Volume 181, number 1 FEBS 2232 February 1985

Essential structure for full enterotoxigenic activity of heat-stable enterotoxin produced by enterotoxigenic *Escherichia coli*

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Received 3 October 1984

Several analogues of heat-stable enterotoxins (ST_h and ST_p) produced by enterotoxigenic *Escherichia coli* were synthesized. Peptides (ST_h[6–18] and ST_p[5–17]) consisting of 13 amino acid residues from the Cys residue near the N-terminus to the Cys residue near the C-terminus and linked by three disulfide bonds had the same biological and immunological properties as native ST_h and ST_p, respectively. The results indicated that the sequence with the 13 amino acid residues and three disulfide linkages is essential for full biological activity of ST.

Enterotoxigenic E. coli *Heat-stable enterotoxin* *Structure* *Synthesis*

1. INTRODUCTION

Various strains of enterotoxigenic *Escherichia coli* (ETEC) from animal hosts produce heterologous heat-stable enterotoxins (ST) that cause watery diarrhea in man and various domestic animals [1]. ST is reported to stimulate the guanylate cyclase-cyclic GMP system and raise the level of cyclic GMP in ST-sensitive cells [2–5], although the biochemical processes involved in development of watery diarrhea are still unknown. Recently, we [6–8] and others [9–11] isolated and purified two kinds of STs from human and porcine strains of ETEC and determined their amino acid sequences. As shown in fig.1, the sequences of the two STs from the Tyr residue near the N-terminus to the Tyr residue at the C-terminus are identical except at position 4 from the C-terminus. We synthesized ST_h and ST_p chemically and confirmed that they have the same biological and physicochemical properties as native ST_h and ST_p, respectively [12–14]. Furthermore, we found that 5 and 4 N-terminal amino acid residues of ST_h and ST_p, respectively, are not essential for expression of the enterotoxigenic activity of ST in suckling mice or for stability on heating [15,16]. The toxic activities of ST_h and ST_p were also found to be neutralized by homologous anti-ST antisera raised in rabbits, and to be cross-neutralized by heterologous anti-ST antisera [17]. These findings indicated that the common sequences are important for expression of the biological and immunological activities and heat-stability of ST. Thus, it seemed interesting to elucidate the minimum structure of ST necessary for expression of full biological activity.

This paper reports the preparation of several analogues of ST_h and ST_p lacking the C-terminal Tyr residue. We found that the sequence from the Cys residue near the N-terminus to the Cys residue near the C-terminus with 3 disulfide linkages showed full biological activity.

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

1 5 10 15

$\mathrm{ST_h}^{a)}$ Asn-Ser-Ser-Asn-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys-Tyr

$\mathrm{ST_p}^{b)}$ Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr

1 5 10 15

Fig.1. Amino acid sequences of ST: (a) from [7] and (b) from [8,10,11]. Underlined regions indicate sequence homology in the two STs.

2. MATERIALS AND METHODS

2.1. *Preparation of ST_h and ST_p analogues*

$\mathrm{ST_h}$ and $\mathrm{ST_p}$ analogues ($\mathrm{ST_h}$[1–19], $\mathrm{ST_h}$[5–19], $\mathrm{ST_h}$[6–19], $\mathrm{ST_p}$[1–18], $\mathrm{ST_p}$[4–18], and $\mathrm{ST_p}$[5–18]) were synthesized by reported procedures [12,14,16,18]. Samples of 200 μg of these $\mathrm{ST_h}$ and $\mathrm{ST_p}$ analogues were dissolved in 200 μl of 0.1 M pyridine-acetate, at pH 8.1, and treated with carboxypeptidase B (Boehringer, Mannheim) at a substrate-enzyme ratio of 33:1 at 37°C for 16 h. This commercial enzyme released C-terminal neutral amino acid residues. The digestion was stopped by addition of glacial acetic acid, digests were applied directly to a reversed-phase column of HPLC under the conditions described below, and peak fractions of eluate were separated and lyophilized.

2.2. *Chemical synthesis of ST_h[6–18]*

Protected linear peptide with the amino acid sequence of $\mathrm{ST_h}$[6–18] was synthesized by conventional methods, as described for the synthesis of $\mathrm{ST_h}$[6–19] [15]. The protected peptide was treated with anhydrous liquid HF and air-oxidized, the air-oxidized peptide being then purified by ion-exchange and high-performance liquid chromatographies.

2.3. *High-performance liquid chromatography (HPLC)*

HPLC was performed on a column (6 × 150 mm) of YMC-ODS S-5 (Yamamura Chemical Laboratory Co., Japan). The column was equilibrated with 10% acetonitrile containing 0.05% trifluoroacetic acid (pH 2.35) and then the sample solution was applied. The column was developed with a linear gradient of 10–40% acetonitrile in 0.05% trifluoroacetic acid (pH 2.35) with increase in acetonitrile of 1%/min at a flow rate of 1 ml/min.

2.4. *Biological assay*

Enterotoxigenic activity was assayed by measuring the amount of sample required to cause fluid accumulation in the intestine of 2–4-day-old suckling mice 4 h after its administration, as in [6]. 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 (minimum effective dose) giving a fluid accumulation ratio of over 0.09 was designated as 1 mouse unit. Neutralization of analogues of ST with anti-native ST antiserum was examined as in [17].

2.5. *FAB mass spectrometry*

Mass spectra were recorded with a Jeol JMS-HX100 double-focusing mass spectrometer equipped with an FAB ion source, as in [8].

2.6. *Heat stability*

Synthetic peptides were tested for heat stability by HPLC as in [15].

3. RESULTS AND DISCUSSION

To elucidate the minimum structure of ST essential for its full biological activity and to determine the effect of the C-terminal Tyr residue on ST activity and heat stability, we first prepared several analogues of $\mathrm{ST_h}$ and $\mathrm{ST_p}$ lacking a C-terminal Tyr residue, as shown in table 1. These peptides were obtained by treatment of $\mathrm{ST_h}$ and $\mathrm{ST_p}$ analogues [16,18] with carboxypeptidase and were finally purified to homogeneity by reverse-phase HPLC. An example of the results is shown in fig.2. The products were analyzed by FAB mass spectrometry and by amino acid analysis in the acid hydrolysates, as summarized in table 2. The biological activity of all the analogues of ST was almost the same within the limits of experimental error as native ST in the suckling mouse assay, as

Table 1

Synthetic analogues of ST and their biological properties

	Synthetic Peptides	Minimum effective dose (ng)	Neutralization by anti-native ST antiserum
	1 5 10 15		
ST_h[1-19]	Asn-Ser-Ser-Asn-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys-Tyr	0.8[a)]	+
ST_h[1-18]	Asn-Ser-Ser-Asn-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys	0.4	+
ST_h[5-19]	Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys-Tyr	0.8[a)]	+
ST_h[5-18]	Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys	0.5	+
ST_h[6-19]	Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys-Tyr	0.6[a)]	+
ST_h[6-18]	Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys	0.6	+
	1 5 10 15		
ST_p[1-18]	Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr	1.0[b)]	+
ST_p[1-17]	Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys	1.3	+
ST_p[4-18]	Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr	0.8-1.0[c)]	+
ST_p[4-17]	Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys	1.2	+
ST_p[5-18]	Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr	0.8-1.0[c)]	+
ST_p[5-17]	Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys	0.7	+

[a] Cited from [18]

[b] Cited from [14]

[c] Cited from [16]

seen in table 1. The enterotoxigenic activity of these analogues was neutralized by anti-native ST antiserum. The heat stability of these ST analogues was also tested (not shown). The analogues with the N-terminal sequences before the Tyr residue at positions 5 and 4 in ST_h and ST_p, respectively, showed the same stability as native ST, regardless of the presence of the C-terminal Tyr residue. In contrast, ST analogues lacking N-terminal sequences had higher heat stability than native ST. These results indicate that removal of the C-terminal Tyr residue does not affect the biological or immunological properties of ST_h or ST_p and that the sequences (ST_h[6–18] and ST_p[5–17]) with 13 amino acid residues from the Cys residue near the N-terminus to the Cys residue near the C-terminus have full biological activity.

To obtain sufficient ST_h[6–18] for investigation of the biological properties of the peptide with 13

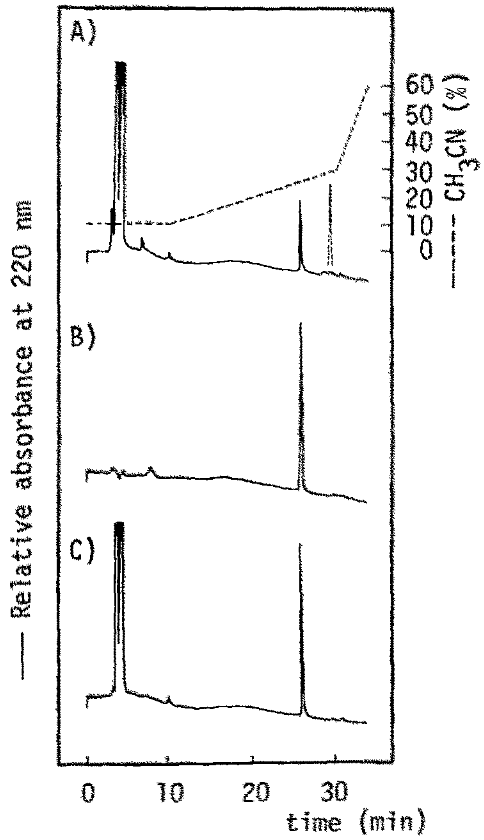

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Fig.2. HPLC on a reversed-phase column (YMC-ODS, 5 μm particle size, 6 × 150 mm): (A) A CPase B digest of ST_h[6–19]; (B) synthetic ST_h[6–18]; (C) a mixture of (A) and (B). The peak shown by a dotted line in (A) denotes the elution position of ST_h[6–19].

Table 2

Amino acid compositions and mass values of synthetic analogues of ST[a]

	ST_h[1-18]	ST_h[5-18]	ST_h[6-18]	ST_h[6-18][b)]	ST_p[1-17]	ST_p[4-17]	ST_p[5-17]
Asp	3.00(3)	1.02(1)	1.06(1)	1.03(1)	1.94(2)	1.08(1)	1.08(1)
Thr	0.95(1)	1.02(1)	1.03(1)	1.01(1)	0.98(1)		
Ser	1.91(2)						
Glu	1.07(1)	1.01(1)	1.03(1)	1.02(1)	1.01(1)	1.03(1)	1.06(1)
Pro	1.13(1)	0.97(1)	1.00(1)	1.03(1)	1.08(1)	1.26(1)	1.30(1)
Gly	1.05(1)	1.05(1)	1.01(1)	1.02(1)	0.98(1)	1.09(1)	0.99(1)
Ala	1.00(1)	1.00(1)	1.00(1)	1.00(1)	2.00(2)	2.00(2)	2.00(2)
½Cys	5.53(6)	5.89(6)	5.33(6)	5.46(6)	5.52(6)	5.00(6)	5.67(6)
Leu	1.07(1)	1.09(1)	1.04(1)	1.03(1)	1.07(1)	1.10(1)	1.00(1)
Tyr	0.97(1)	0.95(1)			0.94(1)	0.95(1)	
Phe					1.04(1)		
$[M+H]^+$	1878.6 (1878.6)	1476.4 (1476.4)	1313.0 (1313.4)	1313.5 (1313.4)	1808.5 (1808.6)	1446.5 (1446.4)	1283.4 (1283.3)

[a] Values were calculated as mol/mol of Ala; numbers in parentheses indicate theoretical values

[b] This peptide was synthesized chemically

amino acid residues, we synthesized ST_h[6–18] chemically by the scheme reported in [15]. STs are linked intramolecularly by 3 disulfide bonds, although the exact positions of the disulfide bonds have not yet been determined. To determine whether synthetic ST_h[6–18] has the same disulfide linkages as native ST_h, we compared synthetic ST_h[6–18] with that with the same secondary structure as native ST_h [18] obtained from ST_h[6–19], by enzymatic digestion. This peptide had the same retention time on HPLC (fig.2) and the same biological activity as ST_h[6–18] obtained from ST_h[6–19] by carboxypeptidase digestion. The peptide can be easily synthesized in large quantities, because it has less amino acid residues than native toxin and is formed in good yield from a synthetic linear peptide by air oxidation [18]. This peptide will be of practical use in an ST-enzyme-linked immunoassay (ELISA) [19].

Previously, we synthesized a peptide with the amino acid sequence of ST proposed by Chan and Giannella [9], in which the Asn and Tyr residues at position 11 and the C-terminus of ST_p are replaced by Tyr and Asn, respectively. The peptide had 30–40-times less enterotoxigenic activity than the native toxin [14], suggesting that this substitution reduces the biological activity of ST partially, but not completely. The residue at position 4 from the C-terminus of ST is Thr in ST_h and Ala in ST_p, but this difference does not influence the activity of ST. On the other hand, the reduction of disulfide linkages abolished completely the toxic activity [9]. Thus, we conclude from this work that at least 13 amino acid residues from the Cys residue near the N-terminus to the Cys residue near the C-terminus with 3 disulfide bonds are necessary for expression of the full biological activity of ST.

ACKNOWLEDGEMENT

This work was partly supported by Grant-in-Aid for Scientific Research no.58122002 from the Ministry of Education, Science and Culture of Japan.

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