Vol. 112, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1983 Pages 320-326

# Chemical Synthesis of a Highly Potent and Heat-stable Analog of an Enterotoxin Produced by a Human Strain of Enterotoxigenic Escherichia coli

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Received March 4, 1983

SUMMARY: A shorter analog of a heat-stable enterotoxin produced by a human strain of enterotoxigenic Escherichia coli SK-1, consisiting of 14 amino acid residues including 6 half-cystine residues, was synthesized by conventional methods. The peptide was evaluated for ability to induce intestinal secretion in suckling mice and for stability at high temperature under various conditions. The peptide was 2-5 times more potent than native toxin and was still toxic after heat-treatment at 120°C for 30 min.

Enterotoxigenic Escherichia coli produces chemically heterologous heat-stable enterotoxins (ST) that cause diarrhea in man and in various domestic animals [1]. Recently, we [2-5] isolated and purified two STs named $ST_h$ and $ST_p$ from strain SK-1 and strain 18D, respectively, of enterotoxigenic E. coli, and determined the sequences of their 19 and 18 amino acid residues, as illustrated in Fig. 1. The amino acid sequence of the 18 amino acid residues of $ST_p$ determined by us [5] from strain 18D was found to be different from that reported by Chan and Giannella [6], but to be the same as that of an ST produced by E. coli strain F11(P155) of porcine origin, determined recently by Lallier et al. [7]. Furthermore, we [8] synthesized $ST_h$, an ST from enterotoxigenic E. coli strain SK-1, and confirmed that the synthetic peptide had the same biological and physico-chemical properties as those of the native ST.

$ST_h$ and $ST_p$ show a great deal of structural similarity, although the sequences of their N-terminal regions differ. The common region in the two sequences from the Cys residue near the N-terminal to the Tyr residue at the

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0006-291X/83 $1.50

$ST_h$ a) Asn-Ser-Ser-Asn-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys-Tyr

$ST_p$ b) Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr

Fig. 1. Amino acid sequences of ST isolated from enterotoxigenic E. coli a) strain SK-1 [4] and b) strain 18D [5].

C-terminus is considered to be important for the biological activity and heat-stability of these toxins for the following reasons. First, 6 half-cystine residues, joined by 3 disulfide linkages, are in identical positions in the sequences of these toxins, suggesting that these toxins have similar molecular conformations, although the positions of the disulfide linkages have not yet been determined. Second, Chan and Giannella [6] reported that removal of the N-terminal amino acid residues of $ST_p$ did not abolish the biological activity, although they did not give experimental details. Thus it seemed interesting to elucidate the babic structure of the active site and the heat-stability of the toxin.

We report here the chemical synthesis of a shorter analog of $ST_h$, which has the same sequence as that of the 14 amino acid residues from the C-terminal end of $ST_h$ and that of $ST_p$ except for the amino acid residue in the 4th position from its C-terminus. We found that this sequence had more biological activity than the whole sequence of the original toxin and that its heat-stability was also higher than that of the native toxin.

## MATERIALS AND METHODS

Chemical synthesis: The short analog of $ST_h$ was synthesized by the scheme illustrated in Fig. 2. The sequence of the peptide was divided into three fragments, whose protected derivatives were synthesized separately by conventional methods. Then these peptides were coupled sequentially from the C-terminus to the N-terminus by an azide method [9] for minimizing undesirable racemization. The protected tetradecapeptide was converted to the free peptide by treatment at 0°C with anhydrous hydrogen fluoride [10] containing 10% anisole as a scavenger. The HF reagent was removed under vacuum at 0°C and then the remaining peptide was dissolved in 99% formic acid and washed three times with hexane. The peptide at a concentration of $5 \times 10^{-5}$ M was air-oxidized in ammonium formate buffer (pH 8.0) as described previously [8]. The solution was applied to a column of DEAE-Sephadex A-25 (acetate form) equilibrated with distilled water and the adsorbed material was eluted with a linear gradient of 0 to 1 M acetic acid. The toxic fractions were collected and lyophilized.

Biological assay: Enterotoxigenic activity was assayed in suckling mice of 2-4 days old, as describe previously [2]. Samples of 0.1 ml, with 0.001% Evans blue as a marker, were administered by gastric tube to mice. Four hours after administration of the samples, unless otherwise indicated, animals were sacrificed by inhalation of chloroform. The presence of the dye in the intestinal lumen was confirmed and the entire intestine was removed. 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 amount of sample giving a fluid accumulation ratio of over 0.09 was designated as 1 mouse unit (MU), as reported in a previous paper [2].

Heat treatment: Separate solutions of 1 mg of purified native $ST_h$ [4] and synthetic $ST_h$ [8] and synthetic $ST_h$(6-19) dissolved in 5 ml of 0.01M phosphate buffer (pH 7.2) were used, because at this concentration variations in HPLC profiles could be followed conveniently. Aliquots of 100 µl of these solutions were sealed in capillary tubes and placed for appropriate times in an electric oven controlled at the required temperature. Then the tubes were rapidly cooled to room temperature and 50 µl of the solution was withdrawn and injected onto a column of HPLC, as described below. The remainder of the sample was diluted 10-fold with the same buffer solution and its toxic activity was assayed as described above.

High-performance liquid chromatography (HPLC): HPLC was performed on a column of LiChrosorb RP-8 (Merck, 5 µm, 4 x 250 mm). The column was equilibrated with 10% acetonitrile containing 0.01M ammonium acetate (pH 5.7) and then the sample solution was injected. The column was developed with a linear gradient of 10-35% acetonitrile in 0.01M ammonium acetate (pH 5.7) by ascending chromatography with increase of 1%/min of acetonitrile at a flow rate of 0.5 ml/min.

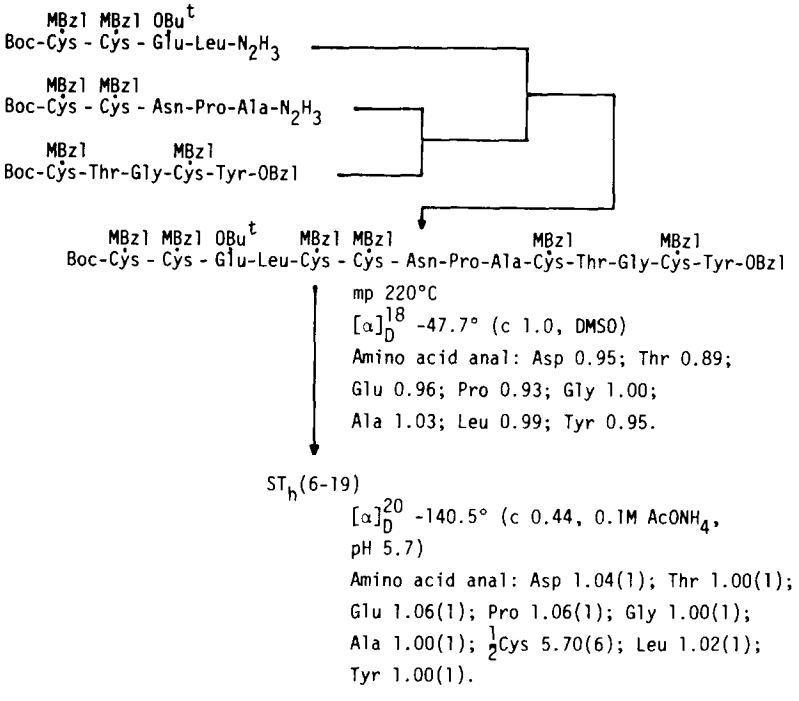


Fig. 2. Scheme of synthesis of a shorter analog of $ST_h$, $ST_h$(6-19), with some data on synthetic materials [13].

## RESULTS AND DISCUSSION

A shorter synthetic analog of $ST_h$, $ST_h(6\text{-}19)$, was purified by chromatography on DEAE-Sephadex A-25 under similar conditions to those for synthetic $ST_h$ [8]. The peptide appeared almost pure on reversed-phase HPLC as shown in Fig. 3. $ST_h$ was synthesized in only about 2.5% yield from the protected peptide by deprotection, air-oxidation, ion-exchange chromatography, and HPLC, but this shorter analog $ST_h(6\text{-}19)$ was obtained in about 23% yield, suggesting that the analog tended to be folded to the thermodynamically stable native structure.

Chan and Giannella [6] reported that the peptide of 14 amino acids remaining after digestion of $ST_p$ with leucine aminopeptidase retained biological activity, although they did not isolate it nor measure the degree of its biological activity. We tested the toxic activity of the shorter analog $ST_h(6\text{-}19)$ in suckling mice in comparison with those of native $ST_h$ [4] and

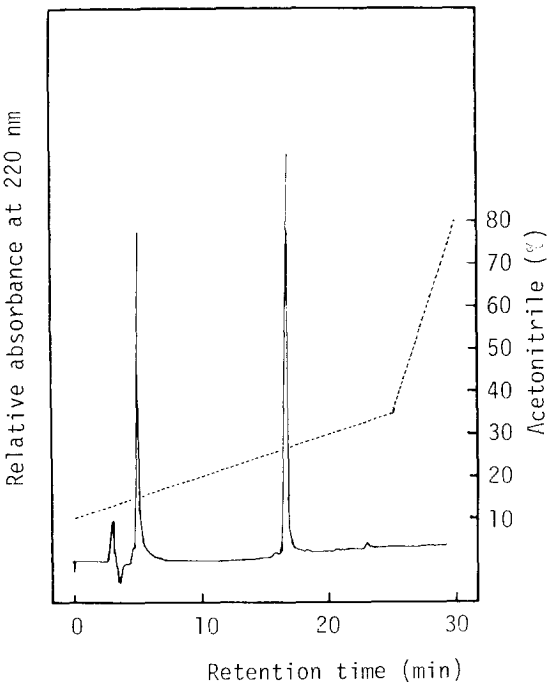


Fig. 3. HPLC profile on a LiChrosorb RP-8 column of $ST_h(6\text{-}19)$ purified on DEAE-Sephadex A-25. A sample was dissolved in ammonium acetate and applied.

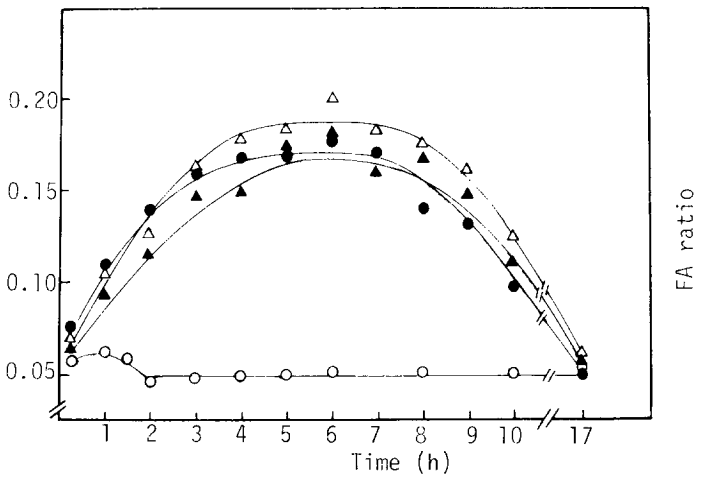


Fig. 4. Time courses of fluid accumulation caused by native $ST_h$ (●), synthetic $ST_h$ (△), synthetic $ST_h$(6-19) (▲), and phosphate buffer saline (○) in suckling mice. 3 MU of each ST was administered to suckling mice. Values are means of six determinations.

synthetic $ST_h$ [8]. The time course of action of $ST_h$(6-19) was found to be similar to that of $ST_h$ (Fig. 4), but the minimum effective dose of $ST_h$(6-19) was 0.4-0.8 ng, indicating that its activity was 2-5 times that of the native $ST_h$ on a molar basis. Fluid accumulation in suckling mice caused by the shorter analog $ST_h$(6-19) was found to be completely inhibited by antiserum against purified native $ST_h$, which was prepared as described previously [11].

Next, we examined the effect of heating on purified native $ST_h$, synthetic $ST_h$ and the shorter analog $ST_h$(6-19) by HPLC and biological assay, because the heat-stability of ST was not quantitated in early studies, although the name "heat-stable enterotoxin" was based on the observation that enterotoxigenic activity was still detectable after heating the toxin in boiling water for 30 min [1, 12]. We were particularly interested in the heat-stability of the shorter analog $ST_h$(6-19), because the analog had more biological activity than $ST_h$, as described above. After heating at 100°C for 30 min, the peak area on HPLC of native $ST_h$ was considerably reduced, as shown in Fig. 5c. The toxic activity of native $ST_h$ was found to be significantly destroyed, as described in [2]. When the shorter analog was heated at 100°C for 30 min, its peak area on HPLC was only reduced to 75% of that of the untreated material and the biological activity remained almost unchanged. Surprisingly, the analog still

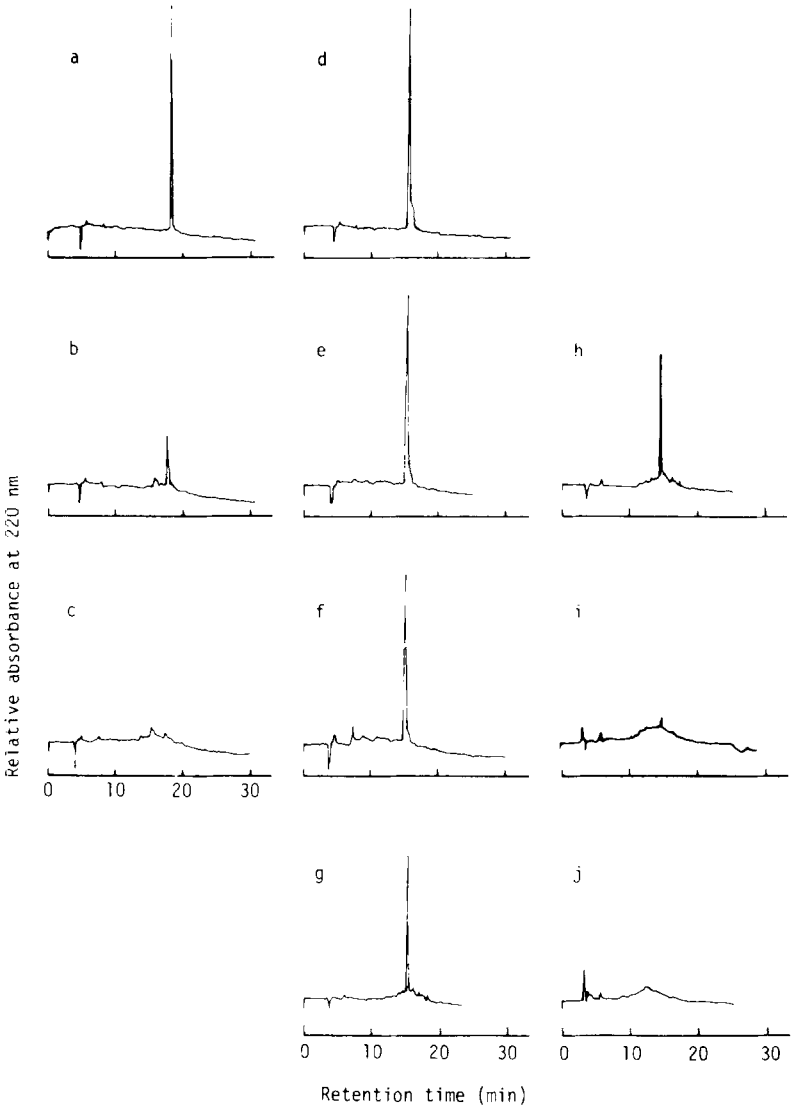


Fig. 5. HPLC profiles on LiChrosorb RP-8 of: (first column) $ST_h$ a) untreated, b) after 10 min at 100°C, c) after 30 min at 100°C, (second and third column) synthetic $ST_h$(6-19) d) untreated, e) after 10 min at 100°C, f) after 30 min at 100°C, g) after 60 min at 100°C, h) after 10 min at 120°C, i) after 30 min at 120°C, and j) after 60 min at 120°C.

showed about two-thirds of the original peak area even after treatment at 100°C for 60 min (Fig. 5g) or 120°C for 10 min (Fig. 5h), although the peak was almost abolished after heating at 120°C for 30 min (Fig. 5i) or at 140°C for 10 min (data not shown). Thus, the shorter analog $ST_h$(6-19) was highly heat-stable. The enhanced structural stability of this shorter analog may be due to decreased

perturbation of the molecule on heating as a result of the absence of the N-terminal sequence.

The present finding that the C-terminal 14 amino acid peptide, synthesized in the present work, was highly potent and heat-stable suggests that this sequence constitutes the thermodynamically stable and biologically active site of the toxin.

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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.