_{1.16}$ (1), $Pro_{1.27}$

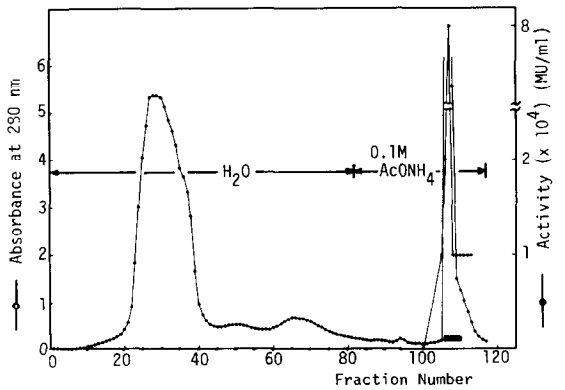

Fig.1. Chromatography on SP-Sephadex C-50 of crude toxin. Column size: 3 × 46 cm, H^+ form. Elution: An aqueous solution of crude toxin was applied, the column was washed with dist. water and then material was eluted with 0.1 M $AcONH_4$ (pH 6.7). Fractionation size: 13.5 ml/tube; flow rate: 150 ml/h.

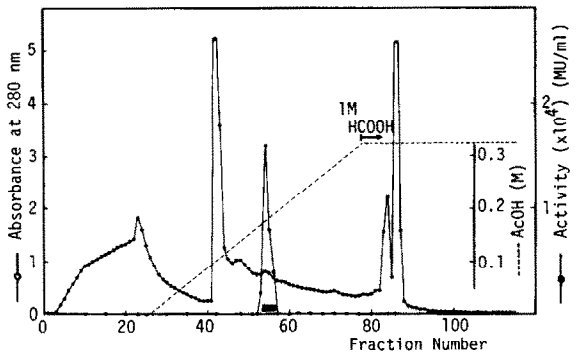

Fig.2. Chromatography on DEAE-Sephadex A-25 of the fraction shown by a horizontal bar in fig.1. Column size: 1.2 × 53 cm, acetate form. Elution: a linear gradient from H_2O to 0.32 M AcOH and then 1 M HCOOH. Fractionation size: 13.5 ml/tube; flow rate: 75 ml/h.

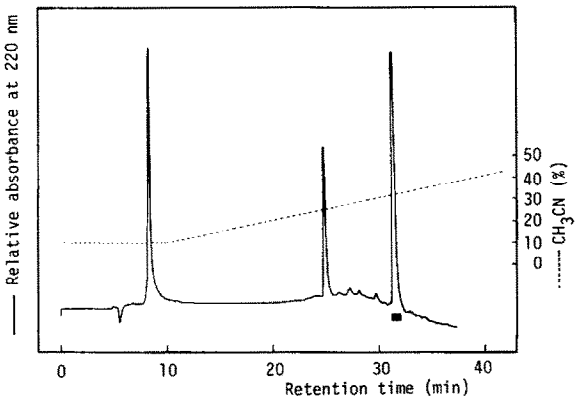

Fig.3. Reversed-phase HPLC of the toxic fraction obtained by DEAE-Sephadex A-25 chromatography (fig.2). Column: LiChrosorb RP-8 (8 × 300 mm, 5 μm). Elution: sample (tube no.54 in fig.2) was applied and material was eluted with 10% CH_3CN in 0.01 M $AcONH_4$ (pH 5.7) for 10 min and then with a linear gradient (----) of 10–35% CH_3CN in 0.01 M $AcONH_4$ (pH 5.7). Flow rate: 2 ml/min. The toxic activity was found in the peak marked by a horizontal bar.

(1), $Gly_{1.16}$ (1), $Ala_{2.00}$ (2), $½Cys_{4.92}$ (6), $Leu_{1.13}$ (1), $Tyr_{1.92}$ (2), and $Phe_{0.99}$ (1) (values were calculated as mol/mol of Ala; numbers in parentheses indicate nearest integer values). The values were identical to those of the ST in [12]. The minimum effective dose of purified ST_p in suckling mice assay was 2.5 ng.

Table 1

Summary of purification of ST_p produced by ETEC 18D

	Total unit	Spec.act. (ng/MU)	Recovery (%)
Culture supernatant (20 L)	3.2×10^6	17800	100
Crude toxin	2.48×10^6	224	77.5
SP-Sephadex C-50	1.89×10^6	172	59.1
DEAE-Sephadex A-25	4.05×10^5		12.7
HPLC			2.5 (1.49 mg)

3.2. *M_r-value*

Purified ST_p was analyzed by FAB mass spectrometry, a method developed in [13] and the results are shown in fig.4. The observed signal at m/z = 1971 indicated that the M_r of ST_p was 1970, because free peptides generally give protonated molecular ions ($[M + H]^+$, where M denotes the M_r) in positive FAB mass spectra. This M_r-value was compatible with the M_r (1969–1972) calculated from the amino acid composition (Asp + Asn and Glu + Gln were analyzed as Asp and Glu, respectively, in the acid hydrolysate).

3.3. *Amino acid sequence*

Recently, we attempted to synthesize ST with the amino acid sequence reported in [7] and found that the synthetic peptide had a different retention time on HPLC from that of the native toxin isolated from the same strain ETEC 18D as that used in [12]. This finding suggested that the purified native toxin used in our work had a different amino acid sequence from that proposed in [7], although it had the same amino acid composition as that of their proposed sequence.

Some of us [8,9] recently developed a new method for sequence determination of peptide mixtures by a combination of field-desorption (FD) mass spectrometry and exopeptidase digestion. We used this method for determination of the sequence of ST_p, but with FAB mass spectrometry instead of FD mass spectrometry [6,14]. Fig.5A shows the positive FAB mass spectrum of reduced and aminoethylated ST_p digested with protease V8, giving intense signals at m/z = 965 and 1289. The

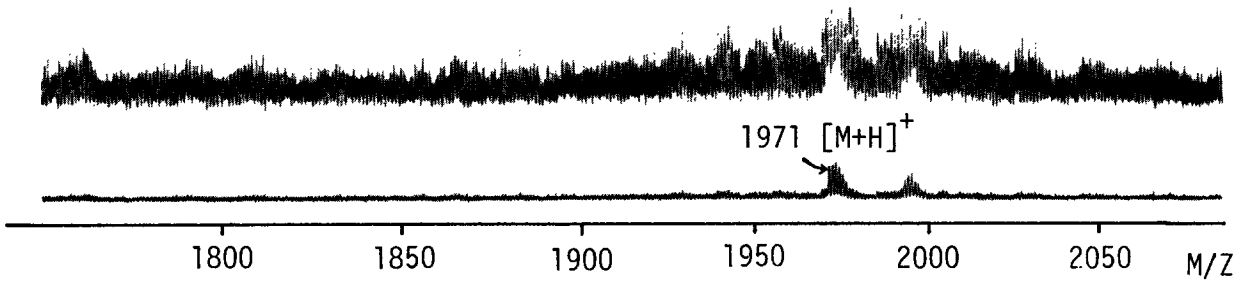

Fig.4. Positive FAB mass spectrum of purified ST_p.

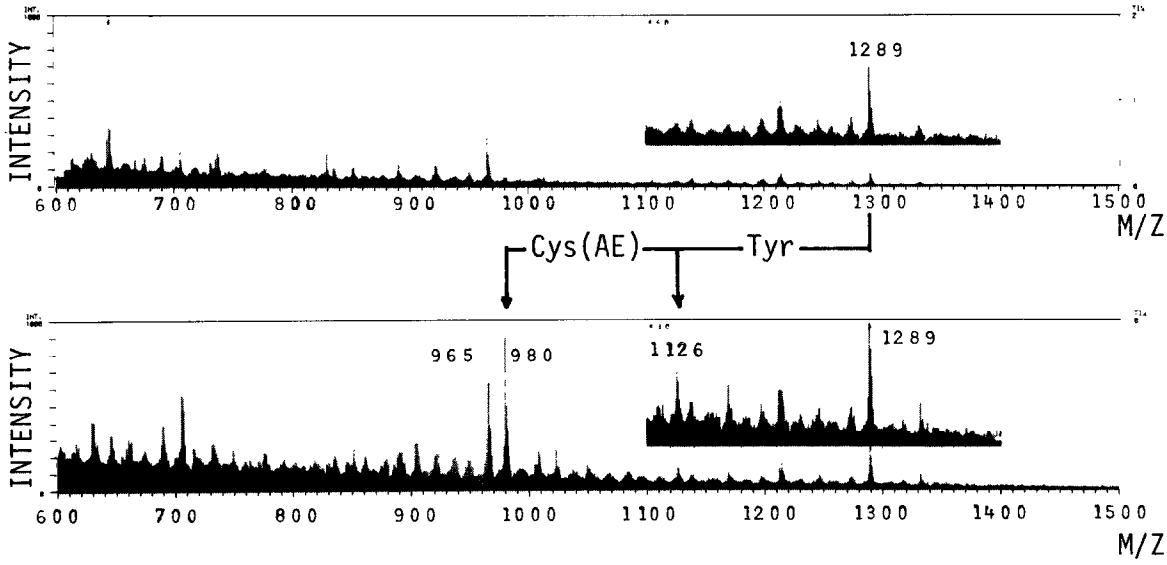

Fig.5. Positive FAB mass spectra of: (A) aminoethylated (AE) ST_p digested with protease V8; (B) a mixture of samples of (A) treated with carboxypeptidase B for 30 s, 1 and 5 min.

results suggest that the resulting two peptide fragments had M_r of 964 and 1288. Fig.5B shows the positive FAB mass spectrum of the digest with carboxypeptidase B of the peptide fragments shown in fig.5A. From the observed signals at m/z = 980 and 1126, the C-terminus of the peptide fragment with the mass value 1289 was -Cys(AE)(146)-Tyr(163)-OH (where AE means an aminoethyl group and numbers in parentheses denote residual weights of amino acids). The FD mass spectrum of the carboxypeptidase B digest gave intense signals at m/z = 165 and 182 together with 1126 (not shown), indicating that aminoethyl-cysteine (165) and tyrosine (182) were released from the peptide fragment (1289).

Reduced and carboxymethylated ST_p was degraded manually by the Edman method, the results revealed the sequence of 16 amino acid residues from the N-terminus. At the C-terminal two residues were uncertain. The sequence was compatible with the data obtained from the mass spectra shown in fig.4,5A and 5B. The sequence data are summarized and compared with reported sequences for ST in fig.6.

In conclusion, the primary structure of ST_p was established. As shown in fig.6, the sequence was

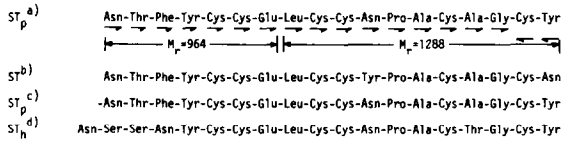

Fig.6. Amino acid sequence of ST: (a) present data: → Edman method, ← a combination of FAB mass spectrometry and carboxypeptidase B digestion and M_r of aminoethylated STP_p digested with protease V8; (b) see [7]; (c) see [3]; (d) see [6].

identical to that deduced from the DNA sequence encoding ST_p [3], but different from that proposed in [7]. In addition, common and different structural features of ST_p and ST_h [6] were established.

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