Chemical Synthesis of Fully Active and Heat-stable Fragments of Heat-stable Enterotoxin of Enterotoxigenic *Escherichia coli* Strain 18D

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(Received December 20, 1983)

Two shorter peptides of a heat-stable enterotoxin (ST_p), which is produced by enterotoxigenic *Escherichia coli* strain 18D, were prepared by two different procedures; i) chemical synthesis and ii) Edman-degradation of ST_p. These peptides (designated as $ST_p(4—18)$ and $ST_p(5—18)$) corresponded to 15 and 14 amino acid residues in the sequence of ST_p without three and four residues, respectively, of the N-terminus. Peptides $ST_p(4—18)$ and $ST_p(5—18)$ prepared by chemical synthesis had the same biological and physicochemical properties as the peptides obtained by the Edman method. Furthermore, they showed almost the same toxicity as native and synthetic ST_p, and their toxicity was neutralized by anti-native ST_p antisera. Synthetic $ST_p(4—18)$ had similar heat-stability to ST_p, but synthetic $ST_p(5—18)$ was much more heat-stable. These findings suggest that the sequence from the Cys residue near the N-terminus to the C-terminal residue is extremely important for the unique characters of toxicity and heat-stability of the toxin.

Various strains of enterotoxigenic *Escherichia coli* (ETEC) from animal hosts produce heterologous low-molecular-weight, heat-stable enterotoxins (ST's), which are responsible for acute diarrhea in man and various domestic animals. Burgess *et al.*[1] reported two distinct ST's, one (ST_a) being methanol-soluble and active in neonatal piglets and infant mice but inactive in weaned pigs, and the other (ST_b) being methanol-insoluble and active in weaned pigs but inactive in infant mice. Recently, we[2-4] isolated two kinds of ST_a (designated as ST_h and ST_p) in pure form from human strains SK-1 and 18D of ETEC, respectively. These ST_a's, which were active in suckling mouse assay, were found to have the amino acid sequences shown in Fig. 1. Slightly before our description,[4] Chan and Giannella[5] reported the amino acid sequence of an ST from ETEC strain 18D to be as shown in Fig. 1. The amino acid sequence of ST_p determined by us was very similar to that of ST proposed by Chan and Giannella,[5] but differed in two residues at positions 11 and 18 from the N-terminus, although we[4] isolated and purified ST_p from the same strain, ETEC 18D, that Chan and Giannella[5] used. To elucidate these discrepancy, we[6] synthesized ST_p and ST with the amino acid sequences proposed by us[4] and Chan and Giannella,[5] respectively, and confirmed that the former had the same biological and physicochemical properties as native ST_p isolated from ETEC strain 18D. Lallier *et al.*[7,8] and Ronnberg *et al.*[9] also purified ST produced by a porcine strain F11 (P155) and human strain C57/26C2 of ETEC, respectively, and reported their amino acid sequences to be as illustrated in Fig. 1. Their amino acid sequences were identical with that of ST_p from ETEC strain 18D determined by us.[4]

These toxins have great structural similarity, although the sequences of their N-terminal regions differ. The sequences from the Tyr residue near the N-terminus to the Tyr residue at the C-terminus are identical, except at the 4th residue from the C-terminus, although the sequence of toxin proposed by Chan and Giannella[5] also differs at the 11th and 18th residues. Six half-cystine residues are conserved in the same positions and intramolecularly linked by disulfide bonds, suggesting that these toxins have similar molecular conformations, although the

(a) Asn-Ser-Ser-Asn-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys-Tyr Strain SK-1

(b) Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr " 18D

(c) Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Tyr-Pro-Ala-Cys-Ala-Gly-Cys-Asn " 18D

(d) Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr " F11(P155)

(e) Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr " C57/26C2

Fig. 1. Comparison of amino acid sequences of ST: (a) ST_h isolated from ETEC strain SK-1, reported in Ref. 3); (b) ST_p isolated from ETEC strain 18D by us;[4] (c) an ST isolated from ETEC strain 18D by Chan and Giannella;[5] (d) an ST isolated from a porcine strain (F11(P155)) of ETEC, reported in Ref. 8); (e) an ST isolated from ETEC strain C57/26C2, reported in Ref. 9).

positions of the disulfide linkages have not yet been determined. Moreover, ST_h and ST_p have the same activities in suckling mouse assay[3,4] and their activity is neutralized by homologous and heterologous *anti*-ST_p and *anti*-ST_h antisera.[10] These findings imply that the common region of the primary structures of these toxin molecules is important for their biological activity and heat-stability.

This paper reports the chemical syntheses of shorter peptides of ST_p, which constitute the common primary sequence of these toxins, but without N-terminal 3 and 4 amino acid residues, respectively, of the N-terminal sequence of ST_p. The preparation of shorter peptides of ST_p by stepwise Edman-degradation of ST_p is also described. Synthetic shorter peptides of ST_p (ST_p (4—18) and ST_p (5—18)) were confirmed to have the same properties as the peptides derived from ST_p by Edman-degradation and to have almost the same biological activities as native and synthetic ST_p. Interestingly, synthetic ST_p (5—18) was more heat-stable than native ST_p.

Experimental

The general experimental and analytical methods used were described in the preceding paper.[6] The abbreviations used in this paper are those recommended by the IUPAC-IUB: *J. Biol. Chem.*, **247**, 977 (1972). Additional abbreviations are: MBzl, *p*-methylbenzyl; TFA, trifluoroacetic acid; DMF, *N,N*-dimethylformamide; TEA, triethylamine.

Boc-Tyr-Cys(MBzl)-Cys(MBzl)-OMe (1). Boc-Cys(MBzl)-Cys(MBzl)-OMe[11] (21.9 g, 40 mmol) was dissolved in TFA (40 ml), stirred at room temperature for 30 min and concentrated to a syrup under reduced pressure. The syrup was mixed with dioxane containing 1 equiv of HCl and concentrated under reduced pressure to a syrup, which was dissolved in DMF (80 ml). Meanwhile, Boc-Tyr-N_2H_3 (13.0 g, 44 mmol) was dissolved in DMF (80 ml), cooled below −20 °C and mixed with 4.39 M HCl (1M=1 mol dm^{-3}) in dioxane (9.1 ml). The solution was stirred with isopentyl nitrite (6.5 ml, 50 mmol) at the same temperature for 35 min and then mixed with the above DMF solution and TEA (20.1 ml). The mixture was stirred at 0 °C for 2 d in a refrigerator. The resulting precipitate was filtered off and the filtrate was concentrated to an oil under reduced pressure. The oil was dissolved in AcOEt and washed successively with 0.1 M HCl, 5% aq $NaHCO_3$ and water. The washed solution was dried over anhydrous Na_2SO_4 and concentrated to a solid under reduced pressure. The solid was crystallized from a mixture of AcOEt, MeOH and hexane; wt 26.0 g (91.6%), mp 160—161 °C, $[\alpha]_D^{23}$ −39.2° (*c* 1.0, DMF).

Found: C, 62.45; H, 6.59; N, 5.72; S, 9.05%. Calcd for $C_{37}H_{47}O_7N_3S_2$: C, 62.61; H, 6.68; N, 5.92; S, 9.02%.

Boc-Tyr-Cys(MBzl)-Cys(MBzl)-N_2H_3 (2). Compound **1** (17.8 g, 25 mmol) was dissolved in a mixture of DMF (50 ml) and MeOH (300 ml). The solution was cooled in an ice-bath, mixed with 100% hydrazine hydrate (50 ml), and then stirred at room temperature for 2 h. The solution was concentrated under reduced pressure and the residue was mixed with water. The solid formed was collected and recrystallized from MeOH and ether; wt 17.0 g (95.5%), mp 194—195 °C, $[\alpha]_D^{23}$ −16.2° (*c* 1.0, DMF).

Found: C, 60.68; H, 6.53; N, 9.76; S, 8.95%. Calcd for $C_{36}H_{47}O_6N_5S_2$: C, 60.91; H, 6.68; N, 9.87; S, 9.02%.

Boc-Tyr-Cys(MBzl)-Cys(MBzl)-Glu(OBu^t)-Leu-OEt (3). Compound **2** (14.2 g, 20 mmol) was dissolved in DMF (100 ml), cooled to −20 °C, and mixed with 4.39 M HCl in dioxane (2.95 ml). The solution was stirred with isopentyl nitrite (2.86 ml, 22 mmol) at the same temperature for 20 min and then mixed with H-Glu(OBu^t)-Leu-OEt·HCl[11] (8.4 g, 22 mmol) and TEA (21.2 ml). The mixture was stirred at 0 °C for 4 d in a refrigerator. The precipitate formed was filtered off and the filtrate was concentrated to an oil under reduced pressure. The oil was dissolved in AcOEt and washed successively with 0.1 M HCl, 5% aq $NaHCO_3$ and water. The washed solution was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was crystallized from AcOEt and hexane; wt 19.0 g (93.1%), mp 153 °C (sintered) and 173 °C (melted), $[\alpha]_D^{23}$ −21.2° (*c* 0.5, DMF).

Found: C, 62.27; H, 7.49; N, 6.83; S, 6.20%. Calcd for $C_{53}H_{75}O_{11}N_5S_2$: C, 62.27; H, 7.40; N, 6.85; S, 6.26%.

Boc-Tyr-Cys(MBzl)-Cys(MBzl)-Glu(OBu^t)-Leu-N_2H_3 (4). Compound **3** (18.4 g, 18 mmol) was dissolved in EtOH (200 ml) and mixed with 100% hydrazine hydrate (40 ml) in an ice-bath. The mixture was stirred at room temperature for 4.5 h. The solution was concentrated under reduced pressure to a solid, which was collected with water. The crude material was crystallized from DMF and ether; wt 15.3 g (83.1%), mp 223 °C (decomp), $[\alpha]_D^{23}$ −11.6° (*c* 0.5, DMF).

Found: C, 59.38; H, 6.89; N, 9.46; S, 6.37%. Calcd for $C_{51}H_{73}O_{10}N_7S_2 \cdot H_2O$: C, 59.69; H, 7.37; N, 9.56; S, 6.24%.

Boc-Tyr-Cys(MBzl)-Cys(MBzl)-Glu(OBu^t)-Leu-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-Tyr-OBzl (5). Boc-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-Tyr-OBzl[6] (3.22 g, 2.0 mmol) was dissolved in TFA (40 ml), stirred at room temperature for 50 min, and then concentrated under reduced pressure. Meanwhile, compound **4** (2.42 g, 2.4 mmol) was dissolved in DMF (14 ml), cooled below −20 °C, and mixed with 4.39 M HCl in dioxane (4.2 ml). The mixture was stirred with isopentyl nitrite (0.35 ml, 2.7 mmol) at the same temperature for 20 min and then mixed with the above residue and TEA (2.87 ml). The mixture was stirred at 0 °C for 2 d in a refrigerator. The precipitate formed was filtered off and the filtrate was concentrated to a solid under reduced pressure. The solid was washed with 0.1 M HCl and water and crystallized first from DMF and ether and then from DMF and EtOH; wt 4.40 g (88.5%), mp 231 °C (decomp), $[\alpha]_D^{23}$ −37.5° (*c* 0.32, DMF). Amino acid ratio in the acid hydrolysate: Asp, 1.05 (1); Glu, 1.05 (1); Pro, 1.00 (1); Gly, 0.98 (1); Ala, 2.00 (2); Leu, 1.02 (1); Tyr, 2.18 (2).

Found: C, 61.54; H, 6.85; N, 9.10; S, 7.60%. Calcd for $C_{128}H_{164}O_{23}N_{16}S_6$: C, 61.82; H, 6.65; N, 9.01; S, 7.72%.

Boc-Cys(MBzl)-Cys(MBzl)-Glu(OBu^t)-Leu-Cys(MBzl)-Cys-MBzl)-Asn-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-Tyr-OBzl (6). This compound was synthesized as described previously.[6] Amino acid ratio in the acid hydrolysate: Asp, 1.06 (1); Glu, 1.00 (1); Pro, 1.07 (1); Gly, 1.00 (1); Ala, 2.02 (2); Leu, 1.01 (1); Tyr, 0.97 (1).

Removal of Protecting Groups and Air-oxidation: Protecting groups of compounds **5** and **6** were removed by treatment with anhydrous liquid hydrogen fluoride[12] and the deprotected peptides in dilute solution (5×10^{-5} M) were air-oxidized at pH 8.0, as described previously.[6]

Edman-degradation: Synthetic ST_p (1-18)[6] (1.4 mg, 0.7 μmol) was dissolved in 50% aq pyridine (300 μl). The solution was adjusted to pH 9.5 by the addition of *N*-methylmorpholine and mixed with phenyl isothiocyanate (30 μl). The mixture was kept at 40 °C for 1 h under a stream of nitrogen. The excess reagent used was removed by three washings with benzene (1 ml) and the aqueous layer was lyophilized. The residue was treated with a few drops of TFA at 40 °C for 20 min. TFA was removed under a stream of nitrogen and the residue was dissolved in water

(200 μl) and washed three times with AcOEt (0.5 ml). The aqueous layer was lyophilized. The lyophilized material was purified by HPLC, as described below, and the purified material was degraded by the Edman method under similar conditions to those described above.

Ion-exchange Chromatography: Ion-exchange chromatography of synthetic peptides was performed on a column (2×33 cm or 1.6×25 cm) of DEAE-Sephadex A-25 (acetate form). The material adsorbed on the ion-exchanger was eluted with a linear-gradient of 0 to 0.5 M acetic acid.

High-performance Liquid Chromatography (HPLC): HPLC was performed on a hand-made column (8×300 mm) packed with LiChrosorb RP-8 (Merck, 5 μm particle size) and on a YMC packed column ODS AM-312 (6×150 mm) (Yamamura Chemical Laboratory Co. Ltd., Kyoto, 5 μm particle size) for semipreparative separation and for analysis, respectively. LiChrosorb RP-8 was equilibrated with 0.01 M ammonium acetate (pH 5.7) containing 10% acetonitrile as an organic modifier. YMC packed column was equilibrated with 0.05% TFA (pH 2.2) containing 10% acetonitrile. Both columns were developed at a flow rate of 1 ml/min with a linear-gradient of 10% to 30% acetonitrile.

Biological Assay: ST activity was assayed in suckling mice of 2—4 d old, as described previously.[2] The minimum toxic activity, designated as 1 mouse unit, was the amount of peptide causing sufficient fluid accumulation in the intestine of the test animal to give a weight ratio of the entire intestine to that of the rest of the body of over 0.09.

Heat-stability: Synthetic peptides were tested for heat-stability with analysis by HPLC as described previously.[6]

Results and Discussion

Syntheses of ST_p (4–18) and ST_p (5–18). Two kinds of heat-stable enterotoxins (ST_h and ST_p) active in suckling mouse were isolated from various strains of ETEC and their amino acid sequences were determined by us[2-4] and others.[5,7-9] These toxins have the same sequence from the Tyr residue near the N-terminus to the C-terminal Tyr residue, except for the 4th residue from the C-terminus, as illustrated in Fig. 1, suggesting that this common sequence is important for their biological activity. On the other hand, we[6] recently obtained ST_p from a chemically synthesized linear peptide with the whole amino acid sequence proposed by us.[4] Since this synthetic ST_p had the same biological and physicochemical properties as native ST_p, it seemed to have the same structure, including disulfide linkages, as native ST_p, although the positions of the three disulfide linkages in ST_p have not yet been determined. Because of the structural similarities of ST_h and ST_p, we attempted to synthesize shorter peptides of ST_p covering the common sequence of the toxin, by the same procedure as that used for synthesis of ST_p. Protected pentadeca- and tetradecapeptides (**5** and **6**) were synthesized by similar procedures to those described previously[6] and then treated with anhydrous liquid hydrogen fluoride[12] to remove all the protecting groups. Then dilute solutions (5×10^{-5} M) of the linear peptides with free thiol groups were air-oxidized at pH 8.0 under similar conditions to those described previously.[6] Toxic peptides were isolated from the air-oxidized solution by successive ion-exchange chromatography (Figs. 2 and 3) and HPLC (Figs. 4 and 5). The purified peptides, ST_p (4—18) and ST_p (5—18), were ob-

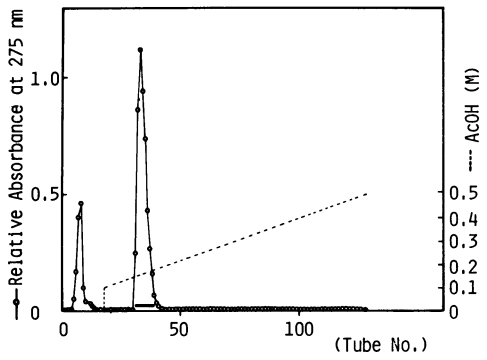

Fig. 2. Chromatography on DEAE-Sephadex A-25 of deprotected and air-oxidized material from compound **5**. Fractions indicated by a horizontal bar showed toxicity and were collected. Column size: 1.3×16.5 cm; fractionation size: 4.5 ml/tube; flow rate: 45 ml/h.

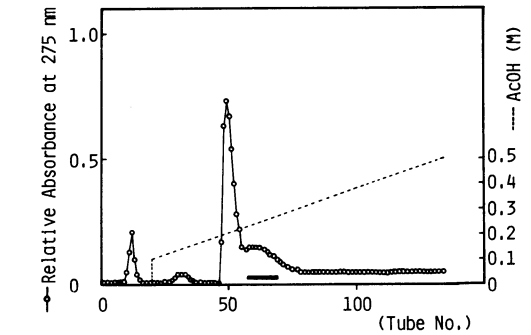

Fig. 3. Chromatography on DEAE-Sephadex A-25 of deprotected and air-oxidized material from compound **6**. The toxic fractions shown by a horizontal bar were collected. Column size: 1.6×25 cm; fractionation size: 4.5 ml/tube; flow rate: 20 ml/h.

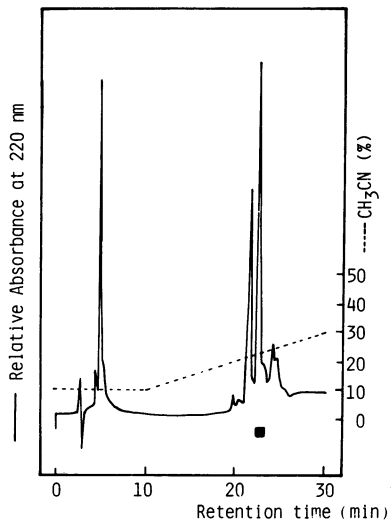

Fig. 4. Semipreparative HPLC of the toxic fraction shown by a horizontal bar in Fig. 2 on a LiChrosorb RP-8 column (5 μm, 8×300 mm). The toxicity was observed in the peak fraction shown by a horizontal bar.

tained in about 6.0% and 10.9% yield from the protected peptides **5** and **6**, respectively.

Preparation of Shorter Peptides of ST_p by Stepwise Edman-degradation. To examine whether synthetic shorter peptides of ST_p (ST_p(4—18) and ST_p(5—18)) were intramolecularly linked by the disulfide bonds in the same way as in native ST_p, we degraded synthetic ST_p (1—18),[6] which has the same biological and physicochemical properties as native ST_p and therefore seems to have the same disulfide linkages as the latter, by the Edman method. Peptides lacking one to four amino acid residues from the N-terminus of ST_p-(1—18) were purified on a semipreparative column of HPLC. The purities of the isolated peptides was confirmed by analytical HPLC, as illustrated in Figs. 6b—e. Removal of N-terminal hydrophilic amino acid residues Asn and Thr from the 1st and 2nd positions of ST_p increased the retention times of the degraded peptides ST_p (2—18) and ST_p (3—18) on HPLC. Further removal of the hydrophobic amino acid residues Phe and Tyr from the 3rd and 4th positions of ST_p gave shorter peptides, ST_p (4—18) and ST_p (5—18), which were eluted earlier than ST_p (1—18). ST_p (1—18) and the degraded peptides were eluted separately from a YMC ODS-column, as shown in Fig. 6f. The recoveries of the degraded peptides in Edman-degradation were 52—56%.

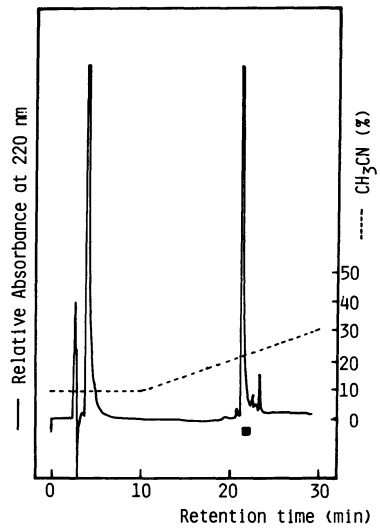

Fig. 5. Semipreparative HPLC on a LiChrosorb RP-8 (5 μm, 8×300 mm) of the fraction shown by a horizontal bar in Fig. 3.

Identification of Synthetic and Edman-degraded Peptides. *i) Amino Acid Analyses*: The amino acid compositions of synthetic peptides (ST_p (4—18) and ST_p (5—18)) and the Edman-degraded peptides (ST_p-(2—18) to ST_p (5—18)) are summarized in Table 1. The results indicate that the N-terminal amino acid residues of ST_p (1—18) were removed stepwise by Edman-degradation.

ii) Mass Spectra: Synthetic and Edman-degraded peptides were subjected to FAB mass spectrometry. As shown in Fig. 7, synthetic ST_p (4—18) and ST_p (5—18), like the Edman-degraded peptides (ST_p(4—18) and ST_p-(5—18)), gave intense signals at m/z=1609 and 1446. The results indicated that the molecular weights of synthetic ST_p (4—18) and ST_p (5—18) were 1608 and 1445, respectively, and hence that these synthetic peptides were intramolecularly linked by three disulfide bonds.

iii) HPLC: The synthetic peptides (ST_p (4—18) and ST_p (5—18)) were coeluted with the respective peptides prepared by the Edman method from LiChrosorb RP-8,

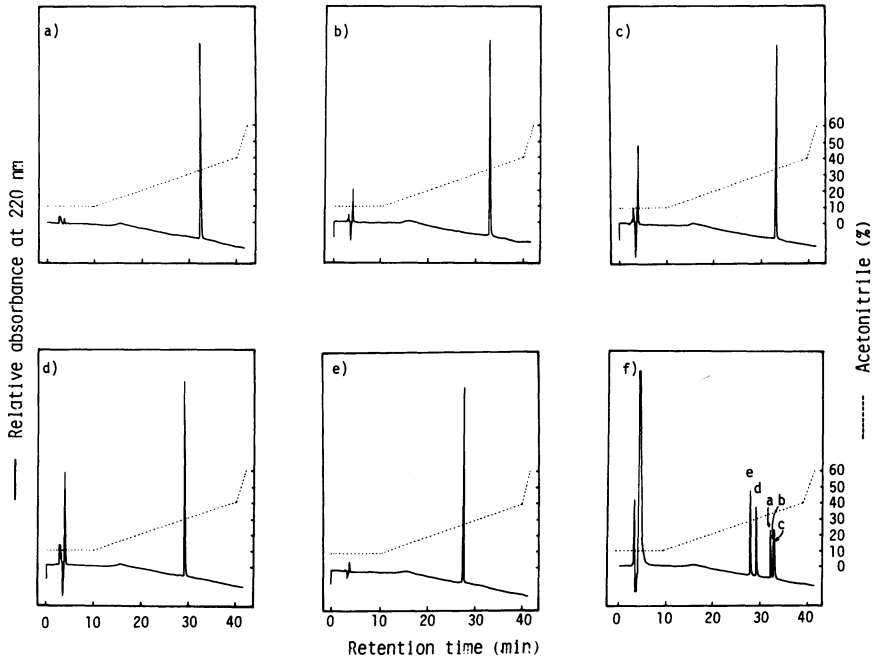

Fig. 6. HPLC profiles on a YMC ODS-column (6×150 mm) of shorter peptides of ST_p prepared by Edman-degradation: a) The starting peptide ST_p (1–18), b) ST_p(2–18), c) ST_p(3–18), d) ST_p(4–18), e) ST_p(5–18), and f) a mixture of a), b), c), d), and e).

Table 1. Amino acid compositions of synthetic peptides and Edman-degraded peptides of ST_p

	Synthetic peptides			Edman-degraded peptides			
	ST_p	ST_p[4-18]	ST_p[5-18]	ST_p[2-18]	ST_p[3-18]	ST_p[4-18]	ST_p[5-18]
Asp	2.00(2)	1.08(1)	1.08(1)	1.03(1)	1.02(1)	1.03(1)	1.04(1)
Thr	1.00(1)			0.95(1)			
Glu	1.11(1)	1.03(1)	1.05(1)	1.01(1)	1.01(1)	1.02(1)	1.06(1)
Pro	1.07(1)	1.22(1)	1.25(1)	0.93(1)	1.09(1)	1.06(1)	0.97(1)
Gly	0.99(1)	1.02(1)	1.02(1)	1.02(1)	0.99(1)	1.04(1)	1.03(1)
Ala	2.00(2)	2.00(2)	2.00(2)	2.00(2)	2.00(2)	2.00(2)	2.00(2)
1/2 Cys	5.25(6)	5.44(6)	5.05(6)	5.16(6)	5.16(6)	5.26(6)	5.18(6)
Leu	0.96(1)	1.09(1)	1.00(1)	1.02(1)	1.01(1)	1.03(1)	1.07(1)
Tyr	1.86(2)	2.05(2)	0.91(1)	1.95(2)	1.95(2)	1.95(2)	0.98(1)
Phe	0.98(1)			0.97(1)	0.94(1)		

Values were calculated as mol/mol of Ala; numbers in parentheses indicate theoretical values.

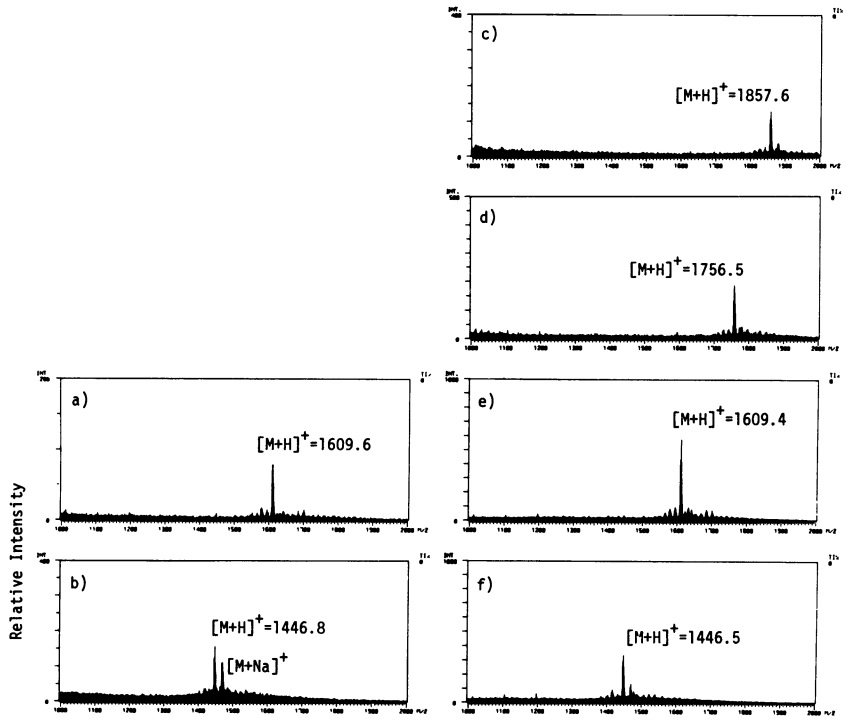

Fig. 7. Positive FAB mass spectra: (first column) synthetic peptides a) ST_p(4–18), b) ST_p(5–18); (second column) Edman degraded peptides c) ST_p(2–18), d) ST_p(3–18), e) ST_p(4–18), and f) ST_p(5–18).

as shown in Fig. 8. These results indicated that the synthetic ST_p (4—18) and ST_p (5—18) were intramolecularly linked by three disulfide bonds in the same positions as in native ST_p.

Biological Activities of Synthetic and Edman-degraded Peptides. The synthetic peptides (ST_p (4—18) and ST_p (5—18)) and Edman-degraded peptides (ST_p (2—18), ST_p (3—18), ST_p (4—18), and ST_p (5—18)) were assayed by the fluid accumulation test using suckling mice.[2] As seen in Table 2, these shorter peptides of ST_p all showed toxicity at a dose of about 1.5—2 ng, which was almost the same value as that of native and synthetic ST_p. Thus the sequence from the Tyr residue near the N-terminus to the C-terminal Tyr residue, which is the same in ST_h and ST_p, except for the 4th residue from the C-terminus, had the same biological

Table 2. Some biological properties of ST_p related peptides

	Minimum effective dose/ng	Neutralization by anti-native ST_p antiserum
Synthetic peptides		
ST_p[1-18]	1	+
ST_p[4-18]	0.8—1	+
ST_p[5-18]	0.8—1	+
Edman-degraded peptides		
ST_p[2-18]	0.5—2	+
ST_p[3-18]	1.5—2	+
ST_p[4-18]	1—2	+
ST_p[5-18]	1	+

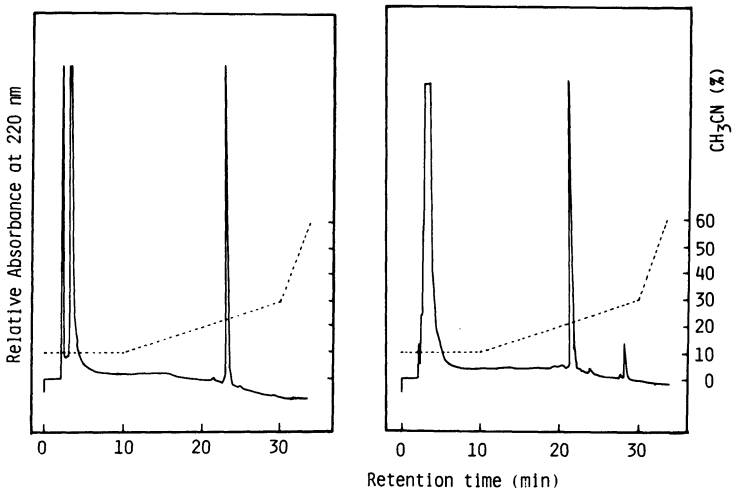

Fig. 8. HPLC profiles on a column (4×250 mm) of LiChrosorb RP-8 of mixtures of a) synthetic ST_p-(4–18) and the Edman-degraded peptide ST_p(4–18) and b) synthetic ST_p(5–18) and the Edman-degraded peptide ST_p(5–18).

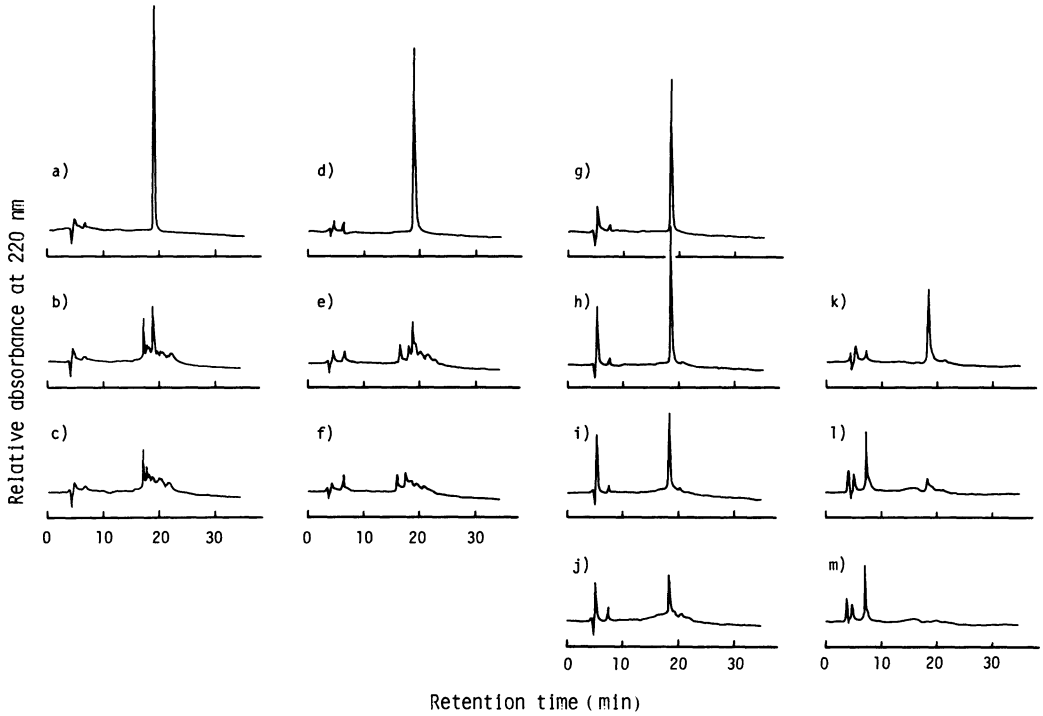

Fig. 9. Comparison of heat-stabilities of synthetic ST_p(1–18) and its shorter peptides: (first column) synthetic peptide ST_p(1–18) a) untreated, b) after 10 min at 100 °C, c) after 30 min at 100 °C; (second column) synthetic ST_p(4–18) d) untreated, e) after 10 min at 100 °C, f) after 30 min at 100 °C; (third and fourth columns) synthetic ST_p(5–18) g) untreated, h) after 10 min at 100 °C, i) after 30 min at 100 °C, j) after 60 min at 100 °C, k) after 10 min at 120 °C, l) after 30 min at 120 °C, and m) after 60 min at 120°C.

activity as that of the whole sequence in ST_p. The results also show that the Tyr residue at the 4th position of ST_p was not necessary for expression of toxicity.

Furthermore, the toxicity of the synthetic and Edman-degraded peptides was found to be neutralized by anti-native ST_p antisera,[10] as shown in Table 2. These results suggest that the three dimensional structure from the Cys residue near the N-terminus to the C-terminal Tyr residue, including the three disulfide linkages, is extremely important for the expression of the toxicity of ST_p, because the reductive cleavage of disulfide linkages destroys the toxicity of the toxin. If the receptor for ST_p is on the epithelial cell membrane in the intestine, the site for binding of ST_p to the receptor may be located in this three dimensional structure. Studies on this possibility are now in progress.

Heat-stabilities of Synthetic Peptides ST_p (4—18) and ST_p (5—18). The name "heat-stable" is based on

the observation[14,15] that the toxicity of this toxin is not lost on heating the toxin for 30 min in boiling water. Recently we[6,13] found that the heat-stability of the toxin can be examined by HPLC. By this HPLC procedure, we compared the heat-stabilities of synthetic ST_p-(4—18) and ST_p (5—18) with that of synthetic ST_p (1—18). ST_p (4—18) showed similar stability to ST_p (1—18) under the conditions examined, as illustrated in Figs. 9a—f. On the other hand, ST_p (5—18) was found to be more stable than ST_p (4—18) and ST_p (1—18) and after heating in boiling water for 60 min, about half its peak height was retained, as shown in Figs. 9g—j, whereas on similar treatment synthetic ST_p (1—18) and ST_p-(4—18) were almost completely destroyed. The HPLC profiles of ST_p (5—18) treated at 120 °C (Figs. 9k—m) were similar to those of ST_p (1—18) and ST_p (4—18) boiled at 100 °C. ST_p (5—18) was completely destroyed by treatment for 60 min at 120 °C. Thus, removal of the four N-terminal amino acid residues, especially the 4th Tyr residue, increased the heat-stability of the toxin. The enhanced structural stability of ST_p (5—18) may be due to decreased perturbation of the molecule on heating as a result of the absence of the N-terminal sequence.

This work was partly supported by a Grant-in-Aid for Scientific Research No. 58122002 from the Ministry of Education, Science and Culture.

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