Chemical Synthesis of a Heat-stable Enterotoxin of *Yersinia enterocolitica*

Shoko YOSHIMURA and Yasutsugu SHIMONISHI*
Institute for Protein Research, Osaka University, Yamada-oka 3-2, Suita, Osaka 565
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A peptide with the amino acid sequence Ser-Ser-Asp-Trp-Asp-Cys-Cys-Asp-Val-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys of heat-stable enterotoxin (ST) produced by *Yersinia enterocolitica* was synthesized by a conventional method. The synthetic peptide was found to be identical with the corresponding natural peptide by chemical and physicochemical criteria, thus establishing the amino acid sequence of an ST of *Yersinia enterocolitica*.

Low-molecular-weight heat-stable enterotoxins (ST's) responsible for acute diarrhea in man and domestic animals are produced by several enteric bacteria such as enterotoxigenic *Escherichia coli* [1] and *Yersinia enterocolitica* [2,3]. The ST's isolated from these two kinds of bacteria have similar biological properties and their toxicities are neutralized by both homologous and heterologous anti-sera against these ST's [4]. These facts imply the similarity of at least the biologically and immunologically active structures of these ST's. Recently, we purified six molecular species of the enterotoxin to homogeneity from the culture supernatant of *Y. enterocolitica* by high-performance liquid chromatography and determined their amino acid sequences [5,6], as shown in Fig. 1. The sequence of the 13 amino acid residues in the C-terminal portion except for Asp and Val at positions 20 and 21, respectively, was identical with those of ST's produced by enterotoxigenic *E. coli* from the near-N-terminal Cys residue to the Cys residue at the 2nd position from the C-terminus, which were previously determined [7—10] and are shown in Fig. 1. These findings provided evidence that ST's of *Y. enterocolitica* and enterotoxigenic *E. coli* have common biological and immunological properties. However, the N-terminal sequences of the ST's isolated from these bacteria were quite different. These results raised the question of whether the divergence in the N-terminal portions reflects solely mutation in the transposon gene encoding ST [11] or a difference of the biological and physicochemical properties of the ST's. Therefore, it seemed interesting to examine the effect of the N-terminal sequence on various properties of these ST's.

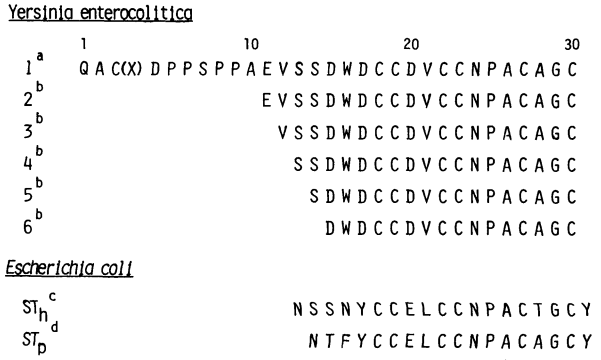

Fig. 1. Amino acid sequences of ST's of *Y. enterocolitica* and enterotoxigenic *E. coli*: a) cited from Ref. 5, b) from Ref. 6, c) from Ref. 7, and d) from Ref. 8—10.

In this work, we synthesized peptide 4 in Fig. 1, which consists of 18 amino acid residues from Ser at position 13 from the N-terminus to the C-terminal Cys, and compared the chemical and physicochemical properties of the synthetic peptide with those of natural peptide 4. This study also forms part of an investigation of the structure-activity relationship of heat-stable enterotoxins of *Y. enterocolitica* and enterotoxigenic *E. coli*, which will be reported in a subsequent paper [12].

Experimental

The general and analytical methods used were described in the preceding paper [13,14]. All chemicals used for preparative experiments were of reagent grade, those used for analysis were of guaranteed grade and solvents were distilled before use. The general procedure for purification of a product from a reaction mixture was as follows: The precipitate formed in the reaction mixture was filtered off and the filtrate was concentrated to a syrup or solid. The syrup or solid 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 dryness. In general, the concentration of solution was carried out under reduced pressure. Aminopeptidase M was purchased from the Protein Research Foundation (Minoh, Osaka). The HPLC apparatus consisted of a Hitachi 655 Liquid Chromatograph equipped with a multi-wave UV monitor, type 635M (Tokyo). YMC-ODS resin (S-5) was obtained from Yamamura Chemical Laboratories Co. (Kyoto) and was packed in our laboratory into columns of 4×250 mm and 8×300 mm for analytical and preparative experiments, respectively. 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.

*Boc-Gly-Cys(MBzl)-OBzl (**Ia**).* H-Cys(MBzl)-OBzl·TosOH (10.4 g, 33 mmol) was dissolved in CH_2Cl_2 (300 ml) and cooled to 0 °C. The chilled solution was mixed with TEA (4.6 ml, 33 mmol) and Boc-Gly-OH (5.3 g, 30 mmol) with

† 1 M = 1 mol dm^{-3}.

stirring. The solution was then mixed with DCC (6.8 g, 33 mmol) at below −20 °C and stirred at the same temperature for 1 h and at room temperature overnight. The syrup separated from the reaction mixture was crystallized from a mixture of $CHCl_3$, ether, and hexane, wt 9.6 g (67.3%), mp 55—56 °C, $[\alpha]_D^{20}$ −28.2° (*c* 1.0, DMF).

Found: C, 63.49; H, 7.11; N, 6.14; S, 6.91%. Calcd for $C_{25}H_{32}O_5N_2S$: C, 63.54; H, 6.83; N, 5.93; S, 6.77%.

*Boc-Cys(MBzl)-Ala-OMe (**Ib**).* Boc-Cys(MBzl)-OH·DCHA (15.2 g, 30 mmol) was dissolved with H-Ala-OMe·HCl (4.2 g, 30 mmol) in CH_2Cl_2 (150 ml), cooled below −20 °C and mixed with DCC (6.9 g, 33 mmol). The solution was stirred at below −20 °C for 1 h and at room temperature for 2.5 h. The syrup obtained from the reaction mixture was crystallized from ether and hexane; wt 10.3 g (83.7%), mp 87—88 °C, $[\alpha]_D^{20}$ −35.2° (*c* 1.0, DMF).

Found: C, 58.50; H, 7.49; N, 6.75; S, 7.68%. Calcd for $C_{20}H_{30}O_5N_2S$: C, 58.52; H, 7.37; N, 6.83; S, 7.80%.

*Boc-Cys(MBzl)-Ala-N_2H_3 (**Ic**).* Compound **Ib** (8.2 g, 20 mmol) was dissolved in MeOH (150 ml) and mixed with 100% hydrazine hydrate (30 ml). The solution was stirred at room temperature for 1 h and concentrated to a solid. The solid was recrystallized from MeOH and ether; wt 7.9 g (96.3%), mp 136—137 °C, $[\alpha]_D^{20}$ −14.0° (*c* 1.0, DMF).

Found: C, 55.42; H, 7.42; N, 13.60; S, 7.89%. Calcd for $C_{19}H_{30}N_4O_4S$: C, 55.60; H, 7.37; N, 13.65; S, 7.80%.

*Boc-Cys(MBzl)-Ala-Gly-Gys(MBzl)-OBzl (**I**).* Compound **Ia** (2.36 g, 5.0 mmol) was dissolved in TFA (10 ml) and stirred at room temperature for 30 min. The solution was concentrated to a syrup. Meanwhile, compound **Ic** (2.26 g, 5.5 mmol) was dissolved in DMF (20 ml) and cooled below −20 °C. The solution was stirred with 6.72 M HCl in dioxane (3.5 ml) and isopentyl nitrite (0.78 ml, 6 mmol) at the same temperature for 30 min, and then mixed with a solution of the above syrup in DMF (20 ml) and TEA (3.3 ml) and stirred at 0 °C for 1 h. The solid isolated from the reaction mixture was crystallized from a mixture of MeOH, ether, and hexane; wt 3.0 g (80.0%), mp 144—145 °C, $[\alpha]_D^{20}$ −20.3° (*c* 1.0, DMF).

Found : C, 62.08; H, 6.75; N, 7.40; S, 8.66%. Calcd for $C_{39}H_{50}O_7N_4S_2$: C, 62.38; H, 6.71; N, 7.46; S, 8.52%.

*Boc-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-OBzl (**II**).* Compound **I** (2.63 g, 3.5 mmol) was dissolved in TFA (10 ml) and stirred at room temperature for 30 min. The solution was concentrated to a syrup, which was triturated in ether. Meanwhile, Boc-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-N_2H_3 [14] (3.32 g, 4.0 mmol) was dissolved in DMF (15 ml) and cooled below −20 °C. The chilled solution was mixed with 6.72 M HCl in dioxane (2.6 ml) and isopentyl nitrite (0.59 ml, 4.5 mmol). The mixture was stirred at below −20 °C for 30 min, and then with a solution of the above solid in DMF (15 ml) and TEA (3 ml) at 0 °C for 4 d. The product separated from the reaction mixture was recrystallized from EtOH and ether; wt 5.0 g (98.5%), mp 179—180 °C, $[\alpha]_D^{20}$ −78.4° (*c* 1.0, DMF).

Found: C, 60.16; H, 6.65; N, 9.58; S, 8.78%. Calcd for $C_{73}H_{94}O_{13}N_{10}S_4$: C, 60.57; H, 6.55; N, 9.68; S, 8.84%.

*Boc-Cys(MBzl)-Cys(MBzl)-Asp(OBu^t)-Val-OMe (**IIIa**).* Z-Asp(OBu^t)-OH·DCHA (5.05 g, 10 mmol) and H-Val-OMe·HCl (1.68 g, 10 mmol) were dissolved in CH_2Cl_2 (50 ml) and cooled to −20 °C. The chilled solution was stirred with DCC (2.3 g, 11 mmol) at the same temperature for 1 h and at room temperature for 3 h. The syrup isolated from the reaction mixture was dissolved in MeOH (200 ml) and hydrogenated over 5% palladium-charcoal in the presence of 1 equiv of HCl at room temperature for 3 h. The catalyst was filtered off and the filtrate was concentrated to a syrup. Meanwhile, Boc-Cys(MBzl)-Cys(MBzl)-N_2H_3 [14] (6.0 g, 11 mmol) was dissolved in DMF (15 ml), cooled below −20 °C and stirred with 6.72 M HCl in dioxane (2.62 ml) and isopentyl nitrite (1.56 ml, 12 mmol) for 30 min. The solution was mixed with a solution of the above syrup in DMF (15 ml) and TEA (3.86 ml) and stirred at 0 °C for 3 d. The syrup isolated from the reaction mixture was solidified to an amorphous powder in ether and hexane; wt 8.16 g (100%).

*Boc-Cys(MBzl)-Cys(MBzl)-Asp(OBu^t)-Val-N_2H_3 (**III**).* Compound **IIIa** (8.16 g, 10 mmol) was dissolved in MeOH (200 ml) and cooled to 0 °C. The solution was mixed with 100% hydrazine hydrate, stirred at room temperature for 2 h and then concentrated to a solid. The solid was recrystallized from EtOH; wt 5.8 g (71.6%), mp 184—185 °C, $[\alpha]_D^{20}$ −35.4° (*c* 1.0, DMF).

Found: C, 57.82; H, 7.32; N, 10.57; S, 7.80%. Calcd for $C_{40}H_{60}O_8N_6S_2 \cdot 1/2H_2O$: C, 58.16; H, 7.44; N, 10.18; S, 7.75%.

*Boc-Cys(MBzl)-Cys(MBzl)-Asp(OBu^t)-Val-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-OBzl (**IV**).* Compound **II** (2.17 g, 1.5 mmol) was dissolved in TFA (10 ml) and stirred at room temperature for 30 min. The solution was concentrated to a syrup, which was solidified in ether. Meanwhile, compound **III** (1.47 g, 1.8 mmol) was dissolved in DMF (8 ml) and cooled below −20 °C. The solution was stirred with 6.72 M HCl in dioxane (1.4 ml) and isopentyl nitrite (0.26 ml, 2.0 mmol) at −20 °C for 30 min and then mixed with a solution of the above solid in DMSO (5 ml) and TEA (1.53 ml). The mixture was stirred at 0 °C for 5 d in a refrigerator and then concentrated to a syrup, which was triturated in ice water. The solid was recrystallized from DMF and EtOH; wt 2.7 g (83.1%), mp 222 °C (dec), $[\alpha]_D^{20}$ −64.6° (*c* 0.5, DMSO).

Found: C, 60.43; H, 6.81; N, 9.18; S, 9.06%. Calcd for $C_{108}H_{142}O_{19}N_{14}S_6$: C, 60.83; H, 6.71; N, 9.20; S, 9.00%.

*Boc-Asp(OBu^t)-Cys(MBzl)-Cys(MBzl)-Asp-Val-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-OBzl (**V**).* Compound **IV** (2.13 g, 1.0 mmol) was dissolved in TFA (10 ml), stirred at room temperature for 2 h and concentrated to a syrup, which was triturated in ether. The solid was dissolved in a mixture of DMF (5 ml) and DMSO (5 ml), cooled to 0 °C and mixted with TEA (0.28 ml) and a solution of Boc-Asp(OBu^t)-ONSu (0.53 g, 1.5 ml) in 1-methyl-2-pyridone (5 ml). The solution was stirred at room temperature for 1 d and concentrated to a semisolid, which was collected with AcOEt and ether. The solid was recrystallized from EtOH; wt 1.9 g (84.8%), mp 219—220 °C (dec), $[\alpha]_D^{20}$ −63.4° (*c* 0.5, DMSO).

Found: C, 59.57; H, 6.46; N, 9.27; S, 8.42%. Calcd for $C_{112}H_{147}O_{22}N_{15}S_6$: C, 59.85; H, 6.59; N, 9.35; S, 8.54%.

*Z-Asp(OBu^t)-Trp-OMe (**VIa**).* Z-Asp(OBu^t)-OH·DCHA (10.1 g, 20 mmol) and H-Trp-OMe·HCl (5.09 g, 20 mmol) were dissolved in CH_2Cl_2 (200 ml), cooled below −10 °C and mixed with a solution of DCC (4.54 g, 22 mmol) in CH_2Cl_2 (50 ml). The mixture was stirred at room temperature overnight. The resulting precipitate was filtered off and the filtrate was concentrated to a syrup. The syrup was purified as described above and triturated in hexane; wt 10.5 g (100%), mp 55—56 °C, $[\alpha]_D^{20}$ +2.8° (*c* 1.0, DMF).

Found: C, 64.04; H, 6.41; N, 7.95%. Calcd for $C_{28}H_{33}O_7N_3$:

C, 64.23; H, 6.35; N, 8.03%.

*Boc-Ser-Ser-OMe (**VIb**).* Boc-Ser-N_2H_3 (2.20 g, 10 mmol) was dissolved in DMF (30 ml), cooled below −20 °C and mixed with 6.72 M HCl in dioxane (5.24 ml) and isopentyl nitrite (1.43 ml, 11 mmol). The solution was stirred at −20 °C for 30 min, and then mixed with H-Ser-OMe·HCl (1.56 g, 10 mmol) and TEA (6.3 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 syrup. The syrup was washed with ether and hexane and crystalllized from a mixture of AcOEt, ether, and hexane; wt 3.0 g (98.0%), mp 62—64 °C.

Found: C, 46.92; H, 7.20; N, 9.34%. Calcd for $C_{12}H_{22}O_7N_2$: C, 47.05; H, 7.24; N, 9.15%.

*Boc-Ser-Ser-N_2H_3 (**VIc**).* Compound **VIb** (3.0 g, 9.8 mmol) was dissolved in MeOH (150 ml), cooled below 0 °C and mixed with 100% hydrazine hydrate (20 ml). The mixture was stirred at room temperature for 2 h and then concentrated to a solid. The solid was recrystallized from MeOH and ether; wt 2.9 g (94.8%), mp 146—147 °C, $[\alpha]_D^{20}$ +3.8° (*c* 1.0, DMF).

Found: C, 38.42; H, 7.61; N, 16.94%. Calcd for $C_{11}H_{12}O_6N_4 \cdot 2H_2O$: C, 38.59; H, 7.64; N, 16.37%.

*Boc-Ser-Ser-Asp(OBu^t)-Trp-OMe (**VId**).* Compound **VIa** (2.62 g, 5.0 mmol) was dissolved in MeOH (100 ml) and hydrogenated over 5% palladium-charcoal in the presence of 1 eq of HCl at room temperature for 1.5 h. The catalyst was filtered off and the filtrate was concentrated to a syrup. Meanwhile, compound **VIc** (1.84 g, 6.0 mmol) was dissolved in DMF (20 ml) and cooled below −20 °C. The chilled solution was stirred with 6.72 M HCl in dioxane (3.5 ml) and isopentyl nitrite (0.91 ml, 7.0 mmol) at the same temperature for 30 min, and then with a solution of the above syrup in DMF (20 ml) and TEA (4.0 ml) at 0 °C for 1 d. The resulting precipitate was filtered off and the filtrate was concentrated to a syrup. The syrup was purified as described above and triturated in ether and hexane; wt 2.45 g (73.8 %).

*Boc-Ser-Ser-Asp(OBu^t)-Trp-N_2H_3 (**VI**).* Compound **VId** (1.2 g, 1.8 mmol) was dissolved in MeOH (40 ml), cooled to 0 °C and mixed with 100% hydrazine hydrate (4 ml). The solution was stirred at room temperature for 2.5 h and concentrated to a syrup. The syrup was triturated in ether and reprecipitated from EtOH and ether; wt 0.99 g (82.5 %), mp 166—168 °C, $[\alpha]_D^{20}$ −13.4° (*c* 1.0, DMF).

Found: C, 50.31; H, 6.83; N, 17.81%. Calcd for $C_{30}H_{45}O_{10}N_7 \cdot N_2H_4 \cdot H_2O$: C, 50.48; H, 7.19; N, 17.67%.

*Boc-Ser-Ser-Asp(OBu^t)-Trp-Asp-Cys(MBzl)-Cys(MBzl)-Asp-Val-Cys(MBzl)-Cys(MBzl)-Asn-Pro-Ala-Cys(MBzl)-Ala-Gly-Cys(MBzl)-OBzl (**VII**).* Compound **V** (1.12 g, 0.5 mmol) was dissolved in TFA (10 ml) and stirred at room temperature for 2 h. The solution was concentrated to a syrup, which was solidified in ether. Meanwhile, compound **VI** (0.40 g, 0.6 mmol) was dissolved in DMF (4 ml) and cooled below −20 °C. The solution was mixed with 6.72 M HCl in dioxane (0.7 ml) and isopentyl nitrite (0.1 ml, 0.7 mmol), stirred at the same temperature for 30 min, and mixed with a solution of the above solid in 1-methyl-2-pyridone (6 ml) and TEA (0.87 ml). The mixture was stirred at 0 °C for 5 d in a refrigerator. The resulting precipitate was filtered off and the filtrate was concentrated to a solid, which was recrystallized from DMF and EtOH; wt 0.68 g (50.0%), mp 207—208 °C, $[\alpha]_D^{20}$ −62.0° (*c* 0.35, DMSO). Amino acid ratio in the acid hydrolysate: Asp, 3.45 (3); Ser, 1.42 (2); Pro, 0.94 (1); Gly, 1.02 (1); Ala, 2.00 (2); Cys, not determined; Val, 0.66 (1); Trp, 0.58 (1).

Found: C, 57.64; H, 6.10; N, 10.22; S, 7.23%. Calcd for $C_{133}H_{172}O_{30}N_{20}S_6 \cdot 2H_2O$: C, 57.90; H, 6.32; N, 10.15; S, 6.96%.

Removal of Protecting Groups and Air Oxidation. Compound **VII** (68 mg, 25 μmol) was mixed with anisole (0.2 ml) and treated with anhydrous liquid hydrogen fluoride (4 ml) at 0 °C for 1 h [15]. The hydrogen fluoride was removed by evaporation under reduced pressure and the residue was dissolved in 99% formic acid (1.5 ml). The solution was washed twice with hexane and diluted with water to 500 ml. The solution was adjusted to pH 8.0 by adding aqueous ammonia and stood at room temperature for 3 d with occasional stirring. Then it was adjusted to pH *ca.* 7.0 with acetic acid and lyophilized. The residue was purified by high-performance liquid chromatography, as described below.

High-performance Liquid Chromatography (HPLC). The HPLC column was equilibrated with 10% acetonitrile in 0.05% trifluoroacetic acid and after injection of the sample, was developed with a linear-gradient of 10—40% acetonitrile with increase in acetonitrile concentration of 1%/min at a flow rate of 1 ml min^{-1}. Eluates were monitored simultaneously for absorbance at 220 and 280 nm using a double-wavelength flow-through spectrophotometer.

Aminopeptidase M Digestion. Synthetic or natural peptide 4 (60 μg) was dissolved in 0.01 M pyridinium acetate buffer (pH 7.0) (100 μl) and digested with aminopeptidase M (5 μg) at 38 °C for 20 h. The reaction was stopped by addition of acetic acid. The solution was applied to a reversed-phase column of HPLC and the adsorbed material was eluted as described above.

Edman-degradation. Synthetic peptide was degraded by the Edman method, as described previously [16]. The degraded product was analyzed by HPLC, as described above.

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

Results and Discussion

Synthesis. The protected linear peptide **VII** consisting of 18 amino acid residues from Ser at position 13 from the N-terminus to the C-terminal Cys was synthesized by a similar procedure to that used for the synthesis of a heat-stable enterotoxin of enterotoxigenic *E. coli* [13], as shown in Fig. 2. All protecting groups were removed with anhydrous liquid hydrogen fluoride [15], and the resulting free linear peptide was air-oxidized in a dilute solution (5×10^{-5} M) until no free mercapto groups could be detected. The air-oxidized product had the HPLC profile shown in Fig. 3A on a reversed-phase column. The fraction shown by a vertical bar was most toxic and was eluted at the same retention time as natural peptide 4, as shown in Fig. 3B. The separated fraction was confirmed to be pure by repeated HPLC, as shown in Fig. 3C. The yield of the purified peptide was about 2.8% on the basis of the protected peptide **VII**. This low yield was considered to be due to various side-reactions

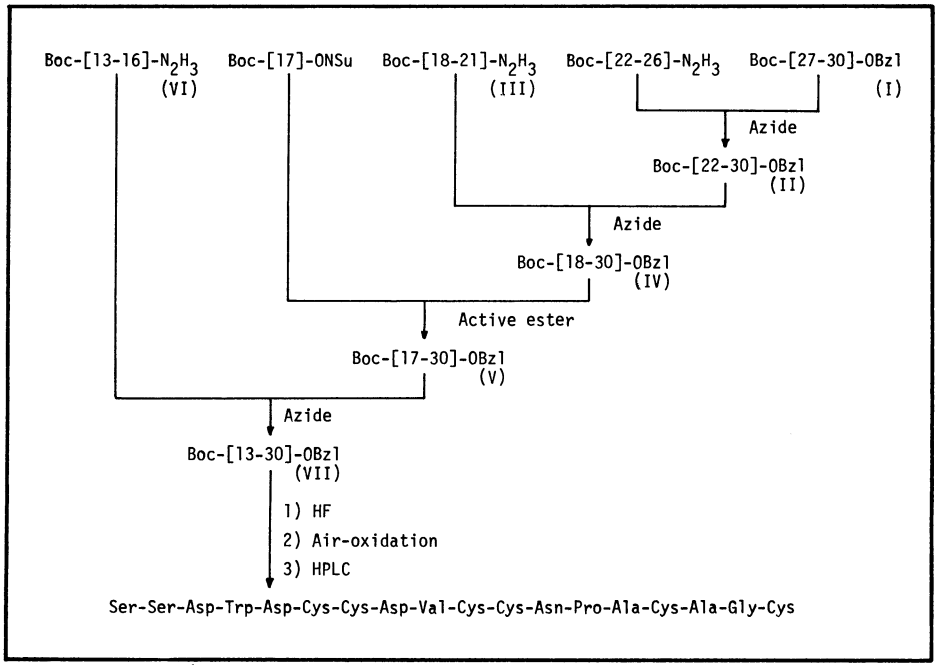

Fig. 2. Scheme for synthesis of a heat-stable enterotoxin of *Y. enterocolitica*.

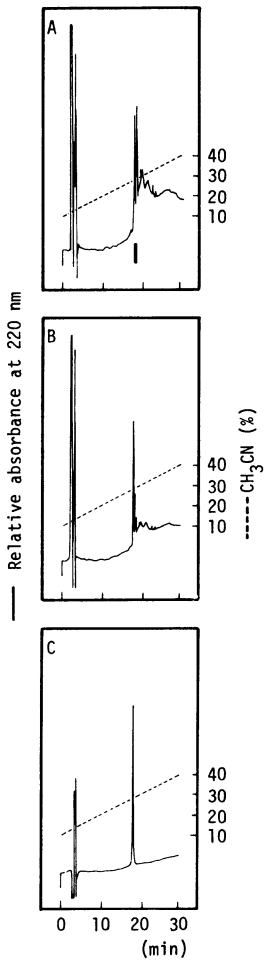

Fig. 3. HPLC profiles on a reversed-phase column (YMC-ODS S-5, 4×250 mm) of A) a deprotected, air-oxidized solution of synthetic peptide, B) a mixture of A) and natural peptide 4, and C) purified synthetic peptide.

during deprotection and air oxidation of the protected peptide **VII**, as shown in Fig. 3A. The amino acid composition and mass value of the purified synthetic peptide were identical with those of natural peptide 4, as shown in Table 1. The toxicity of this synthetic peptide was in the same order as that of natural peptide 4 in a fluid accumulation test in suckling mice [17]. The biological and immunological properties of the synthetic peptide and its shorter peptides will be described elsewhere [12].

Identification of the Synthetic Peptide with the Natural Peptide. To confirm that the synthetic peptide had the same structure as the natural peptide 4, we treated both with aminopeptidase M and compared the digests by HPLC, as shown in Fig. 4. The digest of the synthetic peptide was eluted later than that of the original peptide (Fig. 4B), but had the same retention time as natural peptide 6 lacking the two N-terminal amino acid residues of natural peptide 4 (Fig. 4C). The digest of natural peptide 4 was also eluted in the same position as natural peptide 6 and the digest of the synthetic peptide (not shown). The peak fraction in Fig. 4A was isolated and subjected to amino acid analysis and FAB mass spectrometry, as described in Table 1. Results indicated that the N-terminal Ser-Ser sequence of the synthetic peptide was removed by treatment with aminopeptidase M yielding natural peptide 6.

Then, we subjected the synthetic peptide to one step of Edman degradation. The degraded peptide was eluted at the same retention time on HPLC as natural peptide 5 and had the same amino acid composition and mass value as those of natural peptide 5, as shown in Fig. 5 and Table 1, respectively. Namely, removal of

TABLE 1. AMINO ACID COMPOSITIONS AND MASS VALUES OF SYNTHETIC PEPTIDES AND DEGRADED PEPTIDES AND THEIR COMPARISON WITH THOSE OF NATURAL PEPTIDES[a]

	Synthetic peptide			Natural peptide[b]		
	4	APase digest	Edman degraded	4	6	5
Asp	3.90 (4)	3.83 (4)	3.95 (4)	4.05 (4)	4.23 (4)	3.98 (4)
Thr				0.02	0.07	0.02
Ser	1.82 (2)		1.02 (1)	1.92 (2)	0.23	1.09 (1)
Glu				0.10	0.30	0.10
Gly	0.96 (1)	1.04 (1)	1.01 (1)	0.98 (1)	1.06 (1)	1.02 (1)
Ala	2 (2)	2 (2)	2 (2)	2 (2)	2 (2)	2 (2)
1/2Cys	4.25 (6)	4.74 (6)	4.83 (6)	4.75 (6)	4.76 (6)	4.83 (6)
Val	0.70 (1)	0.69 (1)	0.76 (1)	0.74 (1)	0.83 (1)	0.76 (1)
Met				0.03	0.05	0.02
Ile				0.03	0.09	0.03
Leu				0.02	0.09	0.03
Tyr				0.02	0.03	0.01
Phe				0.02	0.07	—
Lys				0.03	0.07	0.06
His				—	0.03	0.01
Trp	0.51 (1)	0.89 (1)	0.83 (1)	0.95 (1)	0.96 (1)	0.90 (1)
Arg				0.02	0.04	0.03
Pro	1.09 (1)	0.96 (1)	1.09 (1)	1.05 (1)	1.21 (1)	1.04 (1)
$[M+H]^+$	1845.5	1671.4	1757.9	1845.4	1671.4	1758.6

a) Values were calculated as mol/mol of Ala; numbers in parentheses indicate nearest integer values. b) Cited from Ref. 6.

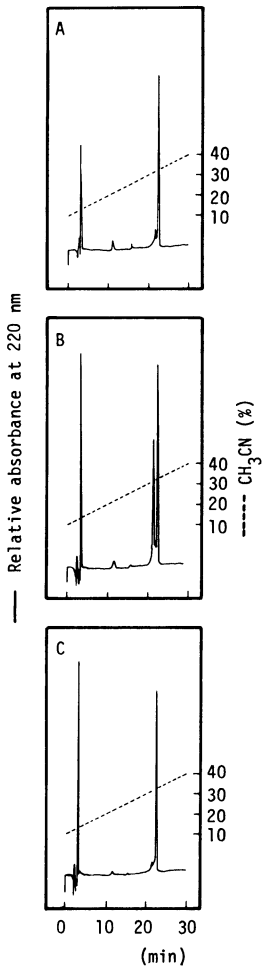

Fig. 4. HPLC profiles on a reversed-phase column (YMC-ODS S-5, 4×250 mm) of A) the aminopeptidase M digest of synthetic peptide, B) a mixture of A) and the undigested synthetic peptide, and C) a mixture of A) and natural peptide 6.

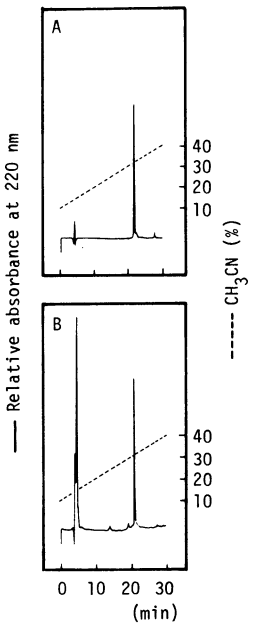

Fig. 5. HPLC profiles on a reversed-phase column (YMC-ODS S-5, 4×250 mm) of A) the synthetic peptide after one step of Edman degradation and B) a mixture of A) and the natural peptide 5.

the N-terminal Ser residue from the synthetic peptide resulted in formation of a peptide identical with natural peptide 5. These results clearly indicate that the synthetic peptide had the same primary and secondary structures as those of natural peptide 4.

Heat Stability. A crude ST isolated from *Y. enterocolitica* has been reported to be more heat-resistant than ST of enterotoxigenic *E. coli*, when assayed in suckling mouse [18]. However, the temperature stability of ST of *Y. enterocolitica* has not yet

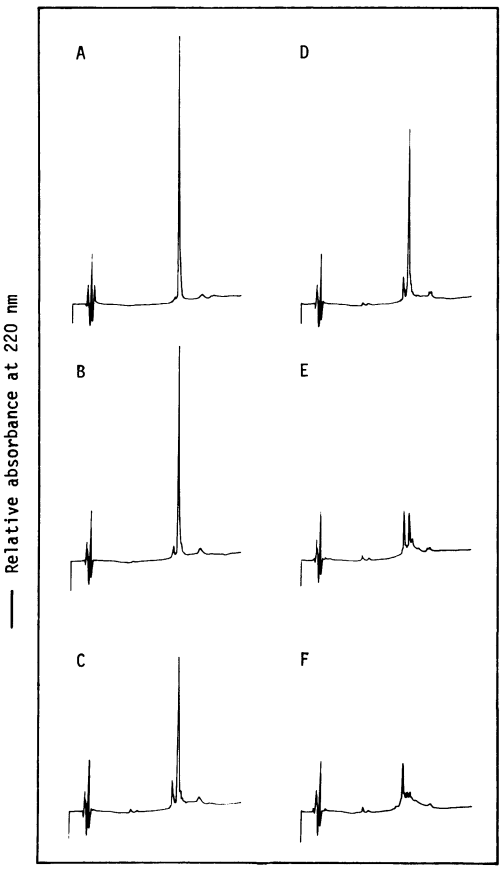

Fig. 6. HPLC profiles on a reversed-phase column (YMC-ODS S-5, 4×250 mm) of heat-treated synthetic peptide: (left column) A) after 10 min at 100 °C, B) after 30 min at 100 °C, C) after 60 min at 100 °C; (right column) D) after 10 min at 120 °C, E) after 30 min at 120 °C, and F) after 60 min at 120 °C.

been examined quantitatively, because purified ST could not be obtained. Since we obtain pure synthetic ST of *Y. enterocolitica* in the present study, we examined its heat stability by the same method as that used for testing the heat stability of ST of enterotoxigenic *E. coli* by HPLC [19]. The stabilities of the natural and synthetic ST's could not be compared, because the natural toxin could be isolated only in minute quantity [6]. Figure 6 illustrates the HPLC profiles of the synthetic toxin after heating at 100 °C and 120 °C for various periods. When the synthetic ST was heated at 100 °C for 30 min, its peak area on HPLC was reduced only to about 75% of that of the untreated ST, while ST of enterotoxigenic *E. coli* was destroyed significantly under the same conditions [19]. Furthermore, after heating at 100 °C for 60 min or at 120 °C for 10 min, about two-thirds of the original peak remained unchanged and the biological activity remained almost unchanged. These results support a previous qualitative experiment [18] which showed that ST of *Y. enterocolitica* was highly heat-resistant, showing no reduction of enterotoxigenic activity after heating for 30 min at 100 °C or 120 °C. This stability of ST of *Y. enterocolitica* was comparable to that of shorter analogues of ST of enterotoxigenic *E. coli*, which consist of 13 and 14 amino acid residues from near the N-terminal Cys residue to the Cys residue at position 2 from the C-terminus and the C-terminal Tyr residue [19,20], respectively. This high heat stability of ST of *Y. enterocolitica* may be due to the difference of its N-terminal sequence from that of enterotoxigenic *E. coli* or the presence of Asp-Val in place of Glu-Leu in ST of enterotoxigenic *E. coli*. We are now examining the relation of the thermodynamic stability and the biological activity.

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