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SYNTHESIS OF A HEAT-STABLE ENTEROTOXIN PRODUCED BY A HUMAN STRAIN OF ENTEROTOXIGENIC *Escherichia coli*

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A heat-stable enterotoxin, produced by a human strain of enterotoxigenic *Escherichia coli*, was synthesized by a solution method. The synthesized peptide has the same biological and physicochemical properties as those of native toxin.

Enterotoxigenic *Escherichia coli* (ETEC) produce two types of enterotoxins that are responsible for severe diarrhea, both in man and domestic animals: a high-molecular weight, heat-labile toxin (LT) and a low-molecular weight, heat-stable toxin (ST).[1] Previous investigations[2-4] provided evidence that several distinct ST molecules are produced by ETEC. Recently, we[5-7] isolated and purified the stable toxins from human and from porcine strains of ETEC, and determined their amino acid sequences, as shown below. We designate them as ST_h and ST_p, respectively, in this paper.

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

These two sequences are quite different in the N-terminal region but similar in the region from the near-N-terminal Cys residue to the Tyr residue at the C-terminus. The latter region is considered to have a unique conformation, resulting from 3 disulfide linkages, important for the biological function of these toxins. However, the properties of the toxins have not yet been defined, other than their possible involvement in the guanylate cyclase-cyclic GMP system.[8-11]

The synthesis of ST_h peptide, dealt with in this paper, forms a part of continuing investigation to elucidate the molecular conformation-activity relationships of the toxin.

Synthesis of Intermediates: ST_h peptide, consisting of 19 amino acid residues, was synthesized in four segments whose protected derivatives were first prepared by conventional solution methods. The segments were coupled sequentially from the C-terminus to the N-terminus, as illustrated in Fig. 1. The coupling reaction was carried out by an azide method[12] for minimizing undesirable racemization. The purity of protected peptide intermediates was estimated by thin layer chromatography, by elementary analysis, and by amino acid analysis of their acid hydrolysates.

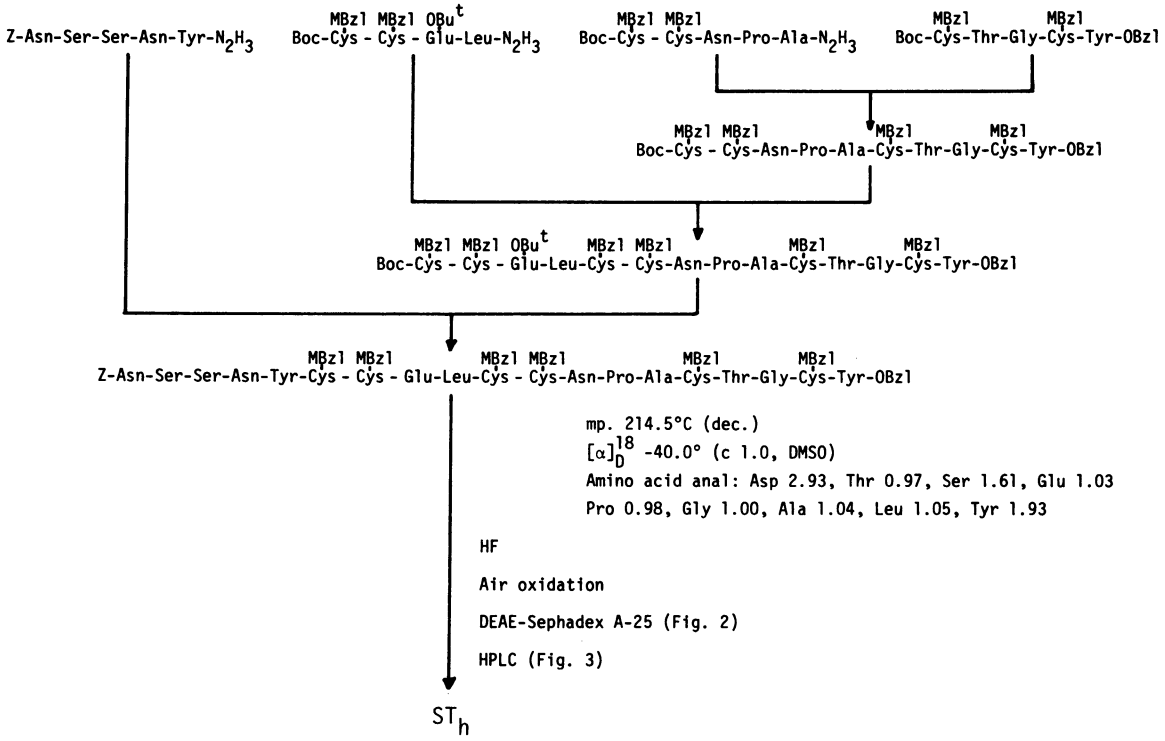

Fig. 1. Synthetic scheme of ST_h[13]

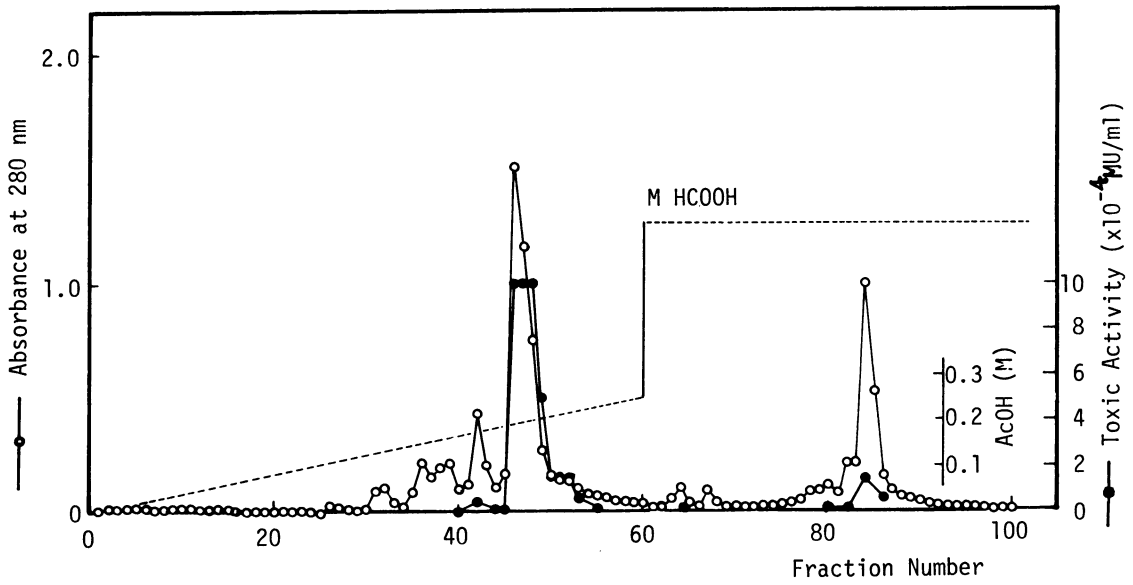

Fig. 2. Chromatogrpahy on DEAE-Sephadex A-25 of the deprotected and air-oxidized peptide.
Fractionation size: 12.5 ml/tube Flow rate: 80 ml/h

Preparation of Toxic Peptide: The protected peptide (290 mg, 0.1 mmol) covering the whole sequence of ST_h was treated with anhydrous hydrogen fluoride (10 ml) in the presence of anisole (0.87 ml, 10 eq. of each protecting group) for 60 min at 0°C.[14] Hydrogen fluoride was evaporated off in vacuum, and the residue was dissolved in 99% formic acid (2.5 ml) and washed with hexane three times. The solution was diluted with distilled water to a final concentration of 10^{-5}M of the peptide and adjusted to pH 8.0 with aqueous ammonia. The solution was allowed to air-oxidize at room temperature with occasional stirring until free thiol groups disappeared, when it was applied to a column of DEAE-Sephadex A-25 (2.2 x 47 cm, acetate form) equilibrated with distilled water. The adsorbed peptide was eluted with a linear gradient of 0 to 0.25M acetic acid as shown in Fig. 2. The main toxic fractions were collected and freeze-dried; yield 28.7 mg. The lyophilized material was further purified on a LiChrosorb RP-8 column (8 x 300 mm) by high-performance liquid chromatography (HPLC), as shown in Fig. 3A. The fraction which eluted with the same retention time as that of native toxin (Fig. 3B) was collected and lyophilized; yield, 5.1 mg (2.5% based on the amount of the protected peptide). The isolated material was compared with native toxin by repeated HPLC, as shown in Fig. 3C. The amino acid composition of the purified material was analyzed as follows: $Asp_{2.82}$, $Thr_{0.92}$,

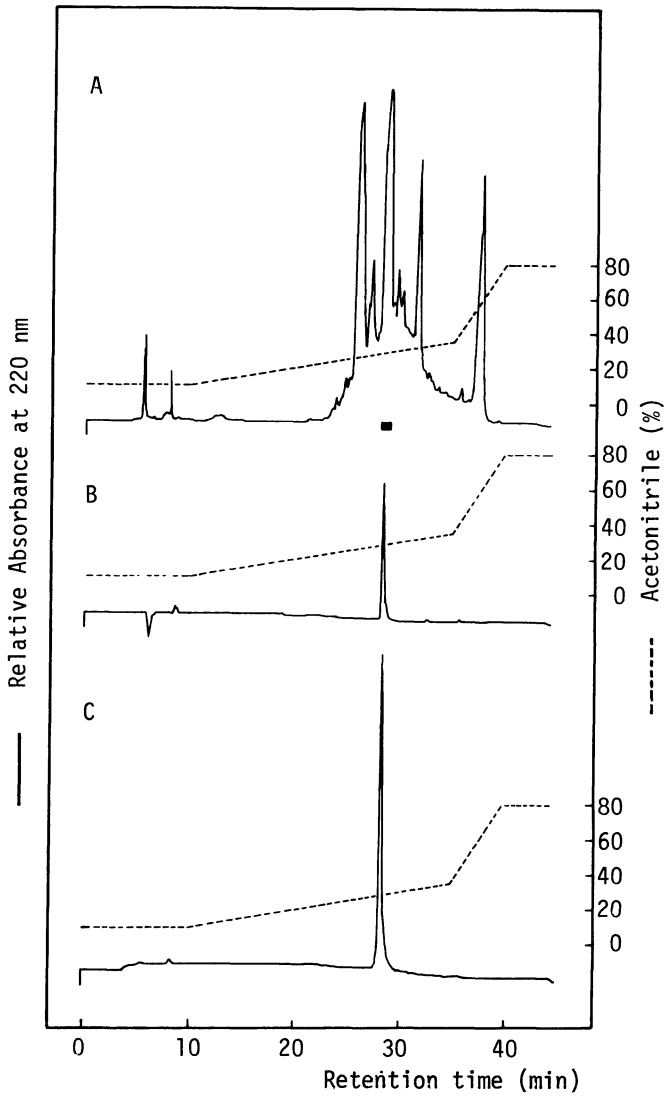

Fig. 3. Reversed-phase HPLC on a LiChrosorb RP-8 of A) the main toxic fraction obtained by DEAE-Sephadex A-25 chromatography (Fig.2), B) purified native ST_h, and C) the fraction shown by a horizontal bar in A). Conditions: the starting solvent was 0.01M $AcONH_4$ (pH 5.7)/acetonitrile. A linear gradient (-----) of 10 - 35% acetonitrile in the solvent was run from 10 min after sample injection. Flow rate: 2 ml/min

$Ser_{1.79}$, $Glu_{1.01}$, $Pro_{1.00}$, $Gly_{1.02}$, $Ala_{1.00}$, $\frac{1}{2}Cys_{4.75}$, $Leu_{1.00}$, $Tyr_{1.88}$ (Values were calculated as mol per mol of Ala).

Biological Activity: ST activity was assayed in suckling mice, 2-4 days old, as described previously.[5] The fluid accumulation ratio of each animal was calculated as the ratio of the weight of the entire intestine to that of the rest of the body. The minimal effective dose giving a fluid accumulation ratio of greater than 0.09 was designated as 1 mouse unit (MU), as described previously.[5] The synthetic peptide obtained after purification on HPLC showed 1.5 ng as the minimum effective dose, a value which is the same as that for native toxin. The toxicity of the synthetic peptide was neutralized by antisera against the native toxin.

Others: Proton n.m.r. spectra of the synthetic and native toxins were obtained by means of a Jeol GX-500 spectrometer. Samples were dissolved in ^2H_2O or d_6-dimethyl sulfoxide to a concentration of 1-2 mM and measured at 25°C. Chemical shifts were measured from the internal standard of acetonitrile. The spectra of the synthetic peptide and native toxin (to be published elsewhere) were superimposable, suggesting that the synthetic peptide has the same tertiary structure as that of native toxin, although the location of 3 disulfide linkages has not yet been determined.

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13) Abbreviations used are those recommended by the IUPAC-IUB: *J. Biol. Chem.*, 247, 977 (1972). Additional abbreviations: MBzl, p-methylbenzyl; DMSO, dimethyl sulfoxide.
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