Structural Requirements for the Spatial Structure and Toxicity of Heat-Stable Enterotoxin (ST_h) of Enterotoxigenic *Escherichia coli*

Shinji YAMASAKI,† Yuji HIDAKA, Hideaki ITO,† Yoshifumi TAKEDA,† and Yasutsugu SHIMONISHI*

Institute for Protein Research, Osaka University, Yamada-oka 3-2, Suita, Osaka 565
†The Institute of Medical Science, The University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108

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The heat-stable enterotoxin (ST_h) of enterotoxigenic *Escherichia coli* consists of 19 amino acid residues and is linked intramolecularly by three disulfide bonds between Cys^6 and Cys^{11}, Cys^7 and Cys^{15}, and Cys^{10} and Cys^{18} (Y. Shimonishi et al., *FEBS Lett.*, **215**, 165 (1987)). The syntheses and biological assays of various analogs of ST_h with one or two of the three disulfide bonds showed that a disulfide bond between Cys^7 and Cys^{15} and another between Cys^6 and Cys^{11} or Cys^{10} and Cys^{18} are necessary for construction and stabilization of the spatial structure of ST_h to express its toxicity.

Various strains of enterotoxigenic *Escherichia coli* from animal hosts produce two kinds of heat-stable enterotoxins (STI and STII) that cause acute diarrhea in man and domestic animals.[1] STI is thought to bind to membrane receptors of intestinal cells[2] and stimulate guanylate cyclase, which is coupled to or present near the receptors, resulting in the induction of fluid accumulation. The mechanism of the biological action of STII is unknown. We have been studying the primary structure of STI (ST_h and ST_p), the positions of disulfide linkages (Fig. 1), and its structure-activity relation for several years.[3-7] We have found that peptides with the same sequences as those of the 13 amino acid residues from Cys near the N-terminus to Cys near the C-terminus in ST_h and ST_p have full enterotoxigenic activities, that the three intramolecular disulfide bonds are between Cys^6 and Cys^{11}, Cys^7 and Cys^{15}, and Cys^{10} and Cys^{18} in ST_h, and that the positions of the disulfide bonds affect the extent of toxicity of ST_h. These findings prompted us to examine which of these three intramolecular disulfide bonds are important for the spatial structure of ST_h and expression of its toxicity. Studies on the toxicities of analogs of ST_h with various combinations of disulfide bonds should be helpful in elucidating the spatial structure necessary for expression of toxicity, the contribution of amino acid residues to the spatial structure of ST_h and its binding site(s) for receptors on the membrane of intestinal cells.

This paper reports that a disulfide bond between Cys^7 and Cys^{15} in ST_h is essential for expression of the toxicity of ST_h and that another disulfide bond between Cys^6 and Cys^{11} or Cys^{10} and Cys^{18} is important for stabilization of the spatial structure and expression of toxicity.

Materials and Methods

The general and analytical methods used were described in the preceeding papers.[7,9] All chemicals used for preparative experiments were of reagent grade, those used for analysis were of guaranteed grade and solvents were distilled before use. All amino acids used were of the L-configuration except glycine. 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; Acm, acetamidomethyl; TFA, trifluoroacetic acid.

Synthesis of Peptides. Peptides were synthesized manually by the solid-phase method.[10] Briefly, Boc-Cys(MBzl)-, Boc-Cys(Acm)-, or Boc-Ala-polystyrene (2% divinylbenzene) (0.2—0.3 mmol of amino acid per 1 g of resin) was used with the following side-chain protecting groups: Bzl for threonine and glutamic acid and MBzl or Acm for cysteine. All couplings were carried out with 4-fold excess of Boc-amino acid and DCC, and the coupling of Boc-Asn-OH was repeated twice with 2 equiv of 1-hydroxybenzotriazole. The amino acids were coupled one by one in order from the C-terminus of peptide sequences. After coupling the N-terminal amino acid,

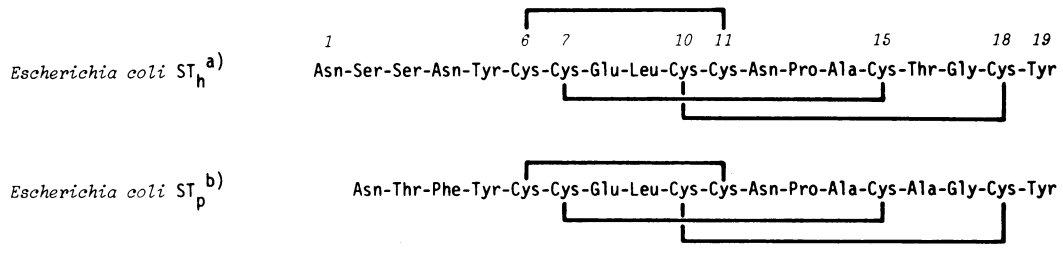

Fig. 1. Amino acid sequences and mode of disulfide bond formation of heat-stable enterotoxins produced by enterotoxigenic *Escherichia coli*: a) see Refs. 3 and 7; b) see Refs. 4 and 8.

the protected peptide-resin was removed from the reaction vessel and treated with the same amount of anisole in anhydrous hydrogen fluoride at 0 °C for 1 h. After removal of the hydrogen fluoride under reduced pressure, the peptide deprotected except for Acm on the mercapto groups of cysteine residues was extracted with 10% formic acid in an ice-water bath and washed three times with hexane. The extract was diluted with water to a final peptide concentration of 5×10^{-5} M (1 M=1 mol dm^{-3}). The solution was adjusted to pH 8.0 by adding aqueous ammonia and stood at room temperature with occasional stirring until no free mercapto groups were detectable. The crude peptide thus obtained was purified by high-performance liquid chromatography, as described below. The purified peptide with Acm groups at given Cys residues was dissolved at 5×10^{-4} M in a mixture of MeOH and H_2O (v/v, 4/1), then mixed with the same volume of a mixture of MeOH and 1M HCl (v/v, 4/1) containing iodine (40 equiv of the sample peptide), and kept for 15 min at room temperature. The reaction was stopped by adding L-ascorbic acid and the reaction product was purified by high-performance liquid chromatography, as described below.

Enzymatic Digestion. Synthetic peptides **16** and **18** were each dissolved in 0.02 M ammonium acetate (pH 7.0) and digested with aminopeptidase M (Pierce) and carboxypeptidase A (Boehringer, Mannheim), respectively, at a substrate:enzyme ratio (w/w) of 40:1 at 37 °C for 24 h.

High-Performance Liquid Chromatography (HPLC). The synthetic peptide was purified on a reversed-phase column (YMC-ODS, S-5, 8×300 mm, Yamamura Chemical Industries Ltd., Kyoto) using a high-performance liquid chromatography system consisting of a Waters M600 multisolvent delivery system (Milford, MA) and Hitachi 655A variable wavelength UV monitor and D-2000 chromato-integrator (Tokyo). The peptide was eluted with a linear gradient of 10—40% CH_3CN in 0.05% TFA or 0.01 M ammonium acetate (pH 5.7) with increase in CH_3CN of 1% min^{-1} at a flow rate of 2 ml min^{-1}. Fractions of the eluate were monitored for absorbance at 220 nm.

Amino Acid and Fast Atom Bombardment (FAB) Mass Analyses. The amino acid compositions and molecular weights of purified synthetic peptides were examined by amino acid analysis and FAB mass spectrometry, as described in Refs. 7 and 11, respectively.

Biological Assay. Toxicity was assayed in suckling mice (1.7±0.1 g) of 2 days old as described previously.[12]

Results and Discussion

To examine the effects of the three disulfide bonds on the toxicity of ST_h, we synthesized peptides with all possible combinations of two disulfide bonds, as shown in Table 1. Peptides **1**—**9** with two disulfide bonds other than that between the two Cys residues protected by Acm groups were synthesized as intermediates in the syntheses of peptides with three disulfide bonds in a previous study.[7] Peptides **10**—**15**, which had two disulfide bonds between four Cys differing for those in peptides **1**—**9** and in which the other two Cys were replaced by Ala, were newly synthesized in this work. That is, we synthesized protected peptide resins with the sequences listed in Table 1 where MBzl group was employed as a protecting group for mercapto groups of four Cys residues (Fig. 2). All protecting groups were removed

Table 1. Synthetic Analogs of ST_h with All Possible Combinations of Two Disulfide Bonds and Their Toxicities

No.	Peptide[a]													Toxicity[b]
	6 Cys	7 Cys	8 Glu	9 Leu	10 Cys	11 Cys	12 Asn	13 Pro	14 Ala	15 Cys	16 Thr	17 Gly	18 Cys	
1	▲	—	—	—	—	▲	—	—	—	—	—	—	—	+
2	▲	—	—	—	—	—	—	—	—	▲	—	—	—	−
3	▲	—	—	—	—	—	—	—	—	—	—	—	▲	−
4	—	▲	—	—	▲	—	—	—	—	—	—	—	—	−
5	—	▲	—	—	—	▲	—	—	—	—	—	—	—	−
6	—	▲	—	—	—	—	—	—	—	▲	—	—	—	−
7	—	▲	—	—	—	—	—	—	—	—	—	—	▲	−
8	—	—	—	—	▲	—	—	—	—	▲	—	—	—	−
9	—	—	—	—	▲	—	—	—	—	—	—	—	▲	+
10	Ala	Ala	—	—	—	—	—	—	—	—	—	—	—	−
11	Ala	—	—	—	Ala	—	—	—	—	—	—	—	—	−
12	—	—	—	—	Ala	Ala	—	—	—	—	—	—	—	−
13	—	—	—	—	—	Ala	—	—	—	Ala	—	—	—	−
14	—	—	—	—	—	Ala	—	—	—	—	—	—	Ala	−
15	—	—	—	—	—	—	—	—	—	Ala	—	—	Ala	−

a) ▲ is a Cys residue in which the thiol group is blocked by an Acm group and — is the same amino acid residue as that in the actual sequence. b) + and − indicate toxicity and no toxicity, respectively, at a dose of less than 1 μg.

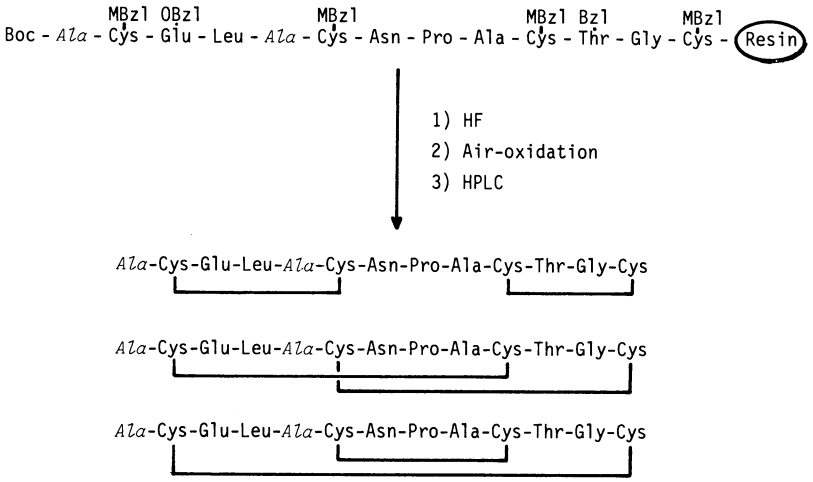

Fig. 2. Scheme for synthesis of an ST_h analog with two disulfide bonds between the four Cys other than those at positions 6 and 10, which are replaced by Ala.

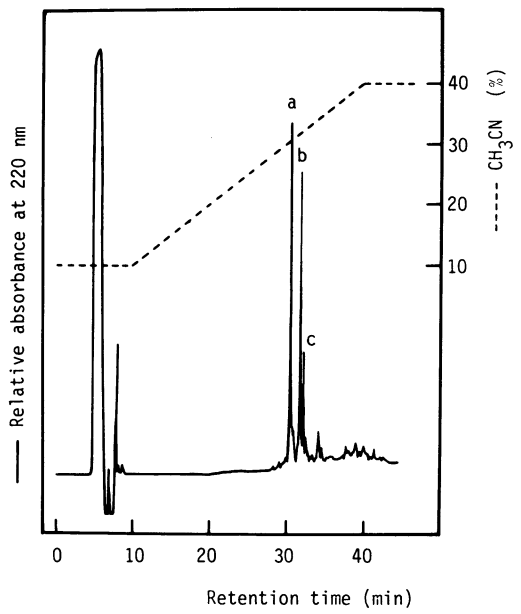

Fig. 3. Reversed-phase HPLC of peptide **11**. For chromatographic conditions, see text.

simultaneously at the stage of cleavage of peptides from the resins by their treatment with anhydrous hydrogen fluoride. The resulting free linear peptides were oxidized by air in dilute aqueous solution (5×10^{-5} M) until no free mercapto groups were detectable. Each peptide should theoretically be a mixture of monomeric peptides with three kinds of disulfide linkages. Figure 3 shows the HPLC profile of peptide **11** with two Ala at positions 6 and 10 and two disulfide bonds between four Cys at positions 7, 11, 15, and 18 (Fig. 2). By analyses of all peaks, monomeric peptides with two disulfide bonds were found in peaks a, b, and c. The results of amino acid and FAB mass spectrometric analyses of these peaks are shown in Table 2. Other peptides were synthesized by a similar procedure to that described in Fig. 2.

We examined the biological activities of all the synthetic peptides shown in Table 1 and found that peptides **1** and **9** had weak, but distinct toxicity, while the other peptides showed no toxicity at a dose of less than 1 μg. Previously[7] we found that peptides **1** and **9** yielded peptides that were identical with standard ST_h peptide in physicochemical and biological properties when the third disulfide bond was formed between Cys^6 and Cys^{11} and Cys^{10} and Cys^{18}, respectively, whereas other peptides did not yield a peptide that was identical with standard ST_h peptide when the third disulfide bond was formed. These findings indicate that peptides **1** and **9** contained peptides with the same two disulfide bonds as those in standard ST_h peptide, that they were between Cys^7 and Cys^{15} and Cys^{10} and Cys^{18} in peptide **1** and between Cys^6 and Cys^{11} and Cys^7 and Cys^{15} in peptide **9**, and that the toxicities of peptides **1** and **9** were due to peptides with these two disulfide bonds. These findings suggest that one or two of these three disulfide bonds are closely related to formation of the spatial structure of the ST_h molecule, which is necessary for expression of its toxicity.

To examine this possibility, we synthesized peptides **16—18** with two disulfide bonds at specific positions between four Cys and two Ala instead of the two remaining Cys, as shown in Table 3. Peptides **16** and **18** correspond to peptides in peptides **1** and **9**, respectively. We synthesized these peptides using two different types of protecting groups and selective and stepwise formation of disulfide bonds. Figure 4 shows the scheme for synthesis of peptide **18**, in which the two Cys at positions 10 and 18 are replaced by two Ala, the mercapto groups of the two Cys at positions 7 and 15 are protected by MBzl groups, and the mercapto groups of the two Cys at positions 6 and 11 are

Table 2. Amino Acid Compositions,[a] Mass Values, and Biological Activities of Peaks of Peptide **11**

	a[b]	b[b]	c[b]	Theoretical value
Asp	1.02	1.04	1.10	1
Thr	1.06	1.08	1.15	1
Glu	1.00	1.02	1.08	1
Pro	1.08	1.09	1.13	1
Gly	0.99	1.02	1.04	1
Ala	3	3	3	3
$\frac{1}{2}$Cys	4.15	4.04	4.39	4
Leu	1.04	1.04	1.04	1
$[M+H]^+$	1251.1	1251.1	1251.1	1251.4
MED[c] (μg)	>17	>14	>14	

a) The synthetic peptide was hydrolyzed in 6M HCl containing 1% phenol in vacuum sealed tubes at 110°C for 24 h. b) a, b, and c correspond to peaks a, b, and c in Fig. 2. c) MED: Minimum effective dose.

Table 3. Synthetic Analogs of ST_h with One or Two Disulfide Bonds and Their Toxicities

No.	Peptide[a]	MED (pmol)[b]
	Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys	0.4
16	*Ala*-Cys-Glu-Leu-Cys-*Ala*-Asn-Pro-Ala-Cys-Thr-Gly-Cys	380
17	Cys-*Ala*-Glu-Leu-Cys-Cys-Asn-Pro-Ala-*Ala*-Thr-Gly-Cys	Inactive
18	Cys-Cys-Glu-Leu-*Ala*-Cys-Asn-Pro-Ala-Cys-Thr-Gly-*Ala*	290
19	Cys-Glu-Leu-Cys-*Ala*-Asn-Pro-Ala-Cys-Thr-Gly-Cys	150
20	Cys-Cys-Glu-Leu-*Ala*-Cys-Asn-Pro-Ala-Cys	110
21	Cys-Glu-Leu-*Ala*-Cys-Asn-Pro-Ala-Cys-Thr-Gly-*Ala*	Inactive
22	Cys*-Cys*-Glu-Leu-*Ala*-Cys-Asn-Pro-Ala-Cys*-Thr-Gly-*Ala*	Inactive
23	Cys*-Cys-Glu-Leu-*Ala*-Cys*-Asn-Pro-Ala-Cys-Thr-Gly-*Ala*	Inactive
24	Cys*-*Ala*-Glu-Leu-Cys-Cys*-Asn-Pro-Ala-*Ala*-Thr-Gly-Cys	Inactive
25	Cys-Glu-Leu-*Ala*-Cys*-Asn-Pro-Ala-Cys-Thr-Gly-*Ala*	Inactive

a) Cys* denotes a Cys residue in which the thiol group is blocked by an Acm group. b) MED: Minimum effective dose.[12]

protected by Acm groups. The first disulfide bond was formed between the two Cys at positions 7 and 15 after removal of the MBzl groups by treatment with HF, and the second disulfide bond was formed between the two Cys at positions 6 and 11 by oxidation with iodine. The HPLC profile of peptide **18** is shown in Fig. 5. Other peptides were synthesized by a similar procedure. We found that peptides **16** and **18** which had two disulfide bonds between residues 7 and 15, and 6 and 11 or 10 and 18 were toxic, while peptide **17** with no disulfide bond at positions 7 and 15 was inactive, as shown in Table 3. Digestion of peptide **16** with aminopeptidase M, and of peptide **18** with carboxypeptidase A gave peptides **19** and **20**, respectively, which lack their N-terminal amino acid residue and C-terminal three amino acid residues. These peptides were about twice as toxic as their original peptides. These results indicated that a disulfide bond between Cys^7 and Cys^{15} is necessary for the toxicity of ST_h. Analytical data on peptides **16—20** are shown in Table 4.

Next, we synthesized a peptide with two disulfide bonds between Cys^6 and Cys^{11} and Cys^7 and Cys^{15}, but without the peptide bond between Cys^6 and Cys^7 (peptide **21**) and three kinds of peptides with only one disulfide bond between Cys^6 and Cys^{11}, Cys^7 and Cys^{15} or Cys^{10} and Cys^{18} (peptides **22—24**). Peptide **21** was prepared by oxidative coupling of peptide **25** and Boc-Cys(Acm)-OH followed by deprotection of the Boc group. Peptides **22** and **23** were synthesized as intermediates in the synthesis of peptide **18**, and peptide **24** as an intermediate in the synthesis of peptide **17**, by a similar procedure to that shown in Fig. 3. Analytical data on these synthetic peptides are presented in Table 4. All these peptides were inactive, as shown in Table 3. Therefore, the spatial structure of ST_h with two disulfide linkages between Cys^7 and Cys^{15}, and Cys^6 and Cys^{11} or Cys^{10} and Cys^{18} is essential for the toxicity of ST_h. In other words, the spatial structure of ST_h cannot be maintained by only one disulfide bond between Cys^7 and Cys^{15}, but requires one other disulfide bond between Cys^6 and Cys^{11} or Cys^{10} and Cys^{18}.

Figure 6 shows a CPK-model of a short peptide of ST_h with 13 amino acid residues, which was constructed from its three disulfide linkages[7] and

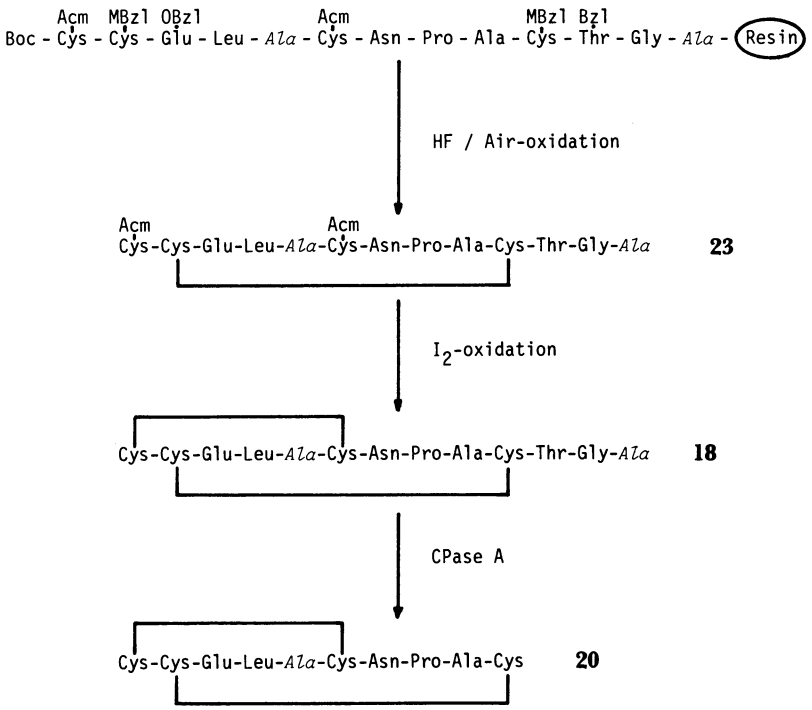

Fig. 4. Scheme for synthesis of an ST_h analog with two disulfide bonds between the Cys at positions 6 and 11 and positions 7 and 15, respectively.

Table 4. Amino Acid Compositions[a] and Mass Values[b] of Synthetic Analogs[c] of ST_h

	16	17	18	19	20	21	22	23	24	25
Asp	1.26(1)	1.00(1)	1.10(1)	0.97(1)	1.22(1)	1.07(1)	1.08(1)	1.09(1)	1.10(1)	0.98(1)
Thr	0.98(1)	1.00(1)	1.00(1)	1.00(1)	—	1.15(1)	1.12(1)	1.21(1)	1.18(1)	1.00(1)
Glu	0.99(1)	1.00(1)	0.98(1)	0.99(1)	0.98(1)	1.06(1)	1.08(1)	1.07(1)	1.03(1)	0.99(1)
Pro	1.20(1)	1.17(1)	1.01(1)	0.99(1)	1.07(1)	1.10(1)	1.16(1)	1.15(1)	1.17(1)	1.00(1)
Gly	1.00(1)	0.98(1)	0.98(1)	0.95(1)	—	1.16(1)	1.00(1)	1.03(1)	1.04(1)	0.97(1)
Ala	3 (3)	3 (3)	3 (3)	2 (2)	2 (2)	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)
$\frac{1}{2}$Cys	2.22(4)	4.12(4)	3.03(4)	3.68(4)	2.77(4)	3.43(4)	3.41(4)	4.04(4)	4.23(4)	2.35(3)
Leu	0.98(1)	0.99(1)	0.98(1)	0.98(1)	1.00(1)	1.03(1)	1.04(1)	1.04(1)	1.06(1)	0.96(1)
$[M+H]^+$	1251.8 (1251.4)	1251.1 (1251.4)	1251.3 (1251.4)	1180.4 (1180.3)	1022.3 (1022.2)	1269.1 (1269.4)	1395.3 (1395.5)	1395.1 (1395.5)	1395.3 (1395.5)	1221.9 (1221.5)

a) Synthetic peptides were hydrolyzed in 6M HCl containing 1% phenol in vacuum sealed tubes at 110°C for 24 h. Numbers in parentheses are theoretical values. b) Numbers in parentheses are theoretical values. c) Numbers of peptides correspond to those in Table 3.

nuclear Overhauser effects (NOE) data measured by two-dimensional ^1H NMR spectroscopy.[13,14] The main chain of the peptide viewed from the front in Fig. 6 is seemingly folded in a twisted "8"-shape and there are two loop structures consisting of two turns between residues 9 to 11 and 12 to 15. The three disulfide linkages are located in the hinge region binding these two loops and presumably stabilize the spatial structure of ST_h. Cleavage of the disulfide bond between Cys^6 and Cys^{11} or Cys^{10} and Cys^{18} does not appreciably disrupt the spatial structure necessary for expression of toxicity, and the twisted "8"-structure shown in Fig. 6 is still maintained, at least partially. However, cleavage of the disulfide bond between Cys^7 and Cys^{15} may completely loosen the spatial conformation of ST_h, because peptide **17** was completely inactive. Cleavage of any two of the disulfide bonds shown in Fig. 6 may completely disrupt the structure fixed by the hinge region. Therefore, a twisted "8"-structure of the main chain seems important for the toxicity of ST_h or for stabilization of its spatial structure. In particular, maintenance of the spatial structure of the peptide chain from Cys^7 to Cys^{15} may be necessary for expression of the toxicity of ST_h. For further elucidation of the effect of the spatial structure of ST_h on its toxicity, attempts should be made to build a reliable molecular model of ST_h and identify the amino acid residues that bind ST_h to membrane proteins of intestinal cells.

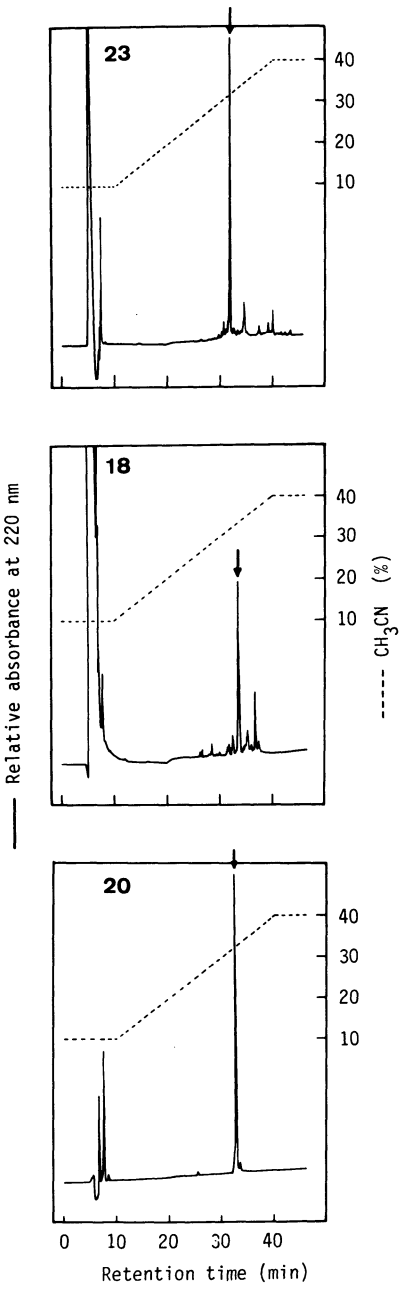

Fig. 5. HPLC profiles of peptides **23**, **18**, and **20**. For chromatographic conditions, see text.

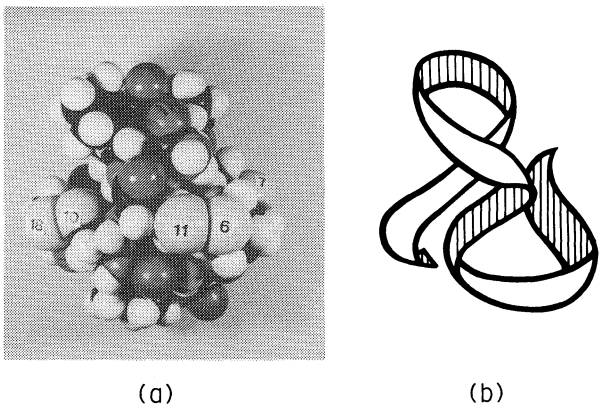

Fig. 6. A CPK-model (a) and schematic ribbon drawing of the polypeptide backbone (b) of a heat-stable enterotoxin (ST_h) consisting of 13 amino acid residues from Cys at position 6 to Cys at position 18, based on the positions of the three disulfide bonds[7] and NOE data measured by ^1H NMR spectroscopy.[13,14]

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Note Added in Proof. After submittion of this paper, Gariepy et al. (*Proc. Natl. Acad. Sci. U.S.A.*, **84**, 8907 (1987)) reported the importance of disulfide linkages in an enterotoxin and the result was the same as described here.