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Purification and Partial Characterization of Heat-Stable Enterotoxin of Enterotoxigenic *Escherichia coli*

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Heat-stable enterotoxin was purified from a strain of enterotoxigenic *Escherichia coli* 53402 A-1 from human intestine. The cells were cultured in Casamino Acids–yeast extract–salts medium, and the purification procedure consisted of protamine sulfate treatment of the culture supernatant, ultrafiltration with an Amicon PM-10 membrane, diethylaminoethyl-cellulose column chromatography, hydroxyapatite column chromatography, Bio-Gel P-10 gel filtration, 90% ethanol extraction, and preparative polyacrylamide gel disc electrophoresis. About 300-fold purification was achieved, with a yield of about 12%. However, the homogeneity of the purified heat-stable enterotoxin was not rigorously demonstrated. The purified heat-stable enterotoxin had an absorption maximum at about 275 nm, and its isoelectric point was about 3.90. The molecular weight of the purified heat-stable enterotoxin was ca. 4,000 by Sephadex G-100 gel filtration. The minimum effective dose of purified heat-stable enterotoxin was about 2.5 ng in the suckling mouse assay. The purified heat-stable enterotoxin gave a positive reaction in not only the suckling mouse assay but also the mouse intestinal loop test and the guinea pig skin permeability test.

Enterotoxigenic *Escherichia coli* produce two distinct enterotoxins: heat-stable, low-molecular-weight toxin (ST) and heat-labile, high-molecular-weight toxin (LT). LT has been purified by several workers (6–8, 10, 14, 19, 22), and some of its properties have been elucidated. Recently, Alderete and Robertson (1) reported the purification of ST from porcine strains of enterotoxigenic *E. coli* and some data on its properties. Mullan et al. (15) also reported the partial purification of ST and demonstrated the existence of two types of ST molecules (3). Moreover, Field et al. (9) studied the mode of action of a purified preparation of ST and found that cyclic guanosine 5′-monophosphate was involved in its action. This finding is supported by the results of Hughes et al. (12). This paper reports the purification and partial characterization of ST from a strain of enterotoxigenic *E. coli* from human intestine. A preliminary report of this work has been presented (14; Y. Takeda, T. Takeda, T. Yano, and T. Miwatani, Abstr. 14th U.S.-Japan Joint Conf. Cholera, Karatsu, Japan, 1978).

MATERIALS AND METHODS

Bacterial strain and culture of cells. *E. coli* 53402 A-1, an ST-producing strain provided by M. Merson, Johns Hopkins University, Baltimore, Md., was cultured in CAYE medium (16) without glucose at 37°C for 24 h with vigorous shaking. CAYE medium consists of 2% Casamino Acids (Difco Laboratories), 0.6% yeast extract (Difco), 0.25% NaCl, 0.871% K_2HPO_4, and 0.1% (vol/vol) of a trace salts solution (5% $MgSO_4$, 0.5% $MnCl_2$, 0.5% $FeCl_3$, and 0.001 N H_2SO_4). The pH of the medium was 8.6.

Preparation of crude toxin. Culture supernatants were collected by centrifugation at 15,000 × *g* for 20 min. Protamine sulfate (50 mg/100 ml) was added in the cold, and the resulting precipitates were discarded after centrifugation at 15,000 × *g* for 15 min. The supernatant was filtered through an Amincon PM-10 membrane, and the filtrate was concentrated by using a rotary evaporator. Then the concentrated material was dialyzed against 0.01 M phosphate buffer (Na_2HPO_4-KH_2PO_4, pH 7.0), using Spectra/por 6 membrane tubing (Spectrum Medical Ind. Inc., Los Angeles, Calif.), which cuts off molecules with molecular weights of less than 1,000. The dialyzed material was used as crude toxin.

Diethylaminoethyl-cellulose column chromatography. Crude toxin was applied to a diethylaminoethyl-cellulose column (4 by 60 cm) equilibrated with 0.01 M phosphate buffer, pH 7.0. Material was eluted with about 1,500 ml of the same buffer and then with 3,000 ml of a linear gradient of 0 to 0.8 M NaCl in the same buffer. Fractions containing toxic activity were collected, concentrated by using a rotary evaporator, and dialyzed against 1 mM phosphate buffer (pH 7.0), using Spectra/por 6 membrane tubing.

Hydroxyapatite column chromatography.

Fractions with toxic activity from the diethylaminoethyl-cellulose column were applied to a hydroxyapatite column (2 by 20 cm) equilibrated with 1 mM phosphate buffer (pH 7.0). Material was eluted with 500 ml of 1 mM phosphate buffer (pH 7.0) and then with 300 ml of 0.2 M phosphate buffer (pH 7.0), and fractions containing toxic activity were collected and concentrated by evaporation.

Bio-Gel P-10 column chromatography. Fractions with toxic activity from the hydroxyapatite column were applied to a Bio-Gel P-10 column (2 by 120 cm) equilibrated with 0.01 M phosphate buffer (pH 7.0). Material was eluted with the same buffer, and fractions with toxic activity were collected and dried by evaporation.

Extraction of the toxin with ethanol. Dried material from the Bio-Gel P-10 column was dissolved in a small volume of distilled water, and then absolute ethanol was added to a final concentration of 90%. The toxin recovered in the 90% ethanol-soluble fraction was dried by evaporation, dissolved in a small volume of distilled water, and dialyzed against 0.01 M phosphate buffer (pH 7.0) by using Spectra/por 6 membrane tubing.

Polyacrylamide gel disc electrophoresis. Polyacrylamide gel disc electrophoresis was carried out essentially as described by either Davis (5) or Williams and Reisfeld (23). The concentrations of acrylamide, *N,N'*-methylenebisacrylamide, and ammonium persulfate were as specified. Electrophoresis was performed at a constant current of 2 mA/tube for 3 to 7 h.

Preparative polyacrylamide gel disc electrophoresis. Preparative polyacrylamide gel disc electrophoresis was carried out on Canalco Prep-Disc (Canal Industrial Co., Rockville, Md.). A PD2/320 column was used. The conditions of the electrophoresis were as described by Williams and Reisfeld (23), except that concentrations of acrylamide, *N,N'*-methylenebisacrylamide and ammonium persulfate were 14, 0.08 and 0.035%, respectively. The gel was 3 cm in length. Electrophoresis was performed at a constant current of 5 mA.

Assay of ST activity in suckling mice. ST activity was assayed in suckling mice as described previously (21). Breeding colonies of white mice (Nippon Clea Co., Osaka, Japan) was established in our laboratory, and suckling mice of 2 to 3 days old were used in assays. A 0.1-ml amount of the samples was administered by gastric tube to each mouse, with about 0.001% Evans blue dye as a marker. At the time indicated after administration of the sample, animals were sacrificed by inhalation of chloroform. After confirming the presence of dye in the intestinal lumen, the entire intestine was removed. The fluid accumulation (FA) 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 ST giving an FA ratio of more than 0.09 after 3 h was tentatively designated as 1 mouse unit.

Determination of molecular weight by Sephadex G-100 gel filtration. The molecular weight of the purified ST was determined by Sephadex G-100 gel filtration by using hen egg albumin, chymotrypsinogen, cytochrome C, and insulin α-chain as standard proteins. The column (2 by 65 cm) was eluted with 0.01 M phosphate buffer (pH 7.0). The absorbance of standard proteins at 280 nm and the ST activity in suckling mice in the eluate were determined.

Isoelectric focusing of purified ST. Electrofocusing of purified ST by using ampholine carrier ampholytes was carried out in an LKB electrofocusing system 8010-10 (LKB Produckter AB, Sweden), with a pH gradient of 3 to 5. About 15,000 mouse units of ST was applied, and electrophoresis was performed at a constant voltage of 450 V for 42 h. The ST activity of each fraction was determined in suckling mice as described above.

Heat treatment of purified ST. The purified ST was heated at 100°C, and remaining activity was assayed in suckling mice.

pH treatment of purified ST. Purified ST (500 ng/ml) in 0.01 M phosphate buffer (pH 7.0) was adjusted to various pH values with 0.1 N HCl or 0.1 N NaOH and incubated at 37°C for 30 min. Then the solution was readjusted to pH 7.0 with NaOH or HCl, and ST activity was assayed in suckling mice after appropriate dilution.

Intestinal loop test in mice. The intestinal loop test in mice was carried out essentially as described previously (24). Male ddO strain mice weighing 20 to 25 g were used without starvation. The mice were anesthesized by intravenous injection (0.1 mg/g of body weight) of sodium pentabarbital, an incision was made along the abdominal midline, and the loop (7 to 14 cm long) of the jejunum was ligated. Then 0.2 ml of the sample was injected into the loop, and the abdomen was sutured. After an appropriate time, the animal was killed by cutting the thoracic aorta under anesthesia, and the abdomen was reopened. The loop was excised, its weight with (*A*) and without (*B*) fluid were measured and the weight of fluid ($A - B$) was calculated. One end of the loop was then attached to a clip and hung with another clip weighing 2 g on the other end, and the length was measured. Toxic activity (W/L ratio) was calculated as the ratio of the weight of fluid accumulated, in mg, to the length of the loop, in cm.

Skin permeability test on guinea pigs. The skin permeability test on guinea pigs was carried out essentially as described by Craig (4) and Ohashi et al. (18). The purified ST in 0.1 ml of phosphate-buffered saline was injected intracutaneously in the back of male albino guinea pigs weighing about 800 to 1,000 g. The back was clipped before injection. The animals were injected intravenously with 2.5% Evans blue in saline (0.2 ml/100 g of body weight) at the indicated time after injection of toxin. One hour later, the animals were sacrificed by bleeding, the skin was removed, and bluing of the skin was examined.

Determination of protein. Protein was determined with the Folin phenol reagent (13). In some cases, especially in purified ST, the method described by Bradford (2) was applied, using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.).

RESULTS

Purification of ST by column chromatography. Crude toxin, prepared as described above, was applied to a diethylaminoethyl-cel-

lulose column. A typical chromatographic profile on the column is shown in Fig. 1. Fractions containing toxin assayed in suckling mice and indicated by bars were collected, concentrated by evaporation, and dialyzed against 1 mM phosphate buffer (pH 7.0) in Spectra/por 6 membrane tubing. The preparation was then chromatographed on a hydroxyapatite column (Fig. 2). Fractions containing toxin (indicated by bars) were collected, concentrated by evaporation, and chromatographed on a Bio-Gel P-10 column (Fig. 3). Fractions containing toxin were collected and dried by evaporation. The dried material was dissolved in a small volume of distilled water, and absolute ethanol was added to a concentration of 90%. The material with toxicity was soluble in 90% ethanol solution. This fraction was then dried by evaporation, dissolved in a small amount of distilled water, dialyzed against 0.01 M phosphate buffer (pH 7.0) by using Spectra/por 6 membrane tubing and was used as partially purified ST in further analyses.

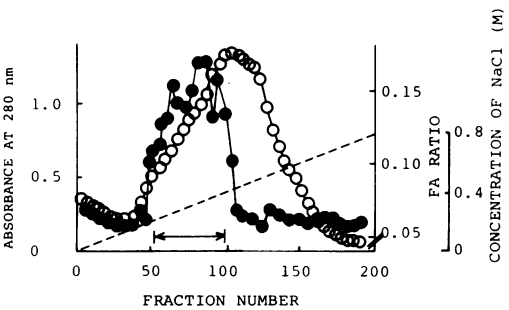

FIG. 1. *Diethylaminoethyl-cellulose column chromatogram of ST. Crude toxin was chromatographed on a diethylaminoethyl-cellulose column as described in the text. Symbols: ○, absorbance at 280 nm; ●, FA ratio determined as described in the text. Broken line indicates concentration of NaCl; bars (|↔|) indicate the fractions concentrated for further chromatography.*

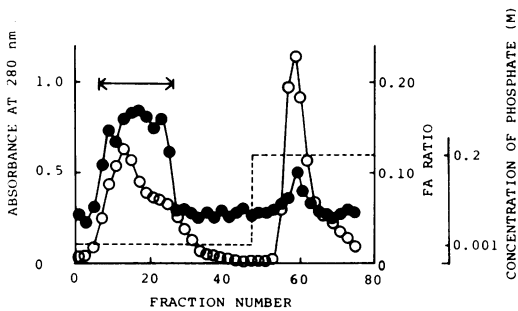

FIG. 2. *Hydroxyapatite column chromatogram of ST. The fraction from diethylaminoethyl-cellulose column was chromatographed on a hydroxyapatite column as described in the text. Symbols: ○, absorbance at 280 nm; ●, FA ratio determined as described in the text. Broken line indicates concentration of phosphate; bars (|↔|) indicate the fractions concentrated for further chromatography.*

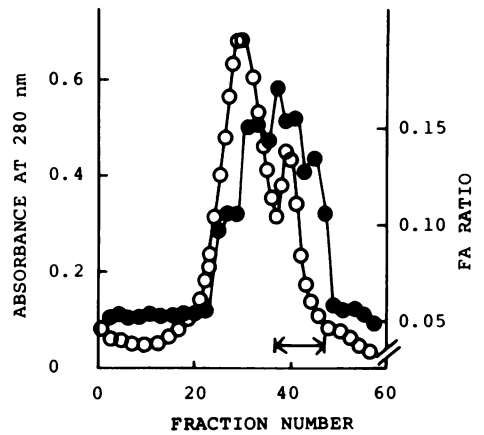

FIG. 3. *Bio-Gel P-10 column chromatogram of ST. The preparation from hydroxyapatite column was chromatographed on a Bio-Gel P-10 column as described in the text. Symbols: ○, absorbance at 280 nm; ●, FA ratio determined as described in the text. Bars (|↔|) indicate the fractions concentrated for further analysis.*

Polyacrylamide gel disc electrophoresis of partially purified ST. The partially purified ST prepared as described above was brown. This material was subjected to polyacrylamide gel disc electrophoresis to see whether the brown material was ST itself. After electrophoresis on gels containing different concentrations of acrylamide, the material was eluted from the gel and its activity was assayed. The brown material migrated with the ST activity in gel containing 7% acrylamide (Fig. 4A), but was separated from the ST activity in gel containing 14% acrylamide (Fig. 4B). Furthermore, when the running pH in the gel was lowered from 9.5 to 8.0, much more separation of ST activity from the brown material was achieved (Fig. 4C).

Purification of ST by preparative polyacrylamide gel disc electrophoresis. The partially purified ST was purified further by preparative polyacrylamide gel disc electrophoresis, using gel containing 14% acrylamide and pH 8.0 as the running pH. ST activity was separated from a major peak of brown material (Fig. 5). Fractions 42 through 58 were collected, concentrated by evaporation, dialyzed against 0.01 M phosphate buffer (pH 7.0), and used as purified ST in further studies.

Recovery of ST activity during purification. Table 1 summarizes typical data on the purification of ST. The specific activity of ST increased about 300-fold, and the recovery was about 12%. The specific activity of ST increased at each step of purification, but there was an

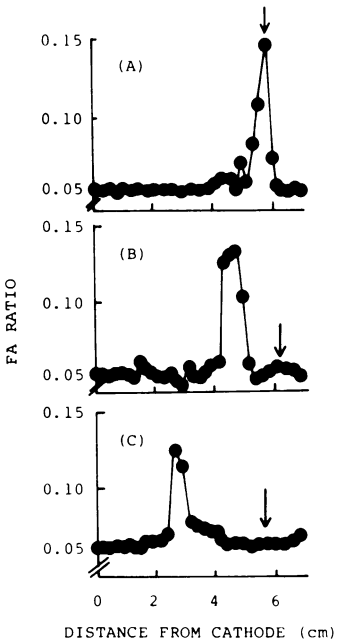

FIG. 4. *Polyacrylamide gel disc electrophoresis of partially purified ST. Polyacrylamide gel disc electrophoresis was carried out as described in the text. (A) Acrylamide, 7%; N,N'-methylenebisacrylamide, 0.18375%; ammonium persulfate, 0.07%; running pH, 9.5. (B) Acrylamide, 14%; N,N'-methylenebisacrylamide, 0.08%; ammonium persulfate, 0.035%; running pH, 9.5. (C) Acrylamide, 14%; N,N'-methylenebisacrylamide, 0.08%; ammonium persulfate, 0.035%; running pH, 8.0. After electrophoresis, the gel was cut into 2 mm thick sections, which were extracted with 0.01 M phosphate-buffered saline for about 16 h. The suspensions were centrifuged at 5,000 × g for 10 min, and the FA ratio of the supernatants was determined as described in the text. The arrows indicate the positions of brown material.*

appreciable loss of ST during the preparation of crude toxin.

Physicochemical properties of purified ST. The absorption spectrum of purified ST in Fig. 6 has a maximum at about 275 nm. Molecular weight was determined to be about 4,000 by Sephadex G-100 gel filtration (Fig. 7). The isoelectric point of the purified ST was abut 3.90 (Fig. 8). Results on the effect of heating purified ST are summarized in Tables 2 and 3. The purified ST was not inactivated by heating at 100°C for 10 min (Table 2). However, it was inactivated upon longer heating (Table 2) or upon heating at 100°C for 10 min at lower concentrations (Table 3). The effect of pH treatment on the purified ST is shown in Table 4; ST was stable at between pH 3 and 9, being more stable at acidic than alkaline pH values.

Biological activities of purified ST. Results of tests on the minimum effective dose of purified ST are shown in Table 5. About 2.5 ng of purified ST gave a positive FA ratio (more than 0.09) in suckling mice. Figure 9 shows the time course of action of ST in suckling mice. The reaction was positive as early as 30 min after the administration of purified ST and was maximal 3 h after the administration of toxin. Upon longer incubation the activity decreased, and the reaction was negative 17 h after the administration of the toxin.

Results of mouse intestinal loop tests on ST activity are shown in Fig. 10 and 11. Positive results were obtained with 16 mouse units of purified ST (Fig. 10). The time course of action of purified ST in the mouse intestinal loop (Fig. 11) was very similar to that in the suckling mouse assay; the reaction was maximal after 3 to 5 h and then decreased.

The purified ST gave a positive skin reaction in guinea pigs. The bluing reaction was positive as early as 1.5 h after administration of the purified toxin and was maximal after 2 to 3 h (Fig. 12). About 4 to 6 h after administration of the toxin, the reaction was not typical bluing, but an erythemal reaction which lasted as long as 18 h, although this is not shown clearly in Fig. 12. Figure 13 shows the results of the skin permeability test at 2 h after administration of various amounts of purified ST; higher concentrations of toxin gave stronger bluing reactions, but as little as 10 ng of purified ST gave a positive reaction.

DISCUSSION

Alderete and Robertson (1) reported extensive purification of ST from porcine strains of enterotoxigenic *E. coli*. They first developed a defined medium for ST production which contained four amino acids (alanine, serine, aspartic acid, and proline), salts, and a metal chelator. ST produced in this medium was purified by sequential

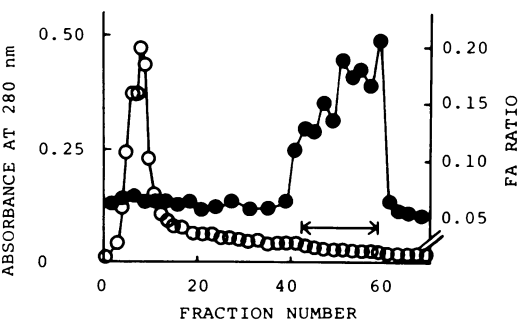

FIG. 5. *Preparative polyacrylamide gel disc electrophoresis of partially purified ST. Carried out as described in the text. Symbols: ○, absorbance at 280 nm; ●, FA ratio determined as described in the text. Bars (|↔|) indicate the fractions concentrated as purified ST.*

TABLE 1. *Recovery of ST activity during purification*

Fraction	Total protein (mg)[a]	Total activity (U)[b]	Sp act (U/mg)	Relative activity	Yield (%)
Culture supernatant	3,656	4,633,600	1,267	1	100
Crude toxin[c]	614	2,641,152	4,301	3.4	57
DEAE-cellulose column eluate	116	1,497,533	12,903	10.2	32.3
Hydroxyapatite column eluate	26.5	1,213,000	45,714	36.1	26.2
Bio-Gel P-10 column eluate	10.9	818,776	75,000	59.2	17.7
90% Ethanol extract	5.35	786,395	147,058	116.0	17.0
Preparative gel disc electrophoresis eluate	1.35	539,467	400,000	315.7	12.1

[a] Material from 2 liters of culture supernatant. Protein was determined with the Folin phenol reagent (13).
[b] One unit is defined as the minimum amount that gives an FA ratio of more than 0.09.
[c] Crude toxin was prepared as described in the text.

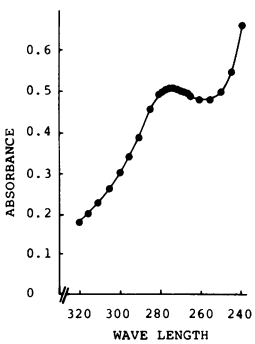

FIG. 6. *Ultraviolet absorption spectrum of purified ST. A solution of 85 μg of purified ST per ml in 0.01 M tris(hydroxymethyl)aminomethane-hydrochloric acid buffer (pH 7.8) was used.*

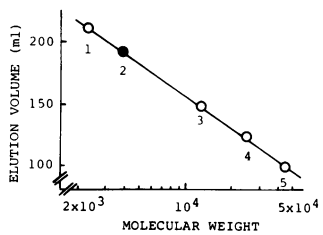

FIG. 7. *Determination of the molecular weight of purified ST by Sephadex G-100 gel filtration. Sephadex G-100 gel filtration of: (1) insulin α-chain (molecular weight = 2,300); (2) purified ST; (3) cytochrome C (molecular weight = 12,500); (4) chymotrypsinogen (molecular weight = 25,000); and (5) hen egg albumin (molecular weight = 45,000) was carried out separately as described in the text. The elution volumes of the standard proteins are plotted against the logarithms of their molecular weight, and the molecular weight of purified ST was calculated from its elution volume.*

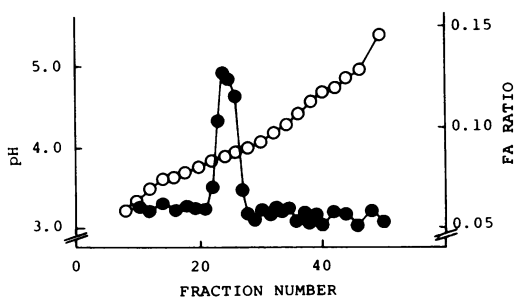

FIG. 8. *Isoelectric focusing of purified ST. Isoelectric focusing of purified ST was carried out as described in the text. Symbols: ○, pH; ●, FA ratio determined as described in the text.*

ultrafiltration, acetone fractionation, preparative gel electrophoresis, diethylaminoethyl-Bio-Gel A ion-exchange chromatography and Bio-Gel P-10 gel filtration. Our method of purification is rather similar to theirs, although some modifications were introduced. We used CAYE medium (16), as described above, and we purified ST by column chromatography on diethylaminoethyl-cellulose, hydroxyapatite and Bio-Gel P-10, and by ethanol extraction and preparative gel disc electrophoresis. It was noted that some properties of our final preparation are different from those reported by Alderete and Robertson (1). A major difference is that our preparation has an absorption maximum at about 275 nm and a ratio of absorbance at 275 nm (A_{275}) to A_{280} of about 1.03 (Fig. 6). Moreover, its A_{260} is less than its A_{280}. The preparation of Alderete and Robertson (1) had an absorption maximum at 270 nm, and the A_{260} was 10 to 20 times the A_{280}. Another difference was that our preparation of ST was separated from much brown material by disc gel electrophoresis on 14% acrylamide gel with running pH 8.0 (Fig. 4 and 5), although the final preparation still contained some colored material judging from its adsorption spectrum shown in Fig. 6. It is possible that these differences are due to differences in the strains used as sources of ST, because Alderete and Robertson (1) noted chemical differences between kinds of the ST produced by *E. coli*

TABLE 2. *Effect of duration of heating at 100°C on the activity of purified ST*

Amt of ST administered/mouse (ng)	FA ratio at the following conditions of heat treatment:[a]				
	37°C, 10 min	100°C, 10 min	100°C, 30 min	100°C, 60 min	100°C, 120 min
20	**0.185 ± 0.014**	**0.146 ± 0.029**	**0.120 ± 0.008**	**0.111 ± 0.010**	**0.093 ± 0.007**
10	**0.166 ± 0.021**	**0.168 ± 0.016**	**0.120 ± 0.012**	0.084 ± 0.008	0.073 ± 0.010
5	**0.115 ± 0.024**	**0.129 ± 0.025**	0.086 ± 0.002	0.071 ± 0.008	0.062 ± 0.005
2.5	**0.120 ± 0.008**	**0.101 ± 0.004**	0.068 ± 0.013	0.059 ± 0.008	0.057 ± 0.007
1.25	0.070 ± 0.003	0.055 ± 0.004	0.057 ± 0.003	0.056 ± 0.002	0.049 ± 0.002

[a] Purified ST at a concentration of 200 ng/ml was used. Each value indicates the mean ± standard error of five determinations. Values in boldface represent positive results.

TABLE 3. *Effect of concentration of ST, during heat treatment, on the activity of purified ST*

Concn of ST during heat treatment (ng/ml)	FA ratio at the following conditions of heat treatment:[a]	
	37°C, 10 min	100°C, 10 min
240	**0.186 ± 0.015**	**0.191 ± 0.018**
120	**0.177 ± 0.017**	**0.175 ± 0.013**
60	**0.143 ± 0.010**	**0.122 ± 0.008**
30	**0.115 ± 0.008**	0.073 ± 0.006

[a] Each value indicates the mean ± standard error of five determinations, using 0.1 ml of heat-treated preparation. Values in boldface represent positive results.

TABLE 4. *Effect of pH treatment on activity of purified ST*

pH	FA ratio with the following amt (ng) of ST administered/mouse:[a]			
	10	5	3	1
1	**0.108±0.008**	**0.098±0.007**	0.068±0.006	0.066±0.002
2	**0.122±0.013**	**0.103±0.018**	0.075±0.009	0.066±0.001
3	**0.135±0.016**	**0.123±0.012**	**0.110±0.011**	0.068±0.010
5	**0.133±0.006**	**0.121±0.013**	**0.099±0.010**	0.060±0.002
7	**0.118±0.013**	**0.125±0.013**	**0.095±0.008**	0.060±0.006
9	**0.127±0.012**	**0.105±0.011**	**0.120±0.010**	0.059±0.002
10	**0.111±0.005**	**0.101±0.008**	0.078±0.005	0.061±0.004
11	**0.128±0.006**	**0.101±0.003**	0.080±0.006	0.058±0.003
12	**0.092±0.002**	0.063±0.005	0.060±0.005	0.062±0.007

[a] Each value indicates the mean ± standard error of five determinations. Values in boldface represent positive results.

TABLE 5. *Activity of purified ST in suckling mice*

Amt of ST administered/mouse (ng)	FA ratio[a]
10	**0.154 ± 0.018**
7.5	**0.145 ± 0.009**
5.0	**0.130 ± 0.008**
2.5	**0.103 ± 0.030**
1.0	0.064 ± 0.003

[a] Each value indicates mean ± standard error of five determinations. Values in boldface represent positive results.

strains isolated from different animal species. The differences could also be due to differences in the media used or to differences in purity of preparations.

Our ST preparation was extensively purified

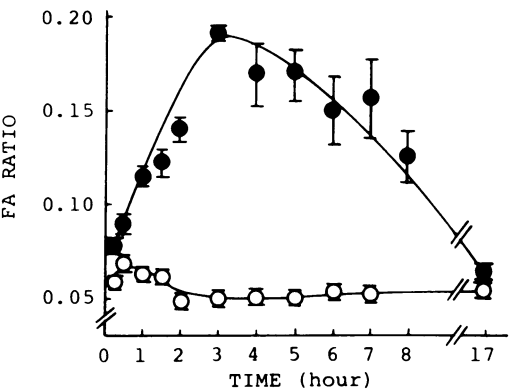

FIG. 9. *Time course of action of ST in suckling mouse assay. Either 3 mouse units of purified ST (●) or phosphate-buffered saline (○) was administered to suckling mice. Values represent means ± standard error of five determinations.*

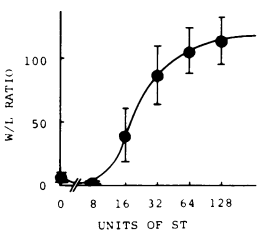

FIG. 10. *Dose response of purified ST in the mouse intestinal loop test. The indicated amount of purified ST was injected into intestinal loops and incubated for 3 h. The W/L ratio was calculated as described in the text. Values represent means ± standard error of 10 determinations.*

(Table 1); the activity of the material was increased about 300-fold, and the minimal effective dose of the purified material in suckling mice was as low as 2.5 ng (Table 4). However, we obtained no data on the homogeneity of the final preparation. The chemical nature of the final preparation was also not determined. As reported by Alderete and Robertson (1), after sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis, the purified ST did not stain with Coomassie brilliant blue. Moreover, we

found that the purified ST did not give any positive reaction in the determination of protein by the method described by Bradford (2), which consists of the binding of Coomassie brilliant blue to the NH_2 residue of the peptides. However, the purified ST gave a positive reaction with the Folin phenol reagent (13). This strange characteristic of the purified ST remains to be elucidated. The possible existence of polysaccharide or lipid or both in the final preparation was not tested, owing to the small amount of material available. Thus, it is uncertain that the final preparation is a homogeneous protein.

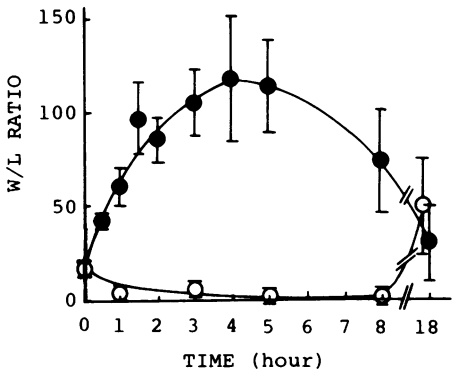

FIG. 11. *Time course of ST activity in the mouse intestinal loop test. Either 64 mouse units of purified ST (●) or phosphate-buffered saline (○) was injected into intestinal loops. After the indicated times, the W/L ratio was calculated as described in the text. Values represent means ± standard error of eight determinations.*

The purified ST gave a positive reaction in the mouse intestinal loop test. The time course of action of the toxin in the mouse intestinal loop test (Fig. 11) was very similar to that in the suckling mouse assay (Fig. 9). These data indicate that the purified ST was active not only in infant mice but also in adult mice. It is also interesting that the purified ST gave a positive reaction in the skin permeability test in guinea pigs (Fig. 12 and 13); typical bluing was observed as early as 1 to 3 h after administration of the toxin, and later the reaction became erythemal. These reactions, that is, early bluing and late erythema, are very similar to those observed with *Clostridium perfringens* type A enterotoxin (11, 17, 20).

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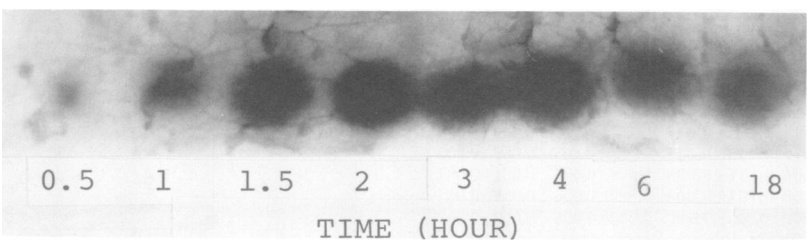

FIG. 12. *Time course of action of purified ST in the guinea pig skin permeability test. The skin permeability test was carried out as described in the text. A 200-ng amount of purified ST was administered, and the dye was injected at various times after the toxin. Typical bluing was seen at 1 to 3 h, and an erythemal reaction was seen at 4 to 18 h.*

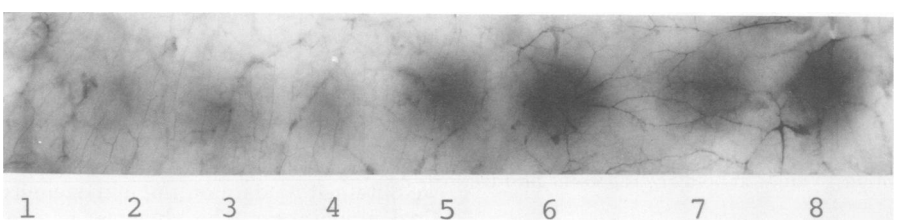

FIG. 13. *Dose response of purified ST in the guinea pig skin permeability test. The skin permeability test, with various amounts of purified ST, was carried out as described in the text. The dye was injected 2 h after administration of the toxin. Injections were as follows: (1) phosphate-buffered saline; (2) ST, 0.1 ng; (3) ST, 0.5 ng; (4) ST, 1 ng; (5) ST, 5 ng; (6) ST, 10 ng; (7) ST, 50 ng; and (8) ST, 100 ng.*

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