# Semi-preparative Purification and Crystallization of Synthetic Analogs of Heat-stable Enterotoxins of Enterotoxigenic *Escherichia coli*

Takashi SATO, Hideaki ITO,† Yoshifumi TAKEDA,† and Yasutsugu SHIMONISHI*
Institute for Protein Research, Osaka University, Yamadaoka 3-2, Suita, Osaka 565
† Faculty of Medicine, Kyoto University, Yoshida-konoe, Sakyo-ku, Kyoto 606
(Received October 30, 1991)

**Synopsis.** The purification by high-performance liquid chromatography of synthetic analogs of heat-stable enterotoxin (ST) of enterotoxigenic *Escherichia coli* from large amounts of their dilute solution on a laboratory scale is described. These analogs were also crystallized by gradually decreasing the temperature of their warm aqueous solution.

Heat-stable enterotoxins (STs), which are responsible for acute diarrhea in infants and domestic animals, are peptides of 18 or 19 amino acid residues.[1-4] ST binds to a receptor(s) on the surface membranes of intestinal epithelial cells, resulting in activation of guanylate cyclase followed by increase of intracellular cGMP concentration and stimulation of fluid secretion.[5-8] We recently crystallized a peptide with the full biological activity of the toxin (**2** in Table 1) and elucidated its molecular structure by X-ray crystallography.[9] The peptide has the amino acid sequence from positions 5 to 17 of one of STs ($ST_p$), which was isolated from a porcine strain of enterotoxigenic *E. coli*, with Mpr at possition 5 instead of Cys.

We are currently examining the mechanism of recognition of ST by its receptor(s) at the molecular level, and the molecular structures of various analogs of ST with different degrees of toxic activity. In this paper we describe the laboratory scale purification of several synthetic analogs of $ST_p$ (exemplified in Table 1) from large amounts of their dilute solutions by HPLC and their crystallization.

## Experimental

All chemicals used in this paper 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 used are: ST, heat-stable enterotoxin; Mpr, 3-mercaptopropionic acid; HPLC, high-performance liquid chromatography.

**Peptide Synthesis:** Protecting peptides bound on a resin were synthesized by the solid-phase method[10-12] and the protecting groups were removed by treatment with anhydrous HF at 0°C for 75 min in the presence of 10 molar excess of anisole over protecting groups. After removal of HF in vacuo, the residues were suspended in $5\times10^{-2}$ M (1 M=1 mol $dm^{-3}$) aqueous formic acid at a final peptide concentration of $5\times10^{-5}$ M and adjusted to pH 8.0 by adding aqueous ammonia. This solutions were gently stirred at room temperature until no free mercapto-groups were detectable by reaction with 5,5′-dithiobis(2-nitrobenzoic acid). Then the crude peptide solutions were purified by reversed-phase HPLC, as described below.

**Purification of Synthetic Peptides:** The HPLC system consisted of a Waters M600 multisolvent delivery system and modified 510P pump (Milford, MA), a Hitachi UV 655A variable wavelength UV monitor and D-2000 chromato-integrator (Tokyo), and a Gilson fraction collector model 201 (Villiers-le-Bil, France), assembled as shown in Fig. 1. The HPLC columns were packed with Cosmosil $5C_{18}$-AR

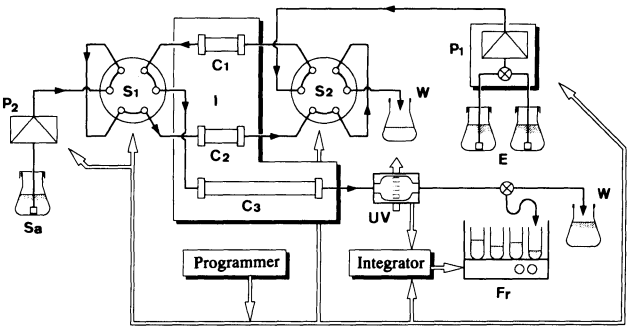


Fig 1. Diagram of HPLC system for continuous separation of peptides from their dilute solutions. The positions of the two valves $S_1$ and $S_2$ for concentrating a peptide solution on $C_2$ and separating a peptide on $C_1$ and $C_3$ columns are shown. $C_1$ and $C_2$: short columns ($5C_{18}$-AR), $C_3$: long column ($5C_{18}$), $S_1$ and $S_2$: high pressure rotatory valves, $P_1$: M600 multisolvent delivery system, $P_2$: modified 510P pump, **UV**: UV detector, **I**: column oven, **E**: eluent, $S_a$: sample solution, **W**: waste.

Table 1. Amino Acid Sequences of the Heat-Stable Enterotoxin ($ST_p$) and Synthetic Analogs and Their Toxic Activities

| | Peptide | MED (ng) |
|---|---|---|
| | 1 5 10 15 18 | |
| **1** | Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-**Ala**-Cys-Ala-Gly-Cys-Tyr | 0.4[a] |
| **2** | Mpr-Cys-Glu-Leu-Cys-Cys-Asn-Pro-**Ala**-Cys-Ala-Gly-Cys | 0.9[b] |
| **3** | Mpr-Cys-Glu-Leu-Cys-Cys-Asn-Pro-**Gly**-Cys-Ala-Gly-Cys | 2.9 |
| **4** | Mpr-Cys-Glu-Leu-Cys-Cys-Asn-Pro-**Abu**-Cys-Ala-Gly-Cys | 116 |
| **5** | Mpr-Cys-Glu-Leu-Cys-Cys-Asn-Pro-**Leu**-Cys-Ala-Gly-Cys | >1000 |

MED: Minimum effective dose. a) Cited from Ref. 3. b) Cited from Ref. 12.

(8×50 mm) for adsorption of crude peptides and Cosmosil $5C_{18}$ (8×250 mm) for separation of the peptides (Nacalai Tesque, Kyoto). The solution (ca. 100 ml) containing a crude peptide (6—10 mg) was passed through the $5C_{18}$-AR columns $C_2$ and the peptide was adsorbed on this column. Then this column $C_2$ was connected with an M600 solvent delivery system in the upper stream of the solvent and the $5C_{18}$ column $C_3$ in the downstream of the solvent by switching the rotatory valves $S_1$ and $S_2$ in Fig. 1. The peptide was eluted from column $C_2$ and transferred to column $C_3$ which was equilibrated with 10% $CH_3CN$ in 0.05% TFA. Column $C_3$ was developed with an increasing $CH_3CN$ concentration of 1% $min^{-1}$ at a flow rate of 2 ml $min^{-1}$ at 45°C. The purified peptides were obtained in the fraction collector $F_r$ and freeze-dried by vaccum centrifugation. After the peptide had been eluted, column $C_3$ was washed with 80% $CH_3CN$ in 0.05% TFA and then conditioned by washing with the starting solvent. Simultaneously during this time, the other part of the solution was concentrated on the other $5C_{18}$-AR column $C_1$. Thus the solution was concentrated alternately on either column $C_1$ or $C_2$ and the adsorbed peptides were continuously separated on column $C_3$.

**Analyses of Purified Peptides:** The purified peptides were examined by fast atom bombardment mass spectrometry and amino acid analysis of their hydrolysates as described previously.[13] The biological activity of the synthetic peptides was examined by assay in suckling mice of 2 days old (1.7±0.1 g) as described previously.[14]

**Crystallization:** A sample of 6 mg of the purified peptide was suspended in 3 ml of water with warming to 70°C in a small capped test tube that has been pretreated with Prosil®-28 (SCM Chemicals/Division of SCM Corp., Gainesville, FL). Acetonitrile was gradually added to the suspension at this temperature until the peptide was completely dissolved. The solution was then cooled with a linear gradient of temperature from 70° to 30°C in 50 h and the solution was kept at 30°C for a further 30 h. Several crystals grew to a maximum size of 2.0×0.2—0.3×0.15—0.2 mm under these conditions and were harvested for X-ray diffraction studies.

## Results and Discussion

**Purification of Synthetic Peptides:** The air-oxidized solution of a synthetic crude peptide was very dilute ($5\times10^{-5}$ M). Therefore, a large amount of the solution was necessary to obtain a sufficiently large amount of the peptide for crystallization and physicochemical study. Moreover, the air-oxidized solution had to be subjected to HPLC directly, because evaporation of the solvent in vacuo resulted in an insoluble precipitate, probably due to irreversible aggregation of the peptide. Therefore, we assembled and used the continuous automatic flow HPLC system as shown in Fig. 1 for processing a large amount of the solution in the laboratory. The solution was concentrated alternately on one of two short columns, $C_1$ and $C_2$, one of which could be connected with the longer column $C_3$ by switching the rotatory valves $S_1$ and $S_2$ in Fig. 1, and the adsorbed peptides were eluted from the short columns $C_1$ or $C_2$ and separated on the longer column $C_3$. The HPLC profiles of crude and purified preparations of [$Mpr^5$, $Gly^{13}$]$ST_p$(5-17) are shown in Fig. 2. In this study, several hundred milligrams of a peptide could be purified in a short time from a large amount of its dilute solution. Pure peptides were recovered in yields of ca. 25% as judged by the amounts of the amino acids bound on the resin.

**Structure-Activity Relationships:** Our previous work[15] indicated that the activities of the peptides decreased with increase in length of the chain of the amino acid residue at position 14 in $ST_h$, which corresponds to position 13 in $ST_p$. In the present work, we synthesized several analogs with side chains of H to $CH_2$–$CH(CH_3)_2$ at position 13 in $ST_p$, as shown in Table 1, and examined their biological activities. The results confirmed our previous finding that the bulkiness of the side chain of the amino acid residue at position 13 in $ST_p$ is related to the toxic activity.

**Crystallization:** The analogs of $ST_p$ synthesized in this work were intermediate in size between small organic compounds and proteins. So, we tried to crystallize them by several methods used for small molecules and

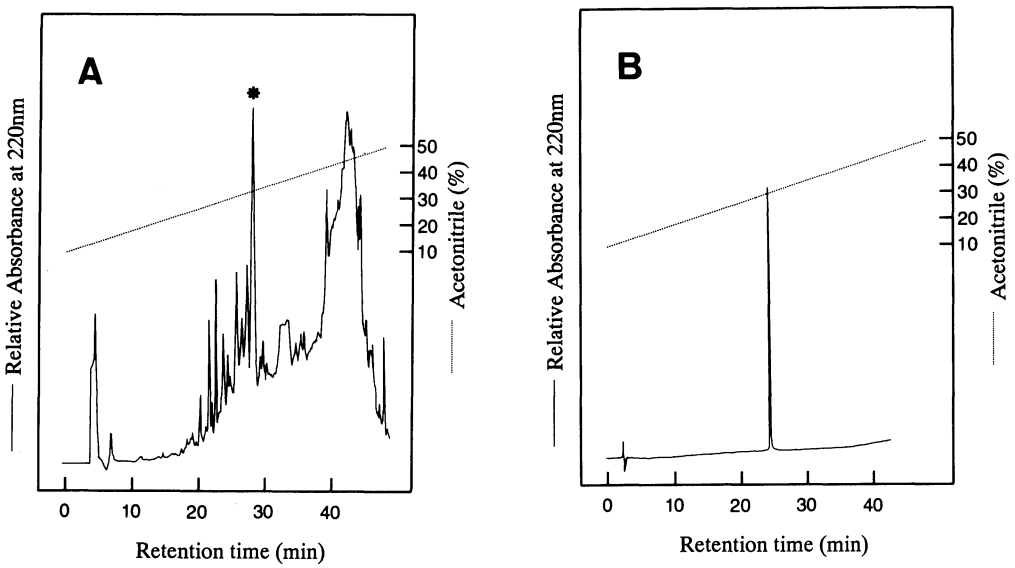


Fig. 2. HPLC profile of [$Mpr^5$,$Gly^{13}$]STp(5-17). A: crude preparation, B: preparation with r.t. 28.40 min(*) in A.

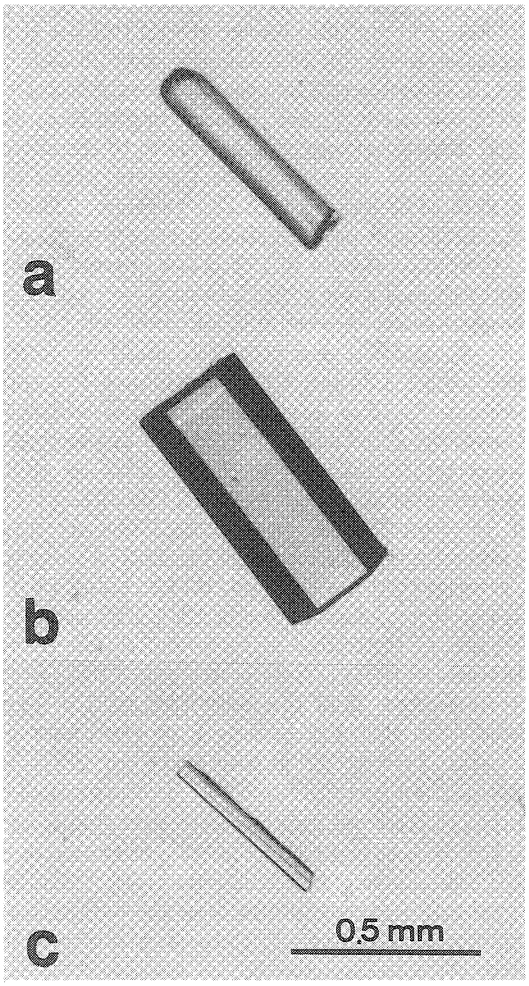


Fig. 3. Photomicrographs of single crystals of a) [Mpr$^5$,Leu$^{13}$]STp(5-17), b) [Mpr$^5$,Gly$^{13}$]STp(5-17), and c) [Mpr$^5$, Abu$^{13}$]STp(5-17).

proteins; namely, 1) equilibrium of vapor pressure between the peptide solution and solutions containing various precipitants, 2) spontaneous vaporization of the solvent from sitting drops of the peptide solution, and 3) cooling of the peptide solution from high temperature to room temperature. We found that method 3) was most suitable for crystallization of the analogs of $ST_p$. These peptides were soluble in $CH_3CN$, but not readily soluble in water. The amount of $CH_3CN$ added to the aqueous suspension of a peptide depended on the solubility of the peptide in water.

Single crystals of the analogs are shown in Fig. 3. The crystal shapes of the analogs were markedly different depending on the amino acid residue at position 13: the crystal of the Leu$^{13}$-analog was a pillar-shape, that of the Gly$^{13}$-analog was a hexagonal pillar-shape, and that of the Abu$^{13}$-analog sheet-like. [Mpr$^5$, Leu$^{13}$]$ST_p$(5-17) formed an orthorhombic crystal with $P2_12_12_1$ space group like [Mpr$^5$]$ST_p$(5-17).[9] On the contrary, the crystal of [Mpr$^5$, Gly$^{13}$]$ST_p$(5-17) showed a monoclinic $P2_1$ space group. [Mpr$^5$, Abu$^{13}$]$ST_p$(5-17) has not yet been grown to a suitable crystal size for X-ray diffraction analysis. The molecular structures of the analogs of $ST_p$ synthesized in this work will be reported in detail elsewhere.

We are indebted to Dr. T. Takao, Institute for Protein Research, Osaka University, for helpful discussion on construction of the semi-preparative high-performance liquid chromatography system. We are also indebted to Mrs. Kazuko Kawaguchi and Aiko Kobatake for preparation of the manuscript. This work was supported in part by a Grant-in-Aid from the ministry of Education, Science and Culture.

**References**

1) H. W. Smith and C. L. Gyles, *J. Med. Microbiol.*, **3**, 387 (1970).
2) S. Aimoto, T. Takao, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani, *Eur. J. Biochem.*, **129**, 257 (1982).
3) T. Takao, T. Hitouji, S. Aimoto, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani, *FEBS Lett.*, **152**, 1 (1983).
4) M. R. Thompson and R. A. Giannella, *Infect. Immun.*, **47**, 834 (1985).
5) M. Field, L. H. Graf, W. J. Laird, and P. L. Smith, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 2800 (1978).
6) R. L. Guerrant, J. M. Hughes, B. Chang, D. C. Robertson, and F. Murad, *J. Infect. Dis.*, **142**, 220 (1980).
7) T. Hirayama, Y. Oku, Y. Takeda, N. Iwata, S. Aimoto, and Y. Shimonishi, "Advances in Cholera and Related Diarrheas," ed by R. B. Sack and Y. Zinnaka, Vol. 7, p. 105 (1990).
8) S. Schulz, C. K. Green, P. S. T. Yen, and D. L. Garbers, *Cell*, **63**, 941 (1990).
9) H. Ozaki, T. Sato, H. Kubota, Y. Hata, Y. Katsube, and Y. Shimonishi, *J. Biol. Chem.*, **266**, 5934 (1991).
10) R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).
11) Y. Shimonishi, Y. Hidaka, M. Koizumi, M. Hane, S. Aimoto, T. Takeda, T. Miwatani, and Y. Takeda, *FEBS Lett.*, **215**, 165 (1987).
12) H. Kubota, Y. Hidaka, H. Ozaki, H. Ito, T. Hirayama, Y. Takeda, and Y. Shimonishi, *Biochem. Biophys. Res. Commun.*, **161**, 229 (1989).
13) T. Takao, N. Tominaga, S. Yoshimura, Y. Shimonishi, S. Hara, T. Inoue, and A. Miyama, *Eur. J. Biochem.*, **152**, 199 (1985).
14) Y. Takeda, T. Takeda, T. Yano, K. Yamamoto, and T. Miwatani, *Infect. Immun.*, **25**, 978 (1979).
15) S. Yamasaki, T. Sato, Y. Hidaka, H. Ozaki, H. Ito, T. Hirayama, Y. Takeda, T. Sugimura, A. Tai, and Y. Shimonishi, *Bull. Chem. Soc. Jpn.*, **63**, 2063 (1990).