Structure-Activity Relationship of a Heat-Stable Enterotoxin Produced by *Yersinia enterocolitica*†

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Several shorter analogues of heat-stable enterotoxin (ST) produced by *Yersinia enterocolitica* were synthesized by a conventional method and their toxicities were examined by using suckling mice. The full enterotoxigenic activity of ST of *Y. enterocolitica* was found to be carried on a peptide, which consists of 13 amino acid residues from the 18th Cys to the C-terminal Cys and has a similar amino acid sequence to those of ST's produced by enterotoxigenic *Escherichia coli* (S. Aimoto et al., *Eur. J. Biochem.*, **129**, 257 (1982)) and *Vibrio cholerae* non-01 (T. Takao et al., *FEBS Lett.*, **193**, 250 (1985)). The results imply that the structural elements for expression of the toxicity are present in the common amino acid sequences of ST's of *Y. enterocolitica*, *E. coli*, and *V. cholerae* non-01.

Some strains of *Yersinia enterocolitica* produce a heat-stable enterotoxin that is responsible for gastroenteritis in children.[1,2] Recently, we isolated six molecular species of the enterotoxin (named *Yersinia*-ST) from the culture supernatant of the bacteria,[3,4] purified them to homogeneity by high-performance liquid chromatography (HPLC) and determined their amino acid sequences, as shown in Fig. 1. These peptides had a common sequence from Asp at position 15 from the N-terminus to the C-terminal residue, although their N-terminal sequences differed in length. This finding suggested that peptides 2—6 in Fig. 1 were not synthesized individually in the cells, but derived from peptide **1** by proteolytic cleavage in the cells or after excretion from the cells. The amino acid sequence of *Yersinia*-ST from the 18th amino acid residue from the N-terminus to the C-terminal residue was very similar to those of STI's of enterotoxigenic *Escherichia coli*.[5,6] However, in *Yersinia*-ST the C-terminal residue is Cys without the additional Tyr residue present in STI's of *E. coli*. Moreover, the N-terminal portion of the sequence of *Yersinia*-ST from the 17th residue is quite different from those of STI's of *E. coli*. The homology and differences in the primary structures of STI's of *E. coli* and *Yersinia*-ST suggest common biological properties of the two but differences in their degrees of activity and physicochemical properties. These observations raised the question of whether most, or all

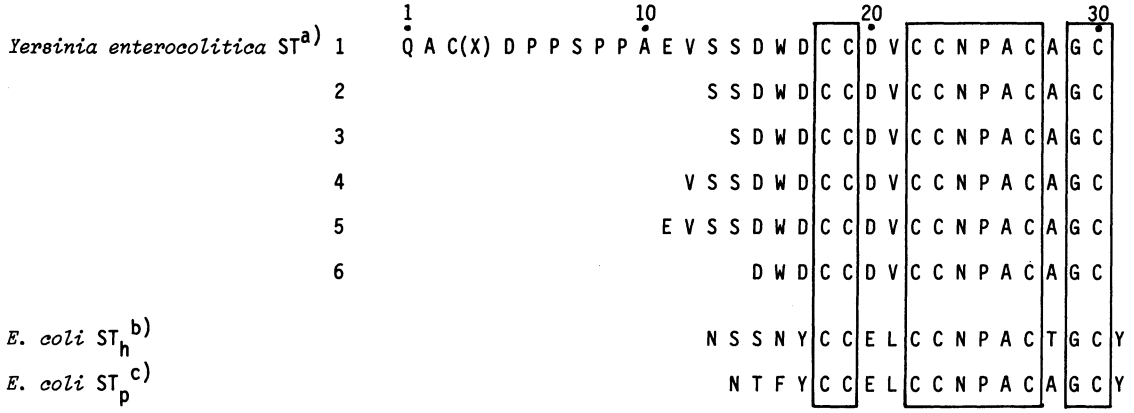

Fig. 1. Amino acid sequences of *Yersinia*-ST's and their comparison with those of ST_h and ST_p of enterotoxigenic *E. coli*. Amino acid residues in boxes are common to all sequences. a) Cited from Refs. 3 and 4. b) Cited from Ref. 5. c) Cited from Ref. 6. The single letter notations of amino acid residues are according to *J. Biol. Chem.*, **261**, 1 (1986).

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the toxicities of these ST's are due to the common sequence while the divergent sequences in the N-terminal and C-terminal regions merely represent the result of mutation, or whether these latter regions are responsible for the specific toxicities of the respective enterotoxins.

To elucidate the structure-activity relationship of *Yersinia*-ST, in this work we synthesized several shorter analogues of *Yersinia*-ST that had no N-terminal sequence or had an additional Tyr at the C-terminus, and examined their properties.

Experimental

The general and analytical methods used were as described previously.[7] All chemicals used for synthetic experiments were of reagent grade, while those used for analysis were of guaranteed grade and solvents were distilled before use. Carboxypeptidase B was obtained from Boehringer-Yamanouchi (Tokyo). A reversed-phase resin (YMC-ODS, S-5) was purchased from Yamamura Chemical Laboratories Co. (Kyoto) and packed in our laboratory into columns of 4×250 mm and 6×150 mm and 8×300 mm for analytical and preparative purposes. The abbreviations used in this paper are those recommended by the IUPAC-IUB [*J. Biol. Chem.*, **261**, 1 (1986)]. Additional abbreviations are: MBzl, *p*-methylbenzyl; TFA, trifluoroacetic acid; DMF, *N,N*-dimethylformamide; TEA, triethylamine; DMSO, dimethyl sulfoxide; HONSu, *N*-hydroxysuccinimide.

Peptide Synthesis. The protected linear peptides with the amino acid sequences of *Yersinia*-ST(16-30), *Yersinia*-ST(15-30), *Yersinia*-ST(14-30), Tyr[31]-*Yersinia*-ST(18-30), and Thr[28], Tyr[31]-*Yersinia*-ST(18-30) were synthesized according to the scheme shown in Fig. 2. Their experimental detail is described below.

Boc-Trp-Asp-Cys(MBzl)-Cys(MBzl)-Asp-Val-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-OBzl (1). Boc-Asp(OBu')-Cys(MBzl)-Cys(MBzl)-Asp-Val-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-OBzl[7] (0.42 g, 0.19 mmol) was dissolved in TFA (5 ml) and stirred at room temperature for 2 h. The solution was concentrated to a syrup, which was triturated in ether. The resulting powder was dissolved with Boc-Trp-ONSu (0.12 g, 0.28 mmol) in a mixture of DMF (1 ml) and 1-methyl-2-pyrrolidone (1 ml) and stirred at room temperature for 2 d. The solution was concentrated under reduced pressure to a solid, which was recrystallized from a mixture of EtOH and ether; 0.43 g (95.6%), mp 179—181 °C, $[\alpha]_D^{25}$ −38.0° (*c* 0.5, DMSO).

Found: C, 59.62; H, 6.41; N, 10.00; S, 8.00%. Calcd for $C_{119}H_{149}O_{23}N_{17}S_6 \cdot H_2O$: C, 59.66; H, 6.35; N, 9.94; S, 8.01%.

Z-Asp(OBu')-Trp-N_2H_3 (2). Z-Asp(OBu')-Trp-OMe[7] (2.62 g, 5.0 mmol) was dissolved in MeOH (100 ml) and mixed with 100% hydrazine hydrate (10 ml) in an ice-water bath. The mixture was stirred at room temperature for 2.5 h, concentrated under reduced pressure, and mixed with water. The resulting precipitate was recrystallized from a mixture of EtOH and ether; 1.8 g (68.7%), mp 195 °C (decomp), $[\alpha]_D^{20}$ −12.2° (*c* 1.0, DMF).

Found: C, 56.66; H, 6.73; N, 20.28%. Calcd for $C_{27}H_{33}O_6N_5 \cdot 3/2H_2O$: C, 56.72; H, 6.87; N, 19.60%.

Z-Asp(OBu')-Trp-Asp-Cys(MBzl)-Cys(MBzl)-Asp-Val-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-

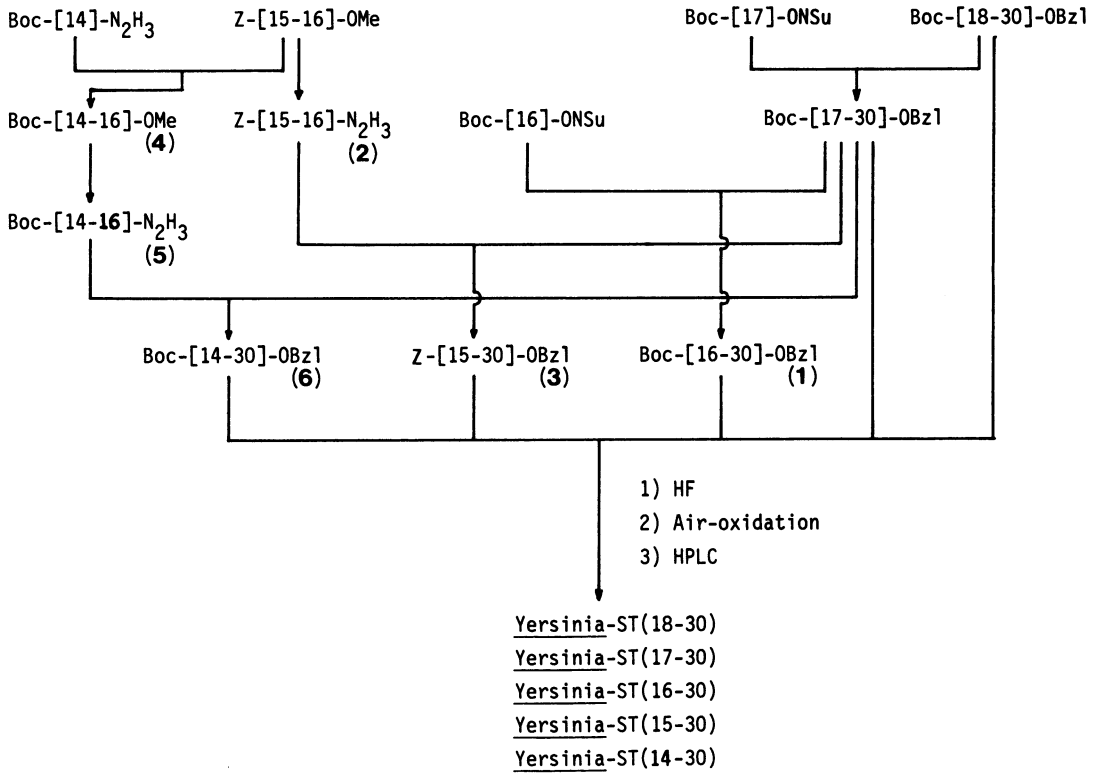

Fig. 2. Scheme for syntheses of shorter analogues of a heat-stable enterotoxin of *Y. enterocolitica*.

Ala-Gly-Cys(MBzl)-OBzl (3). Boc-Asp(OBu')-Cys(MBzl)-Cys(MBzl)-Asp-Val-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-OBzl[7] (1.12 g, 0.5 mmol) was dissolved in TFA (10 ml) and stirred at room temperature for 2.5 h. The residue was triturated in ether. Meanwhile, compound **2** (0.31 g, 0.6 mmol) was dissolved in DMF (4 ml), cooled below −20 °C, and treated with 6.72 M HCl (1 M=1 mol dm^{-3}) in dioxane (0.7 ml) and isopentyl nitrite (0.1 ml, 0.7 mmol) at −20 °C for 30 min. The solution was then mixed with the above powder and TEA (0.87 ml) and stirred at 0 °C for 1 d. The solid isolated from the reaction mixture was recrystallized from a mixture of EtOH, DMF, and ether; 1.1 g (85.3%), mp 194 °C (decomp), $[\alpha]_D^{25}$ −69.4° (*c* 0.5, DMSO).

Found: C, 59.35; H, 6.15; N, 10.09; S, 7.40%. Calcd for $C_{130}H_{160}O_{26}N_{18}S_6 \cdot 2H_2O$: C, 59.62; H, 6.31; N, 9.63; S, 7.33%.

Boc-Ser-Asp(OBu')-Trp-OMe (4). Z-Asp(OBu')-Trp-OMe[7] (3.93 g, 7.5 mmol) was dissolved in MeOH (150 ml) and hydrogenated at room temperature for 40 min over 5% palladium-charcoal in the presence of a solution of 6.72 M HCl in dioxane (1.2 ml). The catalyst was filtered off and the filtrate was concentrated to a syrup under reduced pressure. Meanwhile, Boc-Ser-N_2H_3 (1.98 g, 9.0 mmol) was dissolved in DMF (20 ml) and cooled below −20 °C. The chilled solution was treated with 6.72 M HCl in dioxane (3.5 ml) and isopentyl nitrite (1.4 ml, 11 mmol) at the same temperature for 25 min. The solution was mixed with a solution of the above syrup in DMF (15 ml) and TEA (4.34 ml) and stirred at 0 °C for 1 d. The precipitate was filtered off and the filtrate was concentrated under reduced pressure to a syrup, which was dissolved in ethyl acetate and washed with 0.2 M HCl, 5% aq $NaHCO_3$, and water. The washed solution was dried over anhydrous Na_2SO_4 and then concentrated to dryness; 4.32 g (100%).

Boc-Ser-Asp(OBu')-Trp-N_2H_3 (5). Compound **4** (4.32 g, 7.5 mmol) was dissolved in MeOH (150 ml) and mixed with 100% hydrazine hydrate (15 ml) under cooling. The solution was stirred at room temperature for 2 h and concentrated under reduced pressure to a syrup, which was crystallized from EtOH and ether; 3.4 g (79.1%), mp 200 °C (decomp), $[\alpha]_D^{20}$ −13.7° (*c* 1.0, DMF).

Found: C,53.16; H,7.64; N,18.99%. Calcd for $C_{27}H_{40}O_8N_6 \cdot N_2H_4 \cdot H_2O$: C, 53.27; H, 7.28; N, 18.41%.

Boc-Ser-Asp(OBu')-Trp-Asp-Cys(MBzl)-Cys(MBzl)-Asp-Val-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-OBzl (6). Boc-Asp(OBu')-Cys(MBzl)-Cys(MBzl)-Asp-Val-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-OBzl[7] (0.99 g, 0.44 mmol) was dissolved in TFA (10 ml) and stirred at room temperature for 2 h. The solution was concentrated under reduced pressure to a syrup, which was triturated in ether. Meanwhile, compound **5** (0.32 g, 0.55 mmol) was dissolved in DMF (5 ml) and mixed with 6.72 M HCl in dioxane (0.87 ml) and isopentyl nitrite (0.78 ml, 0.6 mmol) below −20 °C. The mixture was stirred at the same temperature for 30 min, mixed with a solution of the above powder in DMF (10 ml), and stirred at 0 °C for 1 d. The mixture was concentrated to dryness under reduced pressure. The residue was washed with water and then recrystallized from EtOH and ether: 1.0 g (86.2%), mp 185 °C (decomp), $[\alpha]_D^{25}$ −55.4° (*c* 0.35, DMSO).

Found: C, 57.36; H, 6.11; N, 10.57; S, 7.28%. Calcd for $C_{130}H_{167}O_{28}N_{19}S_6 \cdot 4H_2O$: C, 57.63; H, 6.51; N, 9.83; S, 7.09%.

Boc-Cys(MBzl)-Cys(MBzl)-Asp(OBu')-Val-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-Tyr-OBzl (7). Boc-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-Tyr-OBzl[8] (0.32 g, 0.2 mmol) was dissolved with anisole (0.07 ml) in TFA (2 ml) at 0 °C and stirred at room temperature for 50 min. The solution was concentrated under reduced pressure to a syrup, which was triturated in ether. Meanwhile, Boc-Cys(MBzl)-Cys(MBzl)-Asp(OBu')-Val-N_2H_3[7] (0.20 g, 0.25 mmol) was dissolved in DMF (2 ml) and cooled below −20 °C. The solution was treated with 6.72 M HCl in dioxane (0.14 ml) and isopentyl nitrite (0.04 ml) at −20 °C for 60 min. Then the mixture was mixed with *N*-methylmorpholine (0.2 ml) and a solution of the above powder in DMF (1 ml) and stirred at 0 °C for 6 d. The resulting precipitate was filtered off and the filtrate was concentrated under reduced pressure to a solid, which was washed with water and recrystallized from DMF and EtOH: 0.35 g (76.0%), mp 218—220 °C (decomp), $[\alpha]_D^{19}$ −55.6° (*c* 1.0, DMF).

Found: C, 61.03; H, 6.57; N, 9.27; S, 8.24%. Calcd for $C_{117}H_{151}O_{21}N_{15}S_6$: C, 61.20; H, 6.63; N, 9.15; S, 8.38%.

Boc-Cys(MBzl)-Cys(MBzl)-Asp(OBu')-Val-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Thr-Gly-Cys(MBzl)-Tyr-OBzl (8). Boc-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Thr-Gly-Cys(MBzl)-Tyr-OBzl[9] (1.64 g, 1.0 mmol) was treated with TFA (3.5 ml) and allowed to react with Boc-Cys(MBzl)-Cys(MBzl)-Asp(OBu')-Val-N_3, which was prepared from Boc-Cys(MBzl)-Cys(MBzl)-Asp(OBu')-Val-N_2H_3[7] (1.0 g, 1.2 mmol) according to the procedure described in **7**. The crude product was recrystallized from DMF and EtOH: 1.96 g (84.3%), mp 207.5—208.5 °C, $[\alpha]_D^{18}$ −46.5° (*c* 1.0, DMF).

Found: C, 60.76; H, 6.66; N, 8.89; S, 8.42%. Calcd for $C_{118}H_{153}O_{22}N_{15}S_6$: C, 60.93; H, 6.63; N, 9.03; S, 8.27%.

Removal of Protecting Groups and Air Oxidation. The protected peptides (Boc-Cys(MBzl)-Cys(MBzl)-Asp(OBu')-Val-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-OBzl,[7] Boc-Asp(OBu')-Cys(MBzl)-Cys(MBzl)-Asp-Val-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-OBzl,[7] and compounds **1**, **3**, **6**, **7**, and **8**) (25 μmol) were each treated with anhydrous liquid hydrogen fluoride[10] and air-oxidized by the procedure described in Ref. 8. The solutions containing the deprotected and air-oxidized peptides were lyophilized and purified by HPLC, as described below.

High-Performance Liquid Chromatography (HPLC). The HPLC column was equilibrated with 10% CH_3CN in 0.05% TFA or 0.01 M ammonium acetate (pH 5.7) and after injection of the sample solution, was developed with a linear gradient of 10—40% CH_3CN with increase in CH_3CN concentration of 1% min^{-1} at a flow rate of 1 ml min^{-1}. Eluates were monitored for absorbance at 220 and 280 nm using a double-wavelength flow-through spectrophotometer.

Carboxypeptidase Digestion. The sample peptide (ca, 60 μg) was dissolved in 0.01 M pyridine acetate (pH 7.0) (50 μl) and mixed with carboxypeptidase B at a substrate; enzyme ratio (w/w) of 36 : 1. The solution was kept for 17 h at 37 °C and then the digested peptide was separated by HPLC.

Amino Acid Analysis and Fast Atom Bombardment

(FAB) Mass Spectrometry. The amino acid compositions and molecular weights of purified peptides were determined as described in a previous paper.[4]

Biological Assay. Toxicity was assayed in suckling mice of 2—4 d old as described previously.[11]

Heat Stability. The purified peptide was dissolved at a concentration of 200 μg ml^{-1} in 0.01 M phosphate buffered saline (pH 7.2), sealed in a capillary tube and heated for a given period at 100 or 120 °C. Then the solution was cooled in an ice bath and analyzed by HPLC, as described above.

Results and Discussion

Chemical Synthesis. To elucidate the structure-activity relationship of *Yersinia*-ST, in this work we tried to synthesize shorter analogues of *Yersinia*-ST with progressively fewer N-terminal amino acid residues or an additional Tyr residue at the C-terminus or with a replacement by Thr at position 28. In previous work,[7] we synthesized *Yersinia*-ST(13-30) (peptide **2** in Fig. 1), which has the sequence from the 13th residue from the N-terminus to the C-terminal residue, and found that the peptide had the same biological properties as those of the corresponding native peptide. This indicated that the procedure for synthesis of *Yersinia*-ST(13-30) is suitable for the syntheses of *Yersinia*-ST peptides. Therefore, we used this procedure for the syntheses of protected linear peptides **1**, **3**, and **6** which had 15, 16, and 17, respectively, of the amino acid residues in the C-terminal portion of *Yersinia*-ST as shown in Fig. 2. All the protecting groups in these protected peptides **1**, **3**, and **6** were removed by their treatment with anhydrous liquid hydrogen fluoride. Boc-[18-30]-OBzl and Boc-[17-30]-OBzl in Fig. 2, which were intermediates in the syntheses of **1**, **3**, and **6** and had 13 and 14 of the amino acid residues in the C-terminal region of *Yersinia*-ST, were also deprotected by treatment with hydrogen fluoride. The resulting free linear peptides were spontaneously oxidized by air in dilute solution (5×10^{-5} M) until no free mercapto groups were detectable.[12] All the *Yersinia*-ST peptides thus synthesized except *Yersinia*-ST(14-30) were obtained as main products by HPLC after deprotection and air-oxidation, suggesting that peptides with the same disulfide linkages as those

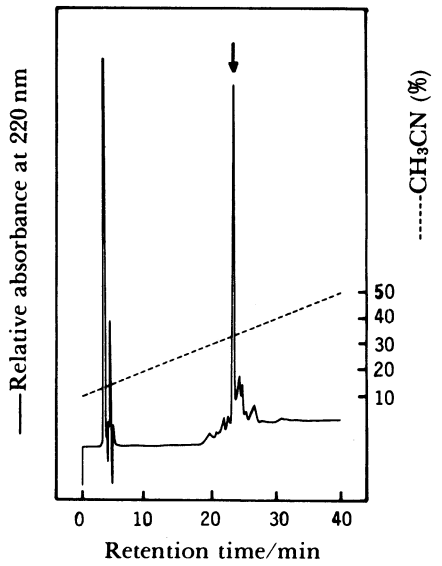

Fig. 3. HPLC profiles on a reversed-phase column (YMC-ODS S-5, 8×300 mm) of a deprotected and air-oxidized solution of compound **1**. The peak fraction marked by an arrow was separated as *Yersinia*-ST(16-30).

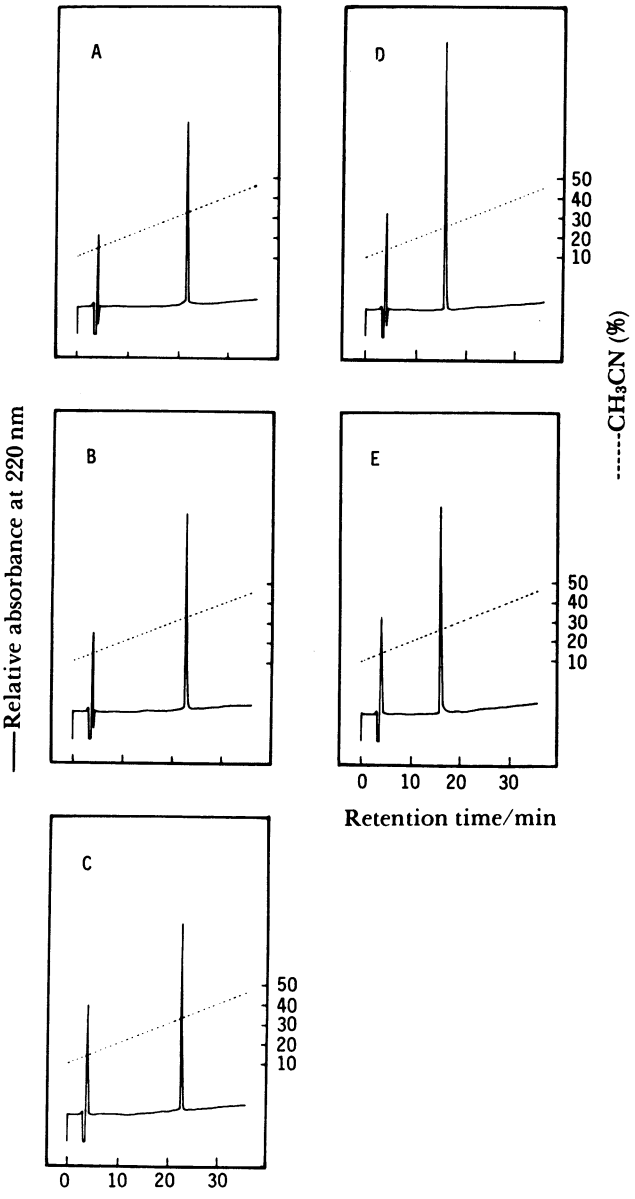

Fig. 4. HPLC profiles of purified synthetic *Yersinia*-ST peptides on a reversed-phase column (YMC-ODS S-5, 6×150 mm): A) *Yersinia*-ST(14-30); B) *Yersinia*-ST(15-30); C) *Yersinia*-ST(16-30); D) *Yersinia*-ST-(17-30); E) *Yersinia*-ST(18-30).

of native *Yersinia*-ST are thermodynamically most stable, as described below. Typical examples of the HPLC profiles of the air-oxidized products are shown in Fig. 3. *Yersinia*-ST(14-30) could be recovered by HPLC only as a minor peak, and it was very difficult to identify its peak on HPLC. Therefore, a deprotected and air-oxidized solution of synthetic *Yersinia*-ST(14-30) was coeluted with the corresponding native peptide (peptide **3** in Fig. 1), and in this way synthetic *Yersinia*-ST(14-30) was isolated. The low yield of *Yersinia*-ST(14-30) after deprotection and air-oxidation is probably due to various side-reactions in the deprotection, because the air-oxidized solution of *Yersinia*-ST(14-30) showed various unidentified peaks on an HPLC (not shown). The HPLC profiles of the purified synthetic peptides are depicted in Fig. 4.

The protected peptides with an additional Tyr residue at the C-terminal position (**7** and **8**) were also synthesized by the same way as that shown in Fig. 2. After treatment of **7** and **8** with hydrogen fluoride, the deprotected peptides were air-oxidized and purified by HPLC, to give Tyr^{31}-*Yersinia*-ST(18-30) and Thr^{28}, Tyr^{31}-*Yersinia*-ST(18-30), respectively (Fig. 5). Thr^{28}-*Yersinia*-ST(18-30) was prepared by digestion of Thr^{28}, Tyr^{31}-*Yersinia*-ST(18-30) with carboxypeptidase B. The amino acid compositions, mass values, and yields of the purified synthetic peptides are summarized in Table 1.

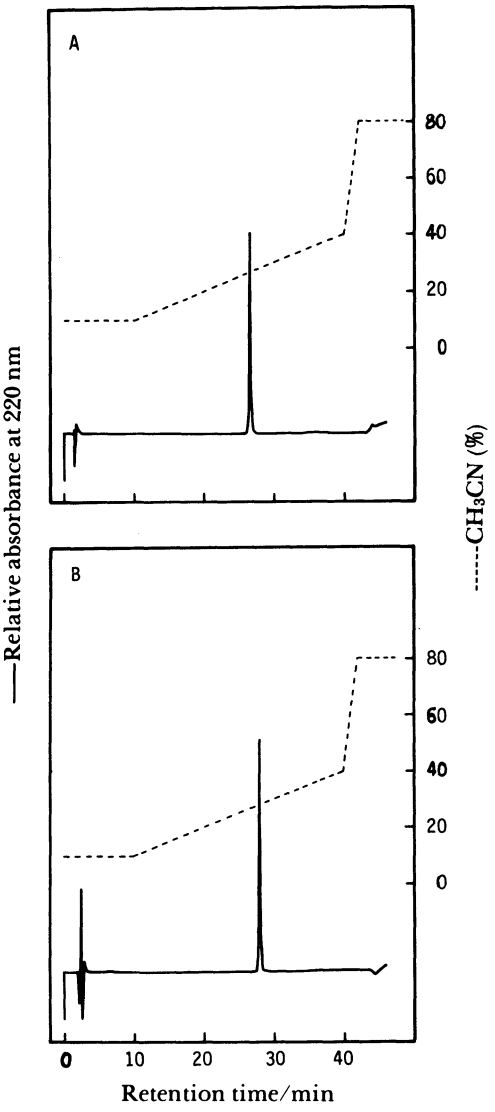

Fig. 5. HPLC profiles of A) purified Tyr^{31}-*Yersinia*-ST(18-30) and B) purified Thr^{28}, Tyr^{31}-*Yersinia*-ST-(18-30) on a reversed-phase column (YMC-ODS S-5, 4×250 mm).

Identification of Synthetic *Yersinia*-ST Peptides with Native Peptides. In a previous paper,[7] we presented evidence that synthetic *Yersinia*-ST(13-30) had the same structure as native peptide **2** in Fig. 1. To confirm that the *Yersinia*-ST peptides synthesized in this work had the same structure as native *Yersinia*-ST, we compared their retention times on HPLC with those of the corresponding native *Yersinia*-ST peptides. One-step Edman degradation of synthetic *Yersinia*-ST(13-30), which had the same structure as native *Yersinia*-ST(13-30),[7] gave a peptide with the same retention time on HPLC as synthetic *Yersinia*-ST(14-30). Synthetic *Yersinia*-ST(15-30) had the same retention time on HPLC as not only native peptide **3** in Fig. 1 but also *Yersinia*-ST(15-30), which was derived from synthetic *Yersinia*-ST(13-30) by digestion with aminopeptidase M. On HPLC synthetic *Yersinia*-ST(16-30) was eluted at the same position as preparations of *Yersinia*-ST(16-30) obtained both from synthetic *Yersinia*-ST(15-30) by the Edman reaction and from synthetic *Yersinia*-ST(17-30) by coupling with Boc-Trp-ONSu. Synthetic *Yersinia*-ST (18-30) was identical on HPLC with synthetic *Yersinia*-ST(17-30) degraded by the Edman reaction. Furthermore, digestions with carboxypeptidase B of synthetic Tyr^{31}-*Yersinia*-ST(18-30) and Thr^{28}, Tyr^{31}-*Yersinia*-ST(18-30), which were prepared from compounds **7** and **8**, gave *Yersinia*-ST(18-30) and Thr^{28}-*Yersinia*-ST(18-30), respectively. The former peptide was identical on HPLC with synthetic *Yersinia*-ST(18-30), which was obtained directly from Boc-[18-30]-OBzl, indicating that Tyr^{31}-*Yersinia*-ST(18-30) had the same structure as *Yersinia*-ST(18-30). A typical example of the correlation of the HPLC profiles of *Yersinia*-ST peptides with each other is shown in Fig. 6. These results demonstrate clearly that the synthetic *Yersinia*-ST peptides had the same structure as the native *Yersinia*-ST peptides. The correlations between these synthetic and native *Yersinia*-ST peptides are summarized in Fig. 7.

Biological Activity. The toxic activities of *Yersinia*-ST peptides synthesized in this paper were examined by the fluid accumulation test in suckling mice.[11] The minimum effective doses of *Yersinia*-ST(14-30), *Yersinia*-ST(15-30), *Yersinia*-ST(16-30), *Yersinia*-ST(17-30), and *Yersinia*-ST(18-30) were all be-

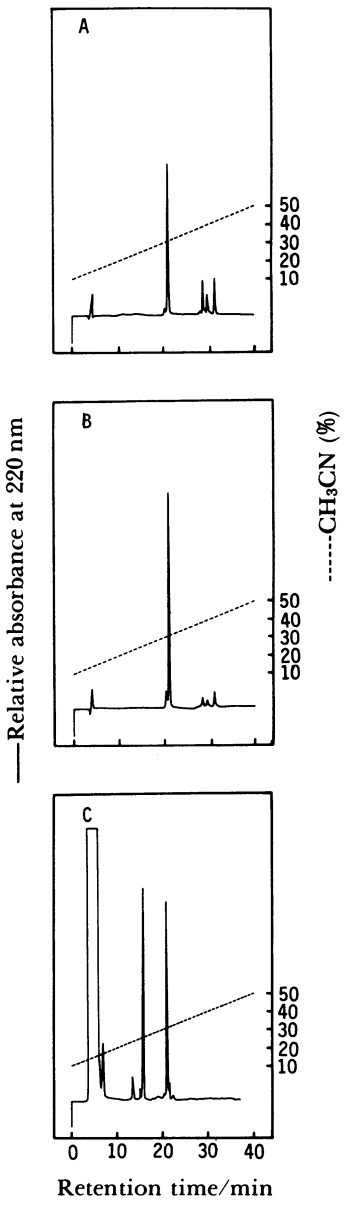

Fig. 6. HPLC profiles on a reversed-phase column (YMC-ODS S-5, 6×150 mm): A) synthetic *Yersinia*-ST(15-30) after one-step Edman degradation, B) a mixture of A) and synthetic *Yersinia*-ST(16-30), C) synthetic *Yersinia*-ST(17-30) after coupling with Boc-Trp-ONSu and removal of the Boc group.

tween 12.5 and 25 ng, as shown in Table 1, indicating that the N-terminal amino acid sequence from the 17th amino acid residue is not concerned in expression of the toxicities of *Yersinia*-ST. Namely, the enterotoxigenic activity of *Yersinia*-ST is carried on *Yersinia*-ST(18-30), which consists of 13 amino acid residues from the 18th Cys to the C-terminal Cys. This observation suggests that the divergency of the amino acid sequences in the N-terminal regions of ST's of *Y. enterocolitica* and *E. coli* is not related to their toxicities, but that the structural elements for expression of their toxicities are present in the commom amino acid sequences of ST's of *Y. enterocolitica* and *E. coli*.

Previously, we synthesized various analogues of ST's of enterotoxigenic *E. coli* and found that the minimum effective doses of their 13 amino acid peptides, which correspond to the sequence of *Yersinia*-ST from Cys at position 18 to the C-terminus, were 0.6 ng.[13] The activity of *Yersinia*-ST(18-30) was one-twentieth to one-thirtieth of those of *E. coli* peptides. The lower toxicity of *Yersinia*-ST(18-30) is considered to be due to the differences of amino acid residues in the common sequences of ST's of *Y. enterocolitica* and *E. coli*, that are at positions 20, 21, and 28 of *Yersinia*-ST(18-30).

The amino acid residue at position 28 is Ala in *Yersinia*-ST, but Thr in ST_h of *E. coli*, as shown in Fig. 1. To elucidate the effect of this amino acid residue on the biological activity of *Yersinia*-ST, we examined the activity of an analogue of *Yersinia*-ST(18-30), Thr^{28}-*Yersinia*-ST(18-30), in which Ala at position 28 is replaced by Thr. As shown in Table 1, Thr^{28}-*Yersinia*-ST(18-30) showed almost the same activity as *Yersinia*-ST(18-30). This result indicated that the toxicity of *Yersinia*-ST is not influenced by replacement of the amino acid residue at position 28 of *Yersinia*-ST by Thr.

The C-terminal amino acid residue, which is Tyr in ST's of *E. coli*, is not related to their toxicities.[13] Since this amino acid residue does not exist in *Yersinia*-ST, it was supposed that the addition of this residue to the C-terminus of *Yersinia*-ST would not effect the toxicity of *Yersinia*-ST. However, we synthesized two analogues of *Yersinia*-ST(18-30) with an additional Tyr at the C-terminus, Tyr^{31}-*Yersinia*-ST(18-30) and Thr^{28}, Tyr^{31}-*Yersinia*-ST(18-30), and found that these peptides had twice and four times the activity of *Yersinia*-ST(18-30), respectively. A monoclonal antibody (ST_h 8G7) raised against native ST_h of *E. coli* recognizes an epitope including the C-terminal Tyr residue of ST_h of *E. coli*, but does not react with synthetic analogues of ST_h lacking its C-terminal Tyr residue.[14,15] Synthetic *Yersinia*-ST peptides do not react with this monoclonal antibody over the range of their concentrations from 0.5 to 0.03 $\mu g\ ml^{-1}$ in an enzyme-linked immunosorbent assay system. However, Tyr^{31}-*Yersinia*-ST(18-30) and Thr^{28}, Tyr^{31}-*Yersinia*-ST(18-30) bind with this antibody. These results suggest that the addition of a Tyr residue to the C-terminus of *Yersinia*-ST(18-30) makes the tertiary structure around the Tyr residue in Tyr^{31}-*Yersinia*-ST(18-30) and Thr^{28}, Tyr^{31}-*Yersinia*-ST(18-30) somewhat similar to that of ST_h of *E. coli*, with resulting increase in toxicity.

The results discussed above imply that the lower toxicity of *Yersinia*-ST(18-30) than of ST_h(6-18) of *E. coli*, which consists of the sequence from position 6 to 18 in ST_h of *E. coli*, is mainly due to the difference in the amino acid residues at positions 20 and 21 of *Yersinia*-ST. In fact, a preliminary experiment showed

Table 1. Amino Acid Compositions[a], Mass Values[b], Yields, and Biological Activities[c] of Synthetic Peptides

	Yersinia-ST (14-30)	*Yersinia*-ST (15-30)	*Yersinia*-ST (16-30)	*Yersinia*-ST (17-30)	*Yersinia*-ST (18-30)	Tyr^{31}-*Yersinia*-ST (18-30)	Thr^{28}, Tyr^{31}-*Yersinia*-ST (18-30)	Thr^{28}-*Yersinia*-ST (18-30)
Asp	3.89(4)	3.84(4)	2.97(3)	3.14(3)	2.10(2)	1.99(2)	2.00(2)	1.97(2)
Thr							1.02(1)	0.97(1)
Ser	1.00(1)							
Pro	1.16(1)	0.99(1)	1.05(1)	1.18(1)	0.99(1)	1.22(1)	1.12(1)	0.96(1)
Gly	1.04(1)	1.05(1)	1.02(1)	0.91(1)	1.03(1)	1.01(1)	1.04(1)	1.05(1)
Ala	2.00(2)	2.00(2)	2.00(2)	2.00(2)	2.00(2)	2.00(2)	1.00(1)	1.00(1)
Cys	4.63(6)	4.34(6)	4.42(6)	4.56(6)	4.72(6)	4.83(6)	4.66(6)	4.54(6)
Val	0.73(1)	0.69(1)	0.71(1)	0.74(1)	0.67(1)	0.87(1)	0.67(1)	0.70(1)
Trp	0.83(1)	0.86(1)	0.94(1)					
Tyr						1.00(1)	0.98(1)	
$[M+H]^+$	1758.1 (1758.5)	1671.2 (1671.4)	1556.2 (1556.4)	1370.2 (1370.3)	1255.3 (1255.3)	1418.3 (1418.4)	1447.9 (1448.4)	1285.0 (1285.3)
Yield/%	0.5	8.3	6.5	8.0	11.8	10.3	14.2	85.8
MED/ng	25	25	25	12.5	12.5	6.25	3.12	12.5

a) Values were calculated as mol/mol of Ala; numbers in parentheses indicate theoretical values. b) $[M+H]^+$, mass value of quasi-molecular ion. c) MED, minimum effective dose.

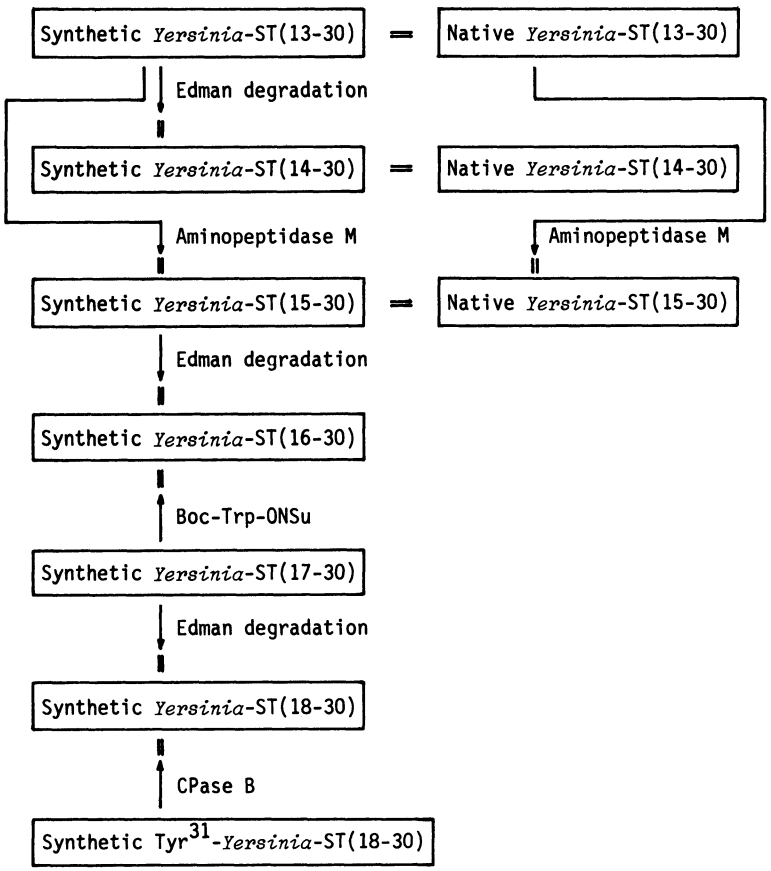

Fig. 7. Structural relationships of synthetic and native *Yersinia*-ST peptides.→=denotes that for example the reaction product of synthetic *Yersinia*-ST(13-30) obtained by Edman degradation is identical as judged by HPLC with synthetic *Yersinia*-ST(14-30).

that substitution of the amino acid residues at positions 20 and 21 in *Yersinia*-ST(18-30) by other amino acid residues influenced the toxicity, as will be reported elsewhere.

Heat Stability. Previously, a crude preparation of *Yersinia*-ST was found to be more heat-stable than a preparation of ST of *E. coli*.[16] In this work, to obtain detailed information about the heat-stability of *Yersinia*-ST peptides, we examined the heat-stabilities of synthetic shorter analogues. Figure 8 shows the HPLC profiles of synthetic *Yersinia*-ST(18-30) after its treatment at 100 and 120 °C for various times. After treatment at 100 °C for 30 min, the peak area of synthetic *Yersinia*-ST(18-30) on HPLC was reduced to about three-quarters of that of the untreated peptide. Under the same conditions the peak area of ST_h of *E. coli* is decreased drastically.[17] Furthermore, after heating at 100 °C for 60 min or at 120 °C for 10 min about two-thirds of the synthetic peptide remained unchanged, and on heating at 120 °C for 60 min the peptide was almost completely destroyed. This heat-stability of *Yersinia*-ST(18-30) was similar to that of synthetic *Yersinia*-ST(13-30) reported previously,[7] indicating that the N-terminal region from position 17 of *Yersinia*-ST does not affect its heat-stability.

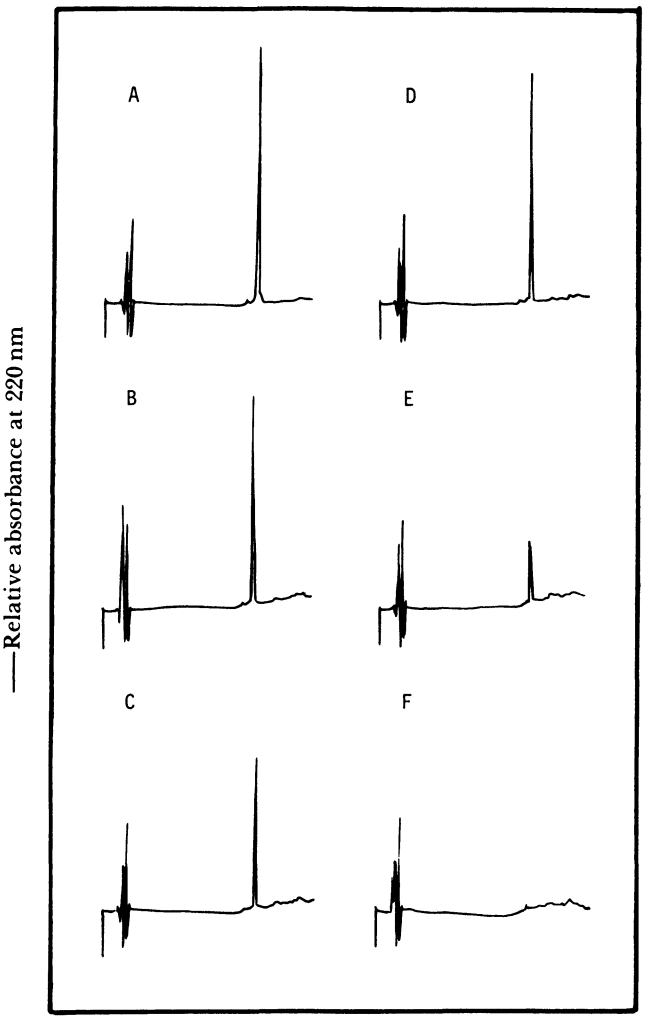

Fig. 8. HPLC profiles on a reversed-phase column (YMC-ODS S-5, 6×150 mm) of synthetic *Yersinia*-ST (18-30) treated under the following conditions: A) at 100 °C for 10 min, B) at 100 °C for 30 min, C) at 100 °C for 60 min, D) at 120 °C for 10 min, E) at 120 °C for 30 min, and F) at 120 °C for 60 min.

Recently, we reported that ST_h(6-19) of *E. coli*, which lacks the five N-terminal amino acid residues of ST_h of *E. coli*, has higher heat-stability than ST_h of *E. coli* with the whole amino acid sequences or ST_h(5-19) of *E. coli*, which lacks the four N-terminal amino acid residues of ST_h of *E. coli*.[17] These results imply that removal of the amino acid residues located at the N-terminal region from Tyr at position 5 results in increase of heat-stability of ST_h of *E. coli*. Moreover, the C-terminal Tyr residue is not related to the heat-stability of ST_h of *E. coli*, because ST_h(6-18) of *E. coli*, which lacks the C-terminal Tyr of ST_h(6-19) of *E. coli*, showed almost the same heat-stability as the latter.

The heat-stabilities of synthetic *Yersinia*-ST(13-30)[7] and *Yersinia*-ST(18-30) were higher than that of ST_h of *E. coli* and comparable with that of synthetic ST_h(6-19) of *E. coli*. Thus the degrees of heat-stability of heat-stable enterotoxins, in other words, the effects of heating in perturbation of their molecules, are due to differences in their N-terminal amino acid sequences.

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