

Characterization of a highly toxic, large molecular size heat-stable enterotoxin produced by a clinical isolate of *Yersinia enterocolitica*

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Abstract A novel heat-stable enterotoxin (ST) designated as Y-STc was purified to homogeneity from the culture supernatant of a pathogenic strain of *Yersinia enterocolitica* serotype O3 and its amino acid sequence was determined. The mature Y-STc was found to consist of 53 amino acid residues, which includes the putative pro-sequence. The molecular weight of Y-STc was 5638 and constituted the largest molecular size in the family of currently known STs. The minimum effective dose of purified Y-STc in the suckling mouse assay was 0.6 ng (0.1 pmol), indicating that, despite the long sequence, Y-STc is the most toxic in the ST family.

Key words: Heat-stable enterotoxin; Amino acid sequence; Purification; Pro sequence; *Yersinia enterocolitica*

1. Introduction

Heat-stable enterotoxins (STs) are peptide toxins produced by bacterial enteropathogens such as enterotoxigenic *Escherichia coli* [1,2], *Yersinia enterocolitica* [3–5] and *Vibrio cholerae* [6–8], and are responsible for acute diarrhea in humans. ST binds to a membrane-bound guanylate cyclase which serve as a specific cell surface receptor for ST, followed by elevation of the intracellular cGMP level [9–11].

Y. enterocolitica is a Gram-negative bacillus frequently associated with human gastrointestinal disorders [12,13]. It is well known that pathogenic strains of *Y. enterocolitica* produce an ST named Y-ST, which is thought to be involved in the virulence of the organism [14–17]. In addition to the known Y-ST, the existence of novel enterotoxins produced by *Y. enterocolitica* was documented by demonstrating that strains which failed to hybridize with a probe for the gene encoding Y-ST still possessed enterotoxic activity [18]. Based on the reactivity with monoclonal antibodies against known STs, the novel Y-STs were classified in to two types. We designated the classical Y-ST as Y-STa and the novel STs as Y-STb and Y-STc. Quite recently, we reported the amino acid sequence of Y-STb [5]. In this paper, we describe the purification and structure of the novel Y-STc produced by a clinical isolate of *Y. enterocolitica* O3. Interestingly, despite having the largest molecular size in the ST family, Y-STc possessed the highest toxic activity.

2. Experimental

2.1. Bacterial strain and culture conditions

A clinical strain (86-11) of *Y. enterocolitica* belonging to the serotype O3 isolated from a patient in Japan was kindly provided by Dr. T. Maruyama, The National Institute of Public Health, Japan. The strain was cultured as previously described [19].

2.2. Purification of Y-STc

The culture supernatant of *Y. enterocolitica* strain 86-11 was fractionated by ammonium sulfate precipitation (80% saturation). After centrifugation (15,000 × g, 30 min, 4°C), the resulting pellets were resolved in buffer A (0.1 M NaCl, 10 mM Tris-HCl, pH 7.2) and dialyzed against buffer A. The dialyzate was applied to a DEAE-Sephacel column (2 × 26 cm) equilibrated with buffer A. The column was washed with 500 ml of buffer A and eluted with a linear gradient from 0.1 to 0.5 M NaCl in a total volume of 500 ml, at a flow rate of 20 ml/h. An aliquot of each fraction was tested for enterotoxicity in the suckling mouse. The active fractions were concentrated, dialyzed against buffer B (0.3 M NaCl, 10 mM Tris-HCl, pH 7.2), and then subjected to gel-filtration on a Sephacryl S-100 HR column (2 × 92 cm) equilibrated with buffer B. The elution was performed using buffer B at a flow rate of 10 ml/h. The toxic fractions were purified further by reverse-phase high-performance liquid chromatography (RP-HPLC). The HPLC instrument used in this study was a high-pressure gradient system which consisted of two Waters model 510 HPLC Pumps. The absorbance of the eluent was monitored at 214 and 280 nm, using Waters Absorbance Detectors model 440 and 441, respectively. The following elution programs were used for the purification of Y-STc. Program I, column: CAPCELL PAK C18 SG120 (4.6 × 250 mm); elution: linear gradient of 10–35% acetonitrile (1%/min) in 10 mM ammonium acetate (pH 5.7), flow rate: 1.0 ml/min. Program II, column: YMC R-ODS-5 (4.6 × 250 mm); elution: linear gradient of 15–45% acetonitrile (1%/min) in 0.1% trifluoroacetic acid (TFA); flow rate: 1.0 ml/min. Program III, column: Develosil ODS-T-5 (4.6 × 150 mm); elution: linear gradient of 10–40% acetonitrile (1%/min) in 10 mM ammonium acetate (pH 5.7); flow rate: 1.0 ml/min. Program IV, column: Develosil ODS-T-5 (4.6 × 150 mm); elution: linear gradient of 5–50% acetonitrile (1%/min) in 0.1% TFA; flow rate: 1.0 ml/min.

2.3. Suckling mouse assay

Toxic activity was assayed in the suckling mouse as previously described [20]. The fluid accumulation (FA) ratio was calculated as the ratio of the weight of the entire intestines of the suckling mouse to that of the rest of the body. An FA ratio of over 0.09 was considered to indicate a positive response. The minimum effective dose (MED) was the minimum amount (weight or mole) of toxin giving the positive response. The amount (mole) of Y-STc was calculated from the data from amino acid analysis of the acid hydrolysate.

2.4. Sequence determination

The purified toxins were digested with endoproteinase Asp-N in 100 mM sodium phosphate buffer (pH 7.9) for 7 h at 37°C as described previously [5]. The digests were separated by RP-HPLC on a Develosil ODS-T-5 column (4.6 × 150 mm) with a linear gradient of 2–50% acetonitrile (1%/min) in 0.1% TFA, at a flow rate of 1.0 ml/min. The amino acid sequences of the resulting peptide fragments were determined by automated Edman degradation using an ABI 437A gas-phase protein sequencer or fast atom bombardment tandem mass spectrometry.

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(FAB-MS/MS) using a JEOL JMS-HX/HX110A four-sector tandem mass spectrometer as previously described [21,22]. The C-terminal fragments were identified by the procedure of Rose et al. with some modifications [5,21,23]. Electrospray ionization mass spectrometry (ESI-MS) and amino acid analysis were carried out as previously described [5].

3. Results and discussion

3.1. Isolation of Y-STc

The culture supernatant of strain 86-11 was partially purified by ammonium sulfate precipitation, DEAE-Sephacel ion-exchange chromatography, Sephacryl S-100 HR gel-filtration and RP-HPLC (Program I), as described in section 2. The toxic fractions obtained by RP-HPLC using Program I were separated further by RP-HPLC using Program II to yield two active peaks, as shown in Fig. 1. Each peak fraction was individually rechromatographed using Program III and then further purified by using Program IV. Two different toxic peptides (T-1 and T-2) were purified from the respective active fractions.

3.2. Sequence determination of Y-STc

ESI-MS showed that the molecular weights of purified toxins were 5638 (T-1) and 5621 (T-2). On the basis of the observed molecular weights and the data from amino acid analysis, the amino acid compositions of the purified toxins were determined as shown in Table 1, indicating that both of the toxins consist of about 50 amino acid residues. Although the deduced amino acid compositions of T-1 and T-2 were nearly identical, their observed mass values were different. The mass difference of 17 atomic mass units strongly suggested that the N-termini of T-1 and T-2 are Gln and 5-oxo-2-pyrrolidinecarboxylic acid (pyroglutamic acid: pGlu) residues, respectively, and the remaining sequences are identical.

The primary structures of purified T-1 and T-2 were established, as shown in Fig. 2, by determination of the amino acid sequences of their endoproteinase Asp-N fragments. As estimated using the data from ESI-MS and amino acid analysis, the amino acid sequences of T-1 and T-2 were identical except for their N-termini which were Gln in T-1 and pGlu in T-2. Thus, we concluded that T-1 is the original Y-STc and that T-2 is a product where the N-terminal Gln residue of T-1 is converted into pGlu during the biosynthesis or purification. It was

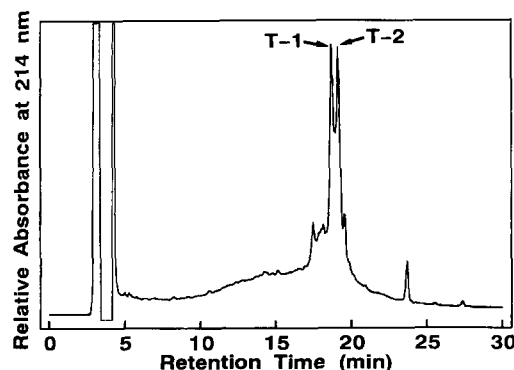


Fig. 1. The second RP-HPLC profile in the purification of Y-STc. The toxic fractions obtained by the first RP-HPLC were applied to a YMC R-ODS-5 column. The separation was carried out with a different solvent system (Program II) from the first RP-HPLC using Program I. See text for the details of chromatographic conditions.

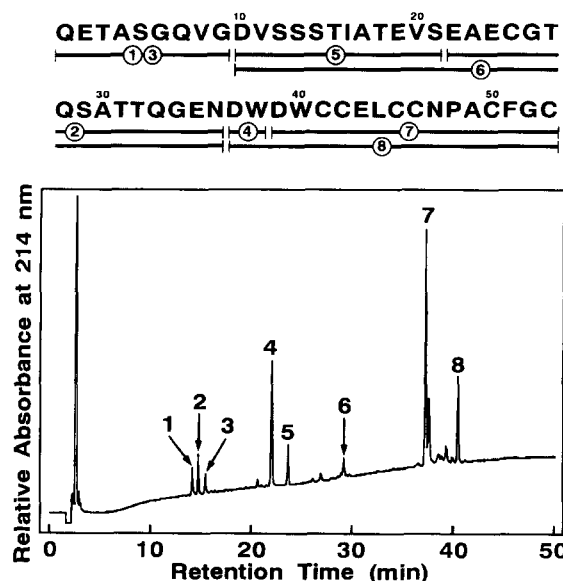


Fig. 2. Separation of the endoproteinase Asp-N digest of purified Y-STc (T-1). See text for chromatographic conditions. The amino acid sequences of the numbered fragments were determined by automated Edman degradation or FAB-MS/MS as shown in the upper panel. The N-terminal amino acid residue of fragment 3 was pGlu and the remaining sequence was identical to that of fragment 1. It appears that the N-terminal Gln residue of fragment 1 was converted into pGlu during the digestion. Based on these data, the amino acid sequence of Y-STc (T-1) was established as shown. The N-terminal sequence was confirmed by automated Edman degradation of intact T-1. Following separation of the endoproteinase Asp-N digest of T-2, fragments 2–8 were found.

demonstrated that the six Cys residues in the C-terminal portion formed three intramolecular disulfide linkages, by FAB-MS of the intact and reduced C-terminal fragments [5,22]. FAB-MS of fragment 6 showed that a component with a molecular weight of 134 linked to Cys²⁵ via a disulfide bond. Although the chemical structure of this component is still unclear, the same modification has been observed in Cys³ of Y-STa [3]. The molecular weight calculated from the determined structure is 5638, which is in good agreement with the molecular weight estimated by ESI-MS.

STs produced by various enteropathogenic bacteria share a highly conserved sequence:



This sequence constitutes the minimal structure essential for toxicity and has been designated as the 'toxic domain' or the 'core sequence' [24]. In Y-STc, the toxic domain is also conserved in its C-terminal portion.

The structural gene of Y-STa encodes a polypeptide with 71 amino acid residues composed of three regions (Fig. 3) [14]. The mature Y-STc purified from the culture supernatant consisted of 53 amino acid residues, which is similar to the length of the pro-form of Y-STa (Fig. 3). The length of the secreted mature polypeptides ranges from only 17 amino acid residues for NAG-ST and O1-ST produced by *V. cholerae* [6,8] to 30 amino acid residues for Y-STa and Y-STb produced by *Y. enterocolitica* [3,5]. To our knowledge, Y-STc has the largest molecular size in the ST family. The alignment of the amino acid

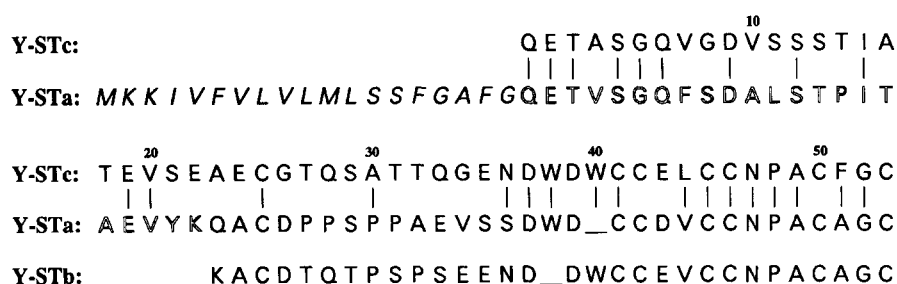


Fig. 3. Alignment of the amino acid sequences of the mature Y-STc, the precursor protein of Y-STa and the mature Y-STb. The precursor protein of Y-STa is composed of three regions which are the pre-sequence of 19 amino acid residues (in italics) corresponding to a signal peptide, the pro-sequence of 22 amino acid residues (in outline) and the mature Y-STa of 30 amino acid residues (in plain letters) [14]. The 26 identical residues between the mature Y-STc and the putative pro-form of Y-STa are indicated by vertical lines. The C-terminal part (23–53) of Y-STc and the mature Y-STb share 19 amino acid residues (61% homology).

sequences of Y-STc and the pro-form of Y-STa revealed that there is a sequence similarity of 49% (26 residues/53 residues) (Fig. 3). Some investigators have examined and discussed the role of the pro-sequence of ST in maturation [25–29]. In the present study, it was shown that the long polypeptide consisting of 53 amino acid residues, including the putative pro-sequence, is secreted as the mature toxin with a highly potent enterotoxigenicity. This implies that the proteolytic processing of a putative intermediate composed of the pro and mature regions appearing in the periplasm is not essential for the secretion of the mature toxin from the periplasmic space to the extracellular space through the outer membrane, and supports the model proposed by Guzmán-Verduzco and Kupersztoch [26,27].

3.3. Toxicity of Y-STc

The purified Y-STc (T-1) showed a MED of 0.6 ng (0.1 pmol) in the suckling mouse assay (Fig. 4). This indicates that Y-STc is 4-fold more potent than Y-STb (MED: 1.2 ng, 0.4 pmol) [5] and *E. coli* STh (MED: 0.8 ng, 0.4 pmol) [30], which have been known as the most toxic STs. The MED of T-2 was nearly identical to that of T-1, indicating that the conversion of N-terminal Gln to pGlu does not affect the enterotoxigenic activity. In ST molecules produced by *V. cholerae* O1, the molecules with the shorter N-terminal sequences showed more potent toxicities, and the MED of the largest one with 28 amino acid

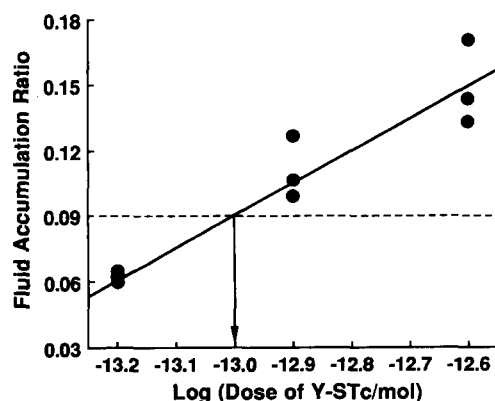


Fig. 4. Determination of the MED of Y-Stc in the suckling mouse assay. The MED was determined as the minimum amount of toxin giving an FA ratio of more than 0.09. By linear regression analysis ($r = 0.95$, $y = 1.964 + 0.144x$) of the data, the MED (in mole) of Y-Stc (T-1) was determined to be 0.1 pmol, which corresponded to 0.6 ng.

residues was ten times more than that of the smallest one with 17 amino acid residues [8]. In contrast, Y-STc was the most toxic in the ST family despite having the longest sequence. ST molecules in which Glu and Leu residues take the positions of acidic and aliphatic amino acid residues between two -Cys-Cys-sequences in the toxic domain, respectively, are known to possess high toxicity [31]. It has been reported that a Trp residue at a position just preceding the toxic domain functions to enhance the toxicity, as shown in Y-STb (Fig. 3) [5]. The highest enterotoxicity of Y-STc could be ascribed to a synergism of both the above properties which are present in Y-STc.

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Table 1
Amino acid compositions and observed mass values of the two enterotoxins (T-1 and T-2) purified from culture supernatant of *Y. enterocolitica* 86-11

	T-1	T-2
Asp	4.69 (5)	4.78 (5)
Thr	5.67 (6)	5.66 (6)
Ser	4.46 (6)	5.19 (6)
Glu	9.69 (10)	9.69 (10)
Pro	1.16 (1)	1.08 (1)
Gly	4.98 (5)	5.23 (5)
Ala	5.00 (5)	5.00 (5)
1/2Cys	ND (7)	ND (7)
Val	2.60 (3)	3.04 (3)
Ile	1.15 (1)	1.04 (1)
Leu	1.30 (1)	1.14 (1)
Phe	1.26 (1)	1.25 (1)
Trp	ND (2)	ND (2)
Total	(53)	(53)
ESI-MS	5638	5621

The amino acid composition is shown as a normalized value with alanine representing five residues. The numbers in parentheses refer to the number of residues in the peptide found by sequencing. 1/2Cys, half-cystine; ND, not determined.

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