  Bull. Chem. Soc. Jpn., 57, 2550—2556 (1984) [Vol. 57, No. 9

Heat-stable Enterotoxin (ST_h) of Human Enterotoxigenic *Escherichia coli* (Strain SK-1). Structure-activity Relationship

Haruo IKEMURA, Hiroyuki WATANABE, Saburo AIMOTO, Yasutsugu SHIMONISHI,* Saburo HARA,† Tae TAKEDA,†† Yoshifumi TAKEDA,††† and Toshio MIWATANI††

Institute for Protein Research, Osaka University, Yamada-oka 3-2, Suita, Osaka 565

†*Department of Chemistry, College of Science, Osaka University, Machikaneyama, Toyonaka, Osaka 560*

††*Research Institute for Microbial Diseases, Osaka University, Yamada-oka 3-1, Suita, Osaka 565*

(Received April 19, 1984)

For examination of the structure-activity relationship of ST_h, five shorter analogs of ST_h lacking one to five N-terminal amino acid residues of ST_h were synthesized. The synthetic peptides were confirmed to have the same intramolecular disulfide linkages as those in native ST_h. These peptides caused fluid accumulation in the intestine of suckling mice at almost the same molar levels as that of native ST_h. The toxicities of these peptides were also neutralized by antisera raised against native ST_h. These results indicated that the toxic and antigenic sites of ST_h are both located in the amino acid sequence between the Cys residue at the 5th position from the N-terminus and the C-terminal Tyr residue.

Heat-stable enterotoxins (named ST_h and ST_p) have been isolated from various strains of enterotoxigenic *Escherichia coli* and their amino acid sequences of 19 and 18 residues, respectively, have been determined[1-7] to be as shown in Fig. 1. These toxins are very similar in primary structure and have the same amino acid sequence from the Tyr residue near the N-terminus to the Tyr residue at the C-terminus, except at position 4 from the C-terminus. Six half-cystine residues are conserved in the same relative positions and are linked intramolecularly by disulfide bonds. These facts suggest that the common sequence is important for the toxicity and heat-stability of the toxins. However, it is difficult to obtain a large amount of the toxin from the culture supernatant of the bacteria, and so apart from these suggestion from the primary structures, little is known about the biological properties of the toxin, except that it seems to act on the guanylate cyclase-cyclic GMP system.[8-11] In the preceding paper,[12] we reported a method for chemical synthesis of ST_h in large quantity. This made it possible to investigate the biochemical and biological properties of the toxin and its analogs.

As part of an investigation of the structure-activity relationship of ST_h, in this work, we synthesized several shorter analogs of ST_h. We also found that a sequence lacking the five N-terminal amino acid residues of ST_h, which is identical to that of ST_p except for the 4th residue from the C-terminus, has the same biological properties as those of native ST_h.

Experimental

The general experimental and analytical methods used were described in the preceding paper.[12] 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; DMSO, dimethyl sulfoxide.

*Z-Ser-Ser-OMe (**Ia**).* Z-Ser-N_2H_3 (12.7 g, 50.0 mmol) was dissolved in DMF (200 ml) and cooled below −20 °C. The solution was mixed with 4.39M HCl (1M=1 mol dm^{-3}) in dioxane (34.2 ml) and isopentyl nitrite (7.40 ml), stirred at the same temperature for 30 min, and then mixed with H-Ser-OMe·HCl (8.6 g, 55 mmol) and *N*-methylmorpholine (16.5 ml). The mixture was stirred at 3 °C for 24 h in a refrigerator. The precipitate formed was removed by filtration and the filtrate was concentrated to dryness under reduced pressure. The residue was triturated and recrystallized from AcOEt; wt 13.6 g (79.9%), mp 142—142.5 °C, $[\alpha]_D^{25}$ +10.1° (*c* 1.1, DMF).

Found: C, 52.94; H, 5.89; N, 8.28%. Calcd for $C_{15}H_{20}O_7N_2$: C, 52.94; H, 5.92; N, 8.23%.

*Z-Ser-Ser-N_2H_3 (**Ib**).* Compound **Ia** (5.1 g, 15 mmol) was dissolved with 100% hydrazine hydrate (7.50 ml) in MeOH. The solution was stirred at room temperature overnight, and the precipitate formed was collected and boiled in hot MeOH. The mixture was cooled and the precipitate was collected by filtration; wt 3.6 g (70.5%), mp 205 °C (dec), $[\alpha]_D^{25}$ +16.0° (*c* 0.9, DMF).

Found: C, 48.99; H, 5.90; N, 16.40%. Calcd for $C_{14}H_{20}O_6N_4$: C, 59.41; H, 5.92; N, 16.46%.

*Z-Ser-Ser-Asn-Tyr-OEt (**Ic**).* Z-Asn-Tyr-OEt[12] (5.95 g, 13.0 mmol) was dissolved in MeOH (100 ml) and hydrogenated over 5% palladium-charcoal at atmospheric pressure. The catalyst was filtered off and the filtrate was concentrated to a solid under reduced pressure. The solid was collected with ether. Meanwhile, compound **Ib** (3.40 g, 10.0 mmol) was dissolved in DMF (80 ml) and cooled below −20 °C. The solution was mixed with 4.39M HCl in

```
                1                   5                  10                  15
ST_h   Asn-Ser-Ser-Asn-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys-Tyr

ST_p       Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr
```

Fig. 1. Amino acid sequences of heat-stable enterotoxins (ST_h and ST_p) isolated from enterotoxigenic *Escherichia coli* strains SK-1 and 18D, respectively.

††† Present address: The Institute of Medical Science, The University of Tokyo, Shirokanedai 4-6-1, Minato-ku, Tokyo 108.

dioxane (8.2 ml) and isopentyl nitrite (1.77 ml) and then stirred at the same temperature for 20 min. The solution was mixed with *N*-methylmorpholine (3.96 ml) and the above solution of the solid in DMF (20 ml). The solution was stirred at 2—3 °C for 3 d and concentrated to dryness under reduced pressure. The residue was triturated in AcOEt and the resulting precipitate was twice reprecipitated from EtOH and AcOEt; wt 3.2 g (50.7%), mp 180.5—183 °C, $[\alpha]_D^{25}$ +7.1° (*c* 1.1, DMF).

Found: C, 53.31; H, 5.95; N, 10.72%. Calcd for $C_{29}H_{37}O_{11}N_5 \cdot H_2O$: C, 53.62; H, 6.05; N, 10.78%.

*Z-Ser-Ser-Asn-Tyr-N_2H_3 (**Id**).* Compound **Ic** (3.5 g, 5.5 mmol) was dissolved in DMF (30 ml) and mixed with 100% hydrazine hydrate (2.75 ml). The mixture was stirred at room temperature overnight and the precipitate formed was collected with EtOH. The precipitate was boiled in EtOH, cooled to room temperature, and filtered; wt 3.3 g (92.0%), mp 218 °C (dec), $[\alpha]_D^{25}$ −6.5° (*c* 1.0, DMSO).

Found: C, 51.56; H, 5.71; N, 15.82%. Calcd for $C_{27}H_{35}O_{10}N_7 \cdot 1/2H_2O$: C, 51.75; H, 5.79; N, 15.65%.

*Z-Ser-Ser-Asn-Tyr-Cys(MBzl)-Cys(MBzl)-Glu-Leu-Cys-(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Thr-Gly-Cys-(MBzl)-Tyr-OBzl (**I**).* Compoud **V**[12] (1.18 g, 0.5 mmol) was dissolved with anisole (0.2 ml) in TFA (10 ml) at 0 °C and stirred at room temperature for 2 h. The solution was concentrated to a solid under reduced pressure. Meanwhile, compound **Id** (463 mg, 0.75 mmol) was suspended in DMF (5 ml) and cooled below −50 °C. The suspension was mixed with 6.72M HCl in dioxane (0.51 ml) and isopentyl nitrite (0.11 ml) and stirred at −20 °C for 30 min. The suspension became clear during this procedure, and was then mixed with *N*-methylmorpholine (1.0 ml) and a solution of the above solid in DMF. The mixture was stirred at 0 °C for 10 d and concentrated to dryness under reduced pressure. The residue was triturated in EtOH; wt 1.23 g (88.4%), mp 193 °C (dec), $[\alpha]_D^{20}$ −43.2° (*c* 1.0, DMSO). Amino acid ratio in the acid hydrolysate: Asp, 1.80 (2); Thr, 0.95 (1); Ser, 1.35 (2); Glu, 0.96 (1); Pro, 1.03 (1); Gly, 1.00 (1); Ala, 1.01 (1); 1/2Cys, not determined; Leu, 0.97 (1); Tyr, 1.68 (2).

Found: C, 57.87; H, 6.67; N, 9.66; S, 6.80%. Calcd for $C_{138}H_{172}O_{30}N_{20}S_6 \cdot 4H_2O$: C, 58.05; H, 6.35; N, 9.81; S, 6.74%.

*Z-Ser-Asn-Tyr-N_2H_3 (**IIa**).* Z-Ser-Asn-Tyr-OEt[12] (5.8 g, 10.7 mmol) was dissolved in DMF (30 ml) and mixed with 100% hydrazine hydrate (10.0 ml). The mixture was stirred at room temperature for 6 h and then concentrated to a solid under reduced pressure. The solid was recrystallized from DMF and EtOH; wt 5.8 g (100%), mp 241.5 °C (dec), $[\alpha]_D^{25}$ −11.5° (*c* 1.0, DMSO).

Found: C, 52.60; H, 5.72; N, 15.60%. Calcd for $C_{24}H_{30}O_8N_6 \cdot H_2O$: C, 52.55; H, 5.88; N, 15.32%.

*Z-Ser-Asn-Tyr-Cys(MBzl)-Cys(MBzl)-Glu-Leu-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Thr-Gly-Cys(MBzl)-Tyr-OBzl (**II**).* Compound **V**[12] (1.18 g, 0.5 mmol) was dissolved with anisole (0.23 ml) in TFA (10 ml) at 0 °C and stirred at room temperature for 2 h. The solution was concentrated to a solid under reduced pressure. Meanwhile, compound **IIa** (318 mg, 0.6 mmol) was suspended in DMF (5 ml) and cooled below −50 °C. The suspension was mixed with 4.39M HCl in dioxane (0.41 ml) and isopentyl nitrite (0.09 ml) and stirred at −20 °C for 30 min. Then the resulting solution was mixed with *N*-methylmorpholine (1.0 ml) and a solution of the above solid in DMF (5 ml). The mixture was stirred at 0 °C for 2 d and concentrated to dryness under reduced pressure. The residue was triturated in 0.1 M HCl and collected. The material was boiled in hot EtOH and filtered after cooling to room temperature; wt 1.22 g (90.5%), mp 195 °C (dec), $[\alpha]_D^{16}$ −46.3° (c 1.0, DMSO). Amino acid ratio in the acid hydrolysate: Asp, 1.90 (2); Thr, 0.93 (1); Ser, 0.79 (1); Glu, 0.97 (1); Pro, 1.02 (1); Gly, 1.00 (1); Ala, 1.10 (1); 1/2Cys, not determined; Leu, 0.94 (1); Tyr, 1.81 (2).

Found: C, 58.82; H, 6.47; N, 9.52; S, 7.02%. Calcd for $C_{135}H_{167}O_{28}N_{19}S_6 \cdot 3H_2O$: C, 58.95; H, 6.34; N, 9.68; S, 7.00%.

*Z-Asn-Tyr-N_2H_3 (**IIIa**).* Z-Asn-Tyr-OEt[12] (4.57 g, 10.0 mmol) was dissolved in DMF (30 ml). The solution was mixed with 100% hydrazine hydrate (15 ml) and stirred at room temperature overnight. The gelatinous precipitate formed was collected with ether and reprecipitated from DMF and EtOH; wt 4.2 g (94.7%), mp 248 °C (dec), $[\alpha]_D^{20}$−9.5° (*c* 0.5, DMSO).

Found: C, 54.91; H, 6.04; N, 15.58%. Calcd for $C_{21}H_{25}O_6N_5 \cdot H_2O$: C, 54.66; H, 5.90; N, 15.18%.

*Z-Asn-Tyr-Cys(MBzl)-Cys(MBzl)-Glu-Leu-Cys(MBzl)-Cys-(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Thr-Gly-Cys(MBzl)-Tyr-OBzl (**III**).* Compound **V**[12] (1.18 g, 0.5 mmol) was dissolved with anisole (0.23 ml) in TFA (10 ml) at 0 °C and stirred at room temperature for 2 h. The solution was concentrated to a solid under reduced pressure. Meanwhile, compound **IIIa** (266 mg, 0.60 mmol) was dissolved in DMF (5 ml) and cooled below −50 °C. The solution was mixed with 4.39M HCl in dioxane (0.41 ml) and isopentyl nitrite (0.09 ml), stirred at −20 °C for 30 min and then mixed with *N*-methylmorpholine (1.0 ml) and a solution of the above solid in DMF (5 ml). The mixture was stirred at 0 °C for 3 d and then evaporated to a solid under reduced pressure. The solid was precipitated from DMF and EtOH; wt 1.16 g (88.9%), mp 203.5 °C (dec), $[\alpha]_D^{16}$ −46.9° (*c* 1.0, DMSO). Amino acid ratio in the acid hydrolysate: Asp, 1.92 (2); Thr, 0.92 (1); Glu, 0.98 (1); Pro, 1.00 (1); Gly, 1.00 (1); Ala, 1.03 (1); 1/2Cys, not determined; Leu, 0.99 (1); Tyr, 1.85 (2).

Found: C, 59.46; H, 6.56; N, 9.38; S, 7.36%. Calcd for $C_{132}H_{162}O_{26}N_{18}S_6 \cdot 3H_2O$: C, 59.53; H, 6.36; N, 9.47; S, 7.22%.

*Z-Tyr-Cys(MBzl)-Cys(MBzl)-Glu-Leu-OEt (**IVa**).* Boc-Cys(MBzl)-Cys(MBzl)-Glu(OBu^t)-Leu-OEt[12] (4.28 g, 5.0 mmol) was dissolved in CH_2Cl_2 (60 ml) and TFA (12.0 ml) was added to the solution. The solution was stirred at room temperature for 40 min and concentrated to a solid under reduced pressure. Meanwhile, Z-Tyr-N_2H_3 (1.98 g, 6.0 mmol) was dissolved in DMF (15.0 ml), cooled below −50 °C and mixed with 4.39M HCl in dioxane (4.1 ml) and isopentyl nitrite (0.88 ml). The solution was stirred at −20 °C for 30 min and then mixed with *N*-methylmorpholine (1.98 ml) and a solution of the above solid in DMF (10 ml). The mixture was stirred at 0—2 °C for 24 h and then concentrated to dryness under reduced pressure. The gelatinous residue was collected with AcOEt and reprecipitated from EtOH; wt 3.8 g (76.0%), mp 186.5—189.0 °C, $[\alpha]_D^{20}$−14.3° (*c* 1.0, DMSO).

Found: C, 61.67; H, 6.48; N, 6.95; S, 6.27%. Calcd for $C_{52}H_{65}O_{11}N_5S_2 \cdot H_2O$: C, 61.33; H, 6.63; N, 6.88; S, 6.30%.

*Z-Tyr-Cys(MBzl)-Cys(MBzl)-Glu-Leu-N_2H_3 (**IVb**).* Compound **IVa** (3.8 g, 3.8 mmol) was dissolved in DMF (30 ml), mixed with 100% hydrazine hydrate (3.8 ml) and stirred at room temperature for 24 h. The precipitate formed was collected with EtOH, boiled in EtOH, cooled to room temperature and filtered; wt 3.0 g (80.0%), mp 212.5 °C (dec), $[\alpha]_D^{20}$ −14.7° (*c* 1.0, DMSO).

Found: C, 60.09; H, 6.69; N, 10.28 S, 6.51%. Calcd for $C_{50}H_{63}O_{10}N_7S_2 \cdot 1/2H_2O$: C, 60.34; H, 6.48; N, 9.85; S, 6.44%.

*Z-Tyr-Cys(MBzl)-Cys(MBzl)-Glu-Leu-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Thr-Gly-Cys(MBzl)-Tyr-OBzl (**IV**).* Compound **V**[12] (1.64 g, 1.0 mmol) was dissolved with anisole (0.46 ml) in TFA (20 ml) at 0 °C and stirred at room temperature for 30 min. The solution was concentrated to a solid under reduced pressure. Meanwhile, compound **IVb** (1.18 g, 1.2 mmol) was dissolved in DMF (5 ml) and cooled below −50 °C. The solution was mixed with 4.39M HCl in dioxane (0.82 ml) and isopentyl nitrite (0.18 ml), stirred at −20 °C for 30 min, and was then mixed with TEA (1.2 ml)

and a solution of the above solid in DMF (7 ml). The mixture was stirred at 0 °C for 5 d and concentrated to dryness under reduced pressure. The residue was collected with EtOH, boiled in EtOH, and cooled to room temperature. The solid material was collected by filtration; wt 1.60 g (64.1%), mp 201.0 °C (dec), $[\alpha]_D^{16}$ −54.4° (*c* 1.0, DMSO). Amino acid ratio in the acid hydrolysate: Asp, 0.96 (1); Thr, 0.87 (1); Glu, 0.97 (1); Pro, 0.99 (1); Gly, 1.00 (1); Ala, 1.01 (1); 1/2Cys, not determined; Leu, 1.02 (1); Tyr, 2.09 (2).

Found: C, 60.93; H, 6.44; N, 8.88; S, 7.69%. Calcd for $C_{128}H_{156}O_{24}N_{10}S_6 \cdot 2H_2O$: C, 60.74; H, 6.37; N, 8.85; S, 7.60%.

Boc-Cys(MBzl)-Cys(MBzl)-Glu(OBu^t)-Leu-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Thr-Gly-Cys(MBzl)-Tyr-OBzl (V). This compound was synthesized as described in the preceding paper.[12] Amino acid ratio in the acid hydrolysate: Asp, 0.95 (1); Thr, 0.89 (1); Glu, 0.96 (1); Pro, 0.93 (1); Gly, 1.00 (1); Ala, 1.03 (1); 1/2Cys, not determined; Leu, 0.99 (1); Tyr, 0.95 (1).

Deprotection, Air-oxidation and Purification of Toxic Peptides. Protected peptides **I**, **II**, **III**, **IV**, and **V** (100 μmol each) were treated with anhydrous liquid hydrogen fluoride (10 ml)[13] and spontaneously air-oxidized under similar conditions to those used for synthesis of ST_h. The air-oxidized materials were purified by ion-exchange chromatography on DEAE-Sephadex A-25 under the conditions described previously.[14] The peak fractions eluted were separated and assayed by the method described below. The toxic fractions were lyophilized and purified further by high-performance liquid chromatography (HPLC) under the conditions used for purification of ST_h, as described in the preceding paper.[12] The highly toxic fractions were isolated and lyophilized. The yields of purified peptides, ST_h[2-19], ST_h[3-19], ST_h[4-19], ST_h[5-19], and ST_h[6-19], were about 4.8, 5.8, 4.8, 5.3, and 27.4% on the basis of the amounts of the protected peptides **I**, **II**, **III**, **IV** and **V**, respectively.

Biological Assay. ST activity was assayed in suckling mice of 2—4 d old, as described previously.[1]

Aminopeptidase M Digestion of ST_h and its Analogs. Native and synthetic ST_h and synthetic analogs of ST_h (*ca.* 100 μg) were digested with aminopeptidase M (5 μg) in 0.1M Tris HCl buffer (100 μl) under the conditions described in the preceding paper.[12] The digested peptides were separated by HPLC as described in the preceding paper.[12]

Edman Degradation. A sample peptide (*ca.* 100μg) was allowed to react with phenyl isothiocyanate (40 μl) in 50% aq pyridine (0.3 ml) adjusted to pH 9.6 with *N*-methylmorpholine at 40 °C for 60 min. The reaction mixture was washed three times with benzene and then lyophilized. The lyophilized material was treated with a few drops of TFA at 40 °C for 20 min and dried under a stream of nitrogen. The residue was dissolved in 0.5M AcOH (0.2 ml) and washed with AcOEt. The aqueous layer was applied directly to a YMC-packed column ODS A-324 (10×300 mm) and the adsorbed material was eluted with a linear gradient of 10—40% acetonitrile in 0.05% TFA (pH 2.35) at a flow rate of 3 ml/min. The peak fractions were separated and lyophilized.

Heat-treatment. Synthetic peptides were each dissolved in 0.01M phosphate buffer (pH 7.2) at a concentration of 200 μg/ml. Sample solutions were sealed in capillary tubes and placed for given periods in an electric oven controlled at the required temperature. Then the tubes were rapidly cooled in an ice-bath and part of the solution (50 μl) was subjected to HPLC. HPLC was performed on a column of LiChrosorb RP-8 (4.6×250 mm). The column was developed with a linear qradient of 10—35% acetonitrile in 0.01M ammonium acetate (pH 5.7) with increase in acetonitrile of 1%/min at a flow rate of 0.5 ml/min.

Results and Discussion

Syntheses. First, protected linear peptides **I** to **V** lacking one to five N-terminal amino acid residues of ST_h, respectively, were synthesized, as shown in the

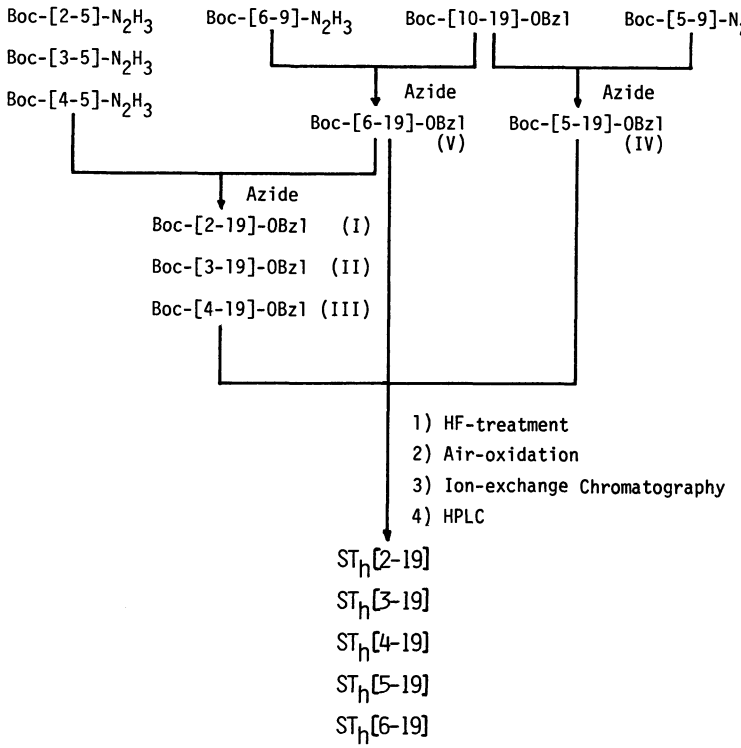

Fig. 2. Scheme for syntheses of shorter analogs of ST_h.

scheme in Fig. 2. Then these protected peptides **I** to **V** were treated with anhydrous liquid hydrogen fluoride[13] and spontaneously air-oxidized in dilute solution (5×10^{-5}M), as described for the synthesis of STh in the preceding paper.[12] The air-oxidized solutions of the deprotected peptides were subjected to HPLC, as shown in Fig. 3, and each fraction was separated and its toxicity was examined by the fluid accumulation test in suckling mice. The peak fractions shown by black bars had the highest toxicity. These fractions were purified by similar methods to those used for STh, as described in the preceding paper.[12] The fractions were confirmed to be pure by HPLC, as shown in Fig. 4, with a different buffer system as solvent from that used for the preparative purification. The amino acid compositions of the synthetic peptides are summarized in Table 1.

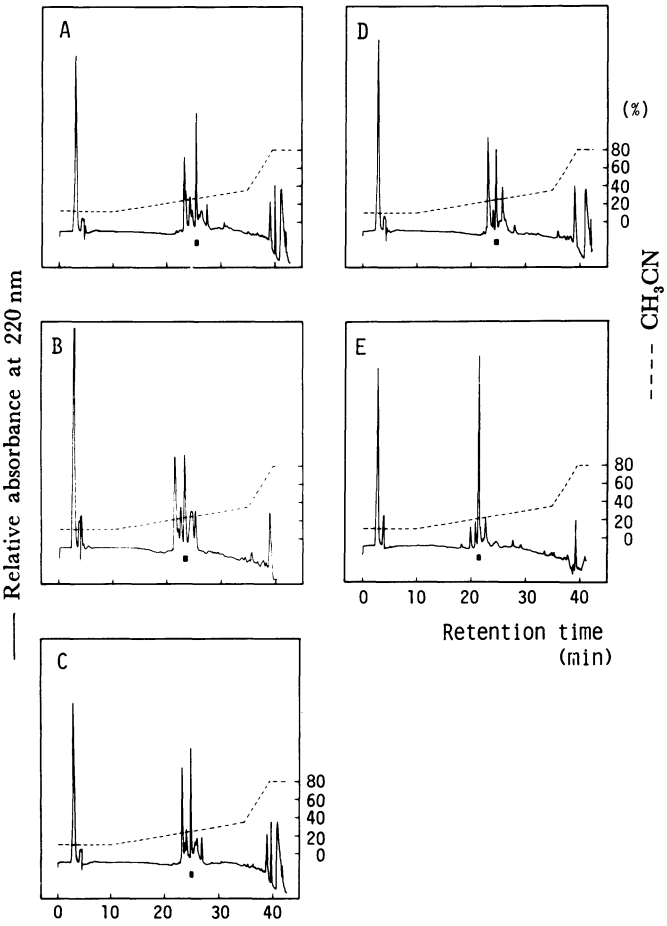

Fig. 3. HPLC profiles of deprotected and air-oxidized solutions of compounds **I**(A), **II**(B), **III**(C), **IV**(D), and **V**(E).
Column: LiChrosorb RP-8 (5 μm, 4×250 mm). The starting solvent was 10% CH_3CN in 0.01 M $AcONH_4$ (pH 5.7), and the flow rate was 1 ml/min.

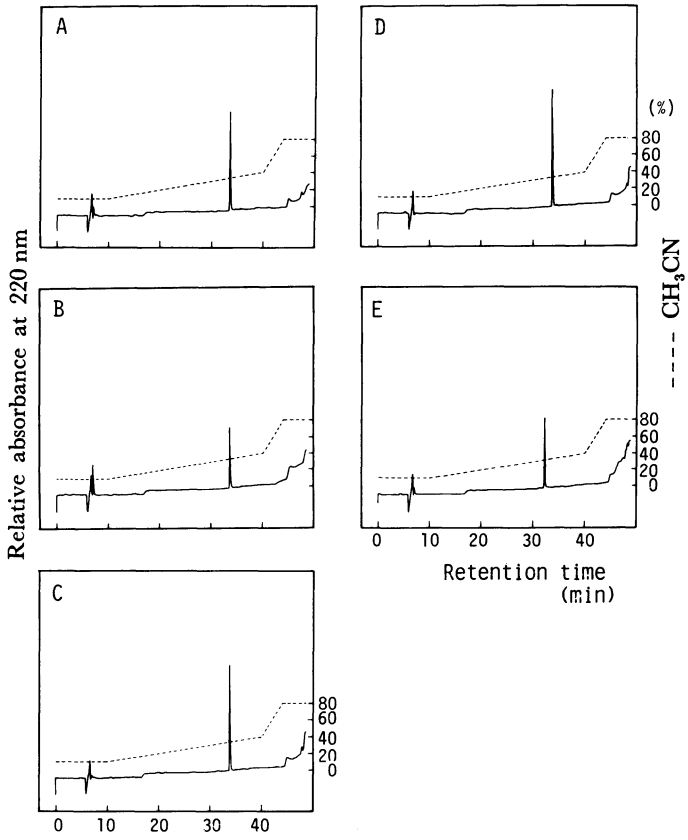

Fig. 4. HPLC profiles of synthetic STh[2-19] (A), STh[3-19] (B), STh[4-19] (C), STh[5-19] (D), and STh[6-19] (E) on a YMC-packed column ODS A-324 (5 μm, 10×300 mm). The starting solvent was 10 % CH_3CN in 0.05 % TFA (pH 2.35), and the flow rate was 2 ml/min.

Table 1. Amino acid compositions of shorter synthetic analogs of STh

	STh[2-19]	STh[3-19]	STh[4-19]	STh[5-19]	STh[6-19]
Asp	1.84(2)	2.02(2)	2.00(2)	1.07(1)	1.02(1)
Thr	0.92(1)	0.99(1)	1.00(1)	1.00(1)	1.01(1)
Ser	1.81(2)	0.96(1)	—	—	—
Glu	1.05(1)	1.02(1)	1.01(1)	1.02(1)	1.01(1)
Pro	0.99(1)	1.12(1)	1.10(1)	1.11(1)	0.99(1)
Gly	1.07(1)	1.03(1)	1.02(1)	1.04(1)	1.02(1)
Ala	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)
1/2Cys	4.82(6)	5.50(6)	5.47(6)	5.51(6)	5.39(6)
Leu	0.97(1)	1.03(1)	1.03(1)	1.03(1)	1.02(1)
Tyr	1.78(2)	1.98(2)	2.09(2)	1.98(2)	1.00(1)

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

High-performance Liquid Chromatography (HPLC) of ST_h Analogs. Shorter synthetic analogs of ST_h could be purified to homogeneity by HPLC under the conditions shown in Fig. 4. However, it was necessary to find conditions for elution of all or almost all the synthetic analogs of ST_h separately on HPLC, because all shorter synthetic analogs of ST_h except ST_h[6-19] had nearly the same retention times on HPLC under the conditions shown in Fig. 4. We tested several commercially available ODS columns and buffer solutions, and found that the following two procedures were satisfactory, although they did not separate all the ST_h analogs completely; i) A YMC-packed ODS column A-324 (10×300 mm) equilibrated with 0.05% heptafluorobutyric acid (pH 2.5) containing 10% CH_3CN and, after sample injection, developed with a gradient of 10% to 40% CH_3CN at a flow rate of

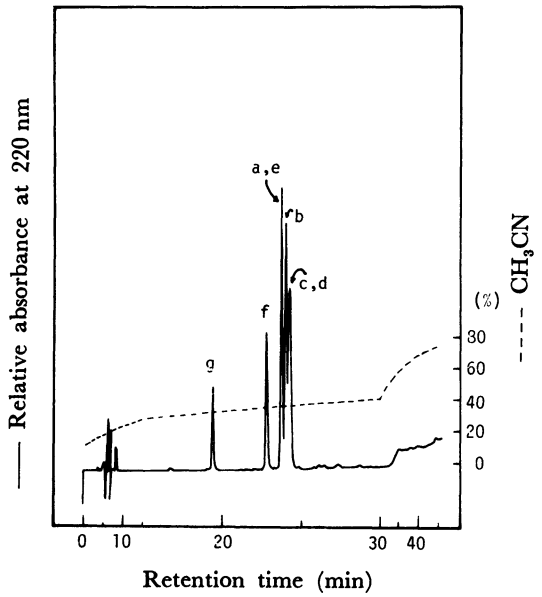

Fig. 5. HPLC profile of a mixture of synthetic ST_h [1-19] (a), ST_h[2-19] (b), ST_h[3-19] (c), ST_h [4-19] (d), ST_h[5-19] (e), and ST_h[6-19] (f) on a YMC-packed column ODS A-324 (5 μm, 10×300 mm). The starting solvent was 10% CH_3CN in 0.05% TFA (pH 2.35) and the flow rate was 3 ml/min. ST_h[6-18] (g) was added as an internal standard.

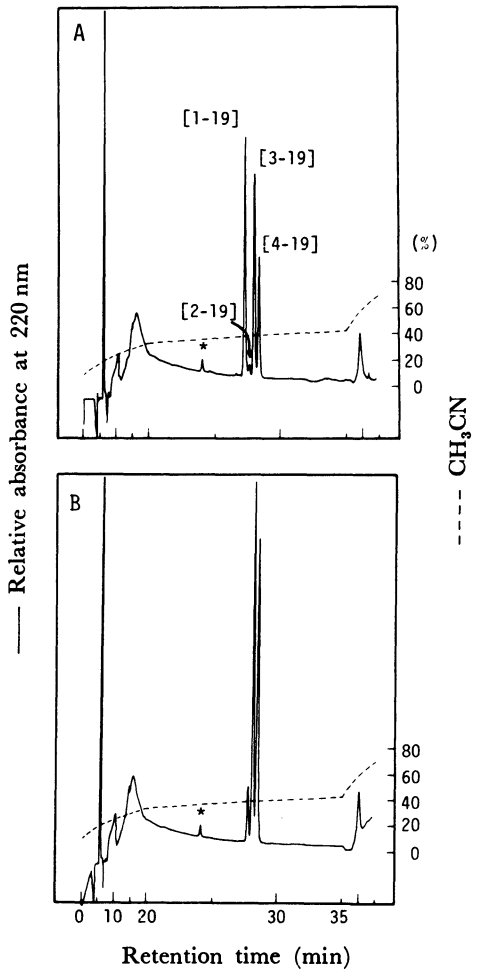

Fig. 6. HPLC profiles of digests of native ST_h (A) and synthetic ST_h[2-19] (B) with aminopeptidase M on a YMC-packed column ODS A-324 (5 μm, 10×300 mm). The starting solvent was 10% CH_3CN in 0.05 % heptafluorobutyric acid (pH 2.51) and the flow rate was 3 ml/min. ST_h[6-18] (peak denoted by*) was added to each digest as an internal standard.

TABLE 2. AMINO ACID COMPOSITIONS OF PEPTIDES OBTAINED BY ENZYMATIC AND EDMAN DEGRADATION OF NATIVE ST_h AND ITS SYNTHETIC ANALOGS

Starting peptide	Native ST_h		Synthetic ST_h[2-19]		Synthetic ST_h[4-19]	Synthetic ST_h[5-19]
Products	ST_h[3-19]	ST_h[4-19]	ST_h[3-19]	ST_h[4-19]	ST_h[5-19]	ST_h[6-19]
Asp	2.00(2)	2.01(2)	2.00(2)	1.98(2)	1.07(1)	1.08(1)
Thr	0.96(1)	1.00(1)	0.96(1)	1.00(1)	1.05(1)	1.03(1)
Ser	0.97(1)	—	0.96(1)	—	—	—
Glu	1.00(1)	1.04(1)	1.01(1)	1.00(1)	1.04(1)	1.02(1)
Pro	1.07(1)	1.11(1)	1.12(1)	1.02(1)	0.99(1)	1.11(1)
Gly	1.03(1)	1.04(1)	1.03(1)	1.04(1)	1.02(1)	1.00(1)
Ala	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)
1/2Cys	5.34(6)	5.36(6)	5.48(6)	5.38(6)	5.58(6)	5.21(6)
Leu	1.05(1)	1.06(1)	1.05(1)	1.06(1)	1.05(1)	1.06(1)
Try	2.00(2)	1.99(2)	2.01(2)	2.00(2)	2.04(2)	1.00(1)

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

3 ml/min; ii) The same column developed under the same conditions as for i) but with 0.05% TFA instead of 0.05% heptafluorobutyric acid as the aqueous phase. The elution profile of a mixture of shorter synthetic analogs by the latter procedure is shown in Fig. 5. With this procedure, synthetic ST_h[1-19] and ST_h[3-19] could not be completely separated from ST_h[5-19] and ST_h[4-19], respectively.

Aminopeptidase M Digestion of Native ST_h and Identification of the Resulting Peptides with Synthetic Analogs. To confirm that shorter synthetic analogs of ST_h had intramolecular disulfide bonds at the same positions as those in native ST_h, we treated native ST_h with aminopeptidase M, isolated the resulting peptides and compared them with the synthetic analogs of ST_h by HPLC. The HPLC profile of the digest of native ST_h showed two major and one minor peak with a peak of undigested peptide, as illustrated in Fig. 6A. The materials in these fractions were separated and submitted to measurement of their molecular weights by fast atom bombardment mass spectrometry (data not shown) and amino acid analysis (Table 2). The two major fractions were identified as those of ST_h[3-19] and ST_h[4-19], which lacked two and three N-terminal amino acid residues, respectively, of ST_h. The minor peak fraction eluted between undigested ST_h and ST_h[3-19] was identified as that of a peptide ST_h[2-19] lacking the N-terminal Asn residue of ST_h, by comparison of its HPLC profile with that of native ST_h digested with aminopeptidase M (Fig. 6B). The shorter ST_h peptides lacking one to three amino acid residues (ST_h[2-19], ST_h[3-19], and ST_h[4-19]) thus prepared by digestion of native ST_h with aminopeptidase M were each mixed with an equal amount of the corresponding synthetic peptide and chromatographed on a reversed-phase column, as shown in Figs. 7A—7C. Each gave a single peak. Thus, synthetic ST_h[2-19], ST_h[3-19], and ST_h[4-19] were confirmed to have disulfide linkages in the same positions as those in native ST_h. The digestion of native ST_h with aminopeptidase M stopped with release of the three N-terminal amino acid residues and did not proceed further even when the ratio of the enzyme to ST_h was raised to 1/10 (w/w). Therefore, Edman degradation was carried out for further removal of the fourth and fifth amino acid residues of ST_h from the N-terminus, as described below.

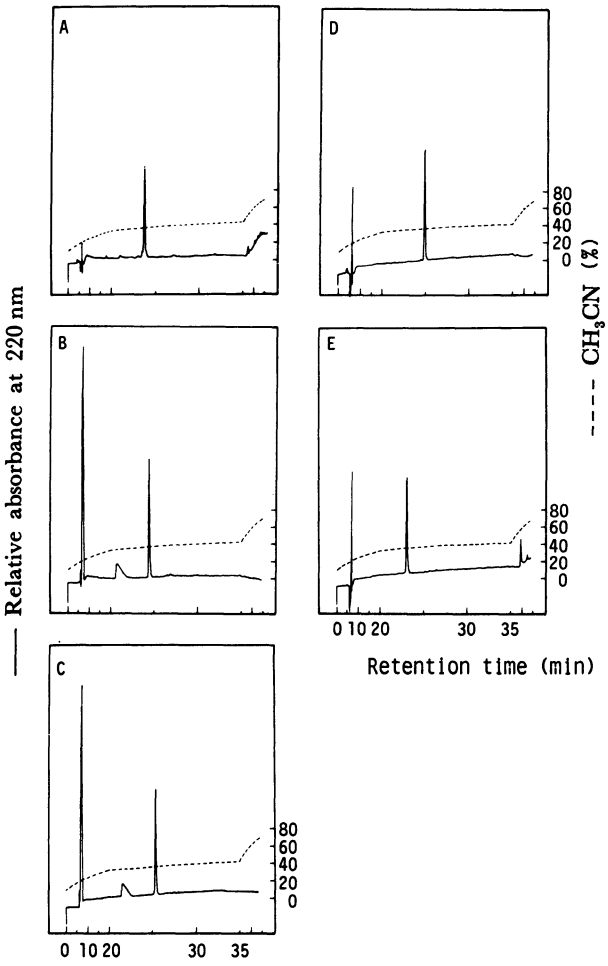

Fig. 7. HPLC profiles of mixtures of equal amounts of ST_h analogs on a YMC-packed column ODS A-324 (5 μm, 10×300 mm). The starting solvent was 10% CH_3CN in 0.05% TFA (pH 2.35), and the flow rate was 3 ml/min. (A) ST_h[2-19] enzymatically derived from native ST_h and synthetic ST_h[2-19]; (B) ST_h[3-19] enzymatically derived from native ST_h and synthetic ST_h[3-19]; (C) ST_h[4-19] enzymatically derived from native ST_h and synthetic ST_h[4-19]; (D) ST_h[5-19] prepared from synthetic ST_h[4-19] by Edman degradation and synthetic ST_h[5-19]; (E) ST_h[6-19] derived from synthetic ST_h[5-19] by Edman degradation and synthetic ST_h[6-19].

Edman Degradation and Comparison of the Degraded Peptides with Synthetic Peptides. Synthetic ST_h[4-19] was confirmed to have intramolecular disulfide linkages in the same positions as those in native ST_h, as described above. Then we subjected synthetic ST_h[4-19] to Edman degradation[14] and obtained ST_h[5-19]. This shortened peptide of ST_h was purified by HPLC and its amino acid composition was analyzed (Table 2). A mixture of equal amounts of this peptide and synthetic ST_h[5-19] gave a single peak on HPLC, as shown in Fig. 7D, indicating that synthetic ST_h[5-19] was linked intramolecularly by disulfide bonds at the same positions as those in ST_h[4-19]. Then we degraded synthetic ST_h[5-19] by a similar method to that used for ST_h[4-19] and purified the degraded peptide by the same procedure as used for degraded ST_h[4-19]. The amino acid composition of the purified peptide is shown in Table 2. When this peptide was mixed with synthetic ST_h[6-19] and subjected to HPLC a single peak was obtained, as shown in Fig. 7E. Thus synthetic ST_h[6-19] had intramolecular disulfide linkages at the same positions as those in ST_h[5-19], and synthetic ST_h[5-19] and ST_h[6-19] were both intramolecularly linked by disulfide bonds in the same way as native ST_h.

Biological Activity. The toxicities of shorter synthetic analogs of ST_h were assayed as described previously.[1] The minimum effective doses of shorter synthetic analogs of ST_h were 1.0±0.4 ng, which were the same as that of native or synthetic ST_h within the limits of experimental error, as shown in Table 3.

These values were also almost the same as those for the corresponding peptides derived from native ST_h by digestion with aminopeptidase M or by Edman degradation. Furthermore, the toxicities of all the shorter synthetic analogs of ST_h were neutralized by anti-native ST_h antiserum. These results indicate that the active site of the toxicity of ST_h is located in the sequence between the Cys residue near the N-terminus and the C-terminal Tyr.

Heat-stability. Synthetic ST_h was found to have the same stability as that of native ST_h on boiling in phosphate buffer, as described in the preceding paper.[12] We examined the heat-stability of shorter synthetic analogs of ST_h by the method as described previously.[15] Synthetic ST_h[5-19] lacking four N-terminal amino acid residues of ST_h showed the same heat-stability as that of synthetic ST_h, as illustrated in Fig. 8. Furthermore, the toxicity of ST_h[5-19] was found to decrease with the duration of heat-treatment. Shorter synthetic analogs of ST_h lacking one to three the N-terminal amino acid residues of ST_h gave similar results (data not shown). On the other hand, synthetic ST_h[6-19] lacking five N-terminal amino acid residues of ST_h showed higher heat-stability than synthetic or native ST_h, as reported in a previous paper.[15] These results suggest that the sequence consisting of 13 amino acid residues from the Cys residue near the N-terminus to the C-terminal Tyr residue is essential for the heat-stability of the toxin. The enhanced structural stability of ST_h[6-19] may be due to decreased perturbation of the molecule on heating as a result of loss of the five N-terminal amino acid residues.

TABLE 3. BIOLOGICAL PROPERTIES OF SYNTHETIC ST_h ANALOGS

	Minimum effective dose (ng/100 μl)[a]	Neutralization by anti-native ST_h antiserum
Native ST_h	1.0 ng (0.5 pmol)[b]	+
Synthetic ST_h	0.8 ng (0.4 pmol)[b]	+
Synthetic ST_h[2-19]	0.8 ng (0.4 pmol)	+
Synthetic ST_h[3-19]	1.3 ng (0.7 pmol)	+
Synthetic ST_h[4-19]	1.1 ng (0.7 pmol)	+
Synthetic ST_h[5-19]	0.8 ng (0.5 pmol)	+
Synthetic ST_h[6-19]	0.6 ng (0.4 pmol)	+

a) Calculated from the recovery of amino acids in acid hydrolysates. b) Cited from Ref. 12.

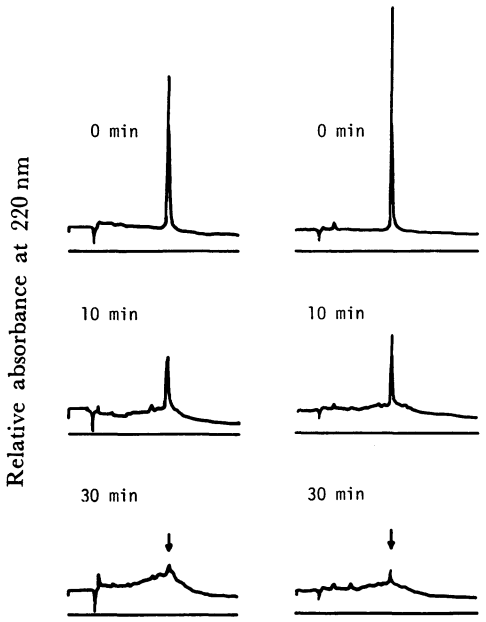

Fig. 8. HPLC profiles on a LiChrosorb RP-8 column (5 μm, 4×250 mm) of heat-treated synthetic ST_h(left column) and ST_h[5-19](right column).

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

References

1) Y. Takeda, T. Takeda, T. Yano, K. Yamamoto, and T. Miwatani, *Infect. Immun.*, **25**, 978 (1979).
2) S. -K. Chan and R. A. Giannella, *J. Biol. Chem.*, **256**, 7744 (1981).
3) S. Aimoto, T. Takao, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani, *Eur. J. Biochem.*, **129**, 257 (1982).
4) T. Takao, T. Hitouji, S. Aimoto, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani, *FEBS Lett.*, **152**, 1 (1983).
5) R. Lallier, F. Bernard, M. Gendreau, C. Lazure, N. G. Seidah, M. Chretien, and S. St-Pierre, *Anal. Biochem.*, **127**, 267 (1982).
6) C. Lazure, N. G. Seidah, M. Chretien, R. Lallier, and S. St-Pierre, *Can. J. Biochem. Cell Biol.*, **61**, 287 (1983).
7) B. Ronnberg, T. Wadstrom, and H. Jornvall, *FEBS Lett.*, **155**, 183 (1983).
8) M. Field, L. H. Graf, Jr., W. J. Laird, and P. L. Smith, *Proc. Natl. Acad. Sci, U.S.A*, **75**, 2800 (1978).
9) J. M. Hughes, F Murad, B. Chang, and R. L. Guerrant, *Nature*, **271**, 755 (1978).
10) P. M. Newsome, M. N. Burgess, and N. A. Mullan, *Infect. Immun.*, **22**, 290 (1978).
11) R. A. Giannella and K. W. Drake, *Infect. Immun.*, **24**, 19 (1979).
12) H. Ikemura, S. Yoshimura, S. Aimoto, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani, *Bull. Chem. Soc. Jpn.*, **57**, 2543 (1984).
13) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jpn.*, **40**, 2164 (1967).
14) P. Edman and A. Henschen, "Protein Sequence Determination" ed by S. B. Needleman, Springer-Verlag, Berlin, Heidelberg, New York, (1975) p. 232.
15) S. Aimoto, H. Watanabe, H. Ikemura, Y. Shimonishi, T. Takeda, Y. Takeda, and T. Miwatani, *Biochem. Biophys. Res. Commun.*, **112**, 320 (1983).