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Purification and Chemical Characterization of the Heat-Stable Enterotoxin Produced by Porcine Strains of Enterotoxigenic *Escherichia coli*

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Heat-stable enterotoxin (ST) produced by porcine strains of enterotoxigenic (ENT+) *Escherichia coli* has been purified to apparent homogeneity by sequential ultrafiltration, acetone fractionation, preparative gel electrophoresis, diethylaminoethyl Bio-Gel A ion-exchange chromatography, and Bio-Gel P-10 gel filtration. The enterotoxin, purified more than 1,500-fold, exhibited a molecular weight of 4,400, as determined by both sodium dodecyl sulfate-gel electrophoresis and gel filtration. A molecular weight of 5,100, representing 47 residues, was calculated from amino acid analysis data. The amino acid content was distinctive, with an unusually high proportion of cystines and few hydrophobic amino acids. A single amino-terminal residue, glycine, was observed. Purified ST was stable to heating (100°C, 30 min) and did not lose biological activity after treatment with Pronase, trypsin, proteinase K, deoxyribonuclease, ribonuclease, and phospholipase C. Periodic acid oxidation and several organic solvents (acetone, phenol, chloroform, and methanol) had no effect on the biological activity of ST. Further, purified ST was stable to acid treatment at pH 1.0 but lost biological activity at pH values greater than 9.0. Neither lipopolysaccharide nor lipid contamination was evident in purified preparations. A characteristic absorption spectrum was observed during the course of the purification, which shifted from a maximum at 260 nm in crude preparations to 270 nm for the purified toxin. Antiserum obtained from rabbits immunized with ST or ST coupled to bovine serum albumin neutralized the action of the enterotoxin in suckling mice; however, passive hemagglutination and hemolysis titer assays suggested that ST is a poor antigen.

Enterotoxigenic (ENT+) strains of *Escherichia coli* have been shown to be responsible for diarrhea in humans and neonatal animals (12, 19, 28, 30-33) through the elaboration of a high-molecular-weight, heat-labile protein (heat-labile enterotoxin, LT) and/or a low-molecular-weight, heat-stable, nonimmunogenic substance (heat-stable enterotoxin, ST) (13, 20, 21, 36). The mechanism of action of LT appears to be similar to that of cholera enterotoxin (24, 32) through activation of membrane-bound adenylate cyclase with subsequent increases in intracellular cyclic adenosine monophosphate in epithelial cells of the small intestine and other sensitive nonintestinal cells. There have been no reports on the activation of adenylate cyclase by ST in various assay systems (32); however, the low levels of ST in culture supernatants and crude preparations complicate interpretation of experiments designed to measure changes in levels of cyclic nucleotides. Alternatively, ST may cause loss of fluid and electrolytes through a unique secretory mechanism.

The purification and characterization of LT have been described by several laboratories (9, 34); however, the purification of ST has been complicated by the complexity of the various growth media used for toxin production. One of the early objectives of our laboratory was to develop a defined medium that would support ST synthesis at levels equivalent to a complex medium (1). A defined medium would reduce contamination by ultraviolet-absorbing substances and reduce potential chemical interactions between the low-molecular-weight ST and components of the culture medium. The purification of porcine ST produced in a defined medium and some of its chemical and immunological characteristics are described in this report. It appears that the small ST produced by ENT+ *E. coli* is a polypeptide with biological activity at nanogram levels.

MATERIALS AND METHODS

Cultures. Porcine strains of ENT+ *E. coli* used in this study were supplied by Harley Moon of the Na-

tional Animal Disease Center, Ames, Iowa. Strain 431 produces only ST, whereas strain 1362 produces both ST and LT. Nonenteropathogenic *E. coli* strain O111B4 used as a control strain was provided by Clarence Buller of this department.

Preparation of media. The minimal salts-tricine medium with 1.0% glucose was prepared as described previously (1, 2). Glucose was prepared as a 25% stock solution and was sterilized by autoclaving. Each component was added aseptically to sterile 250-ml Erlenmeyer flasks, with sterile distilled water added to a final volume of 50 ml.

The defined medium used throughout these studies was composed of basal salts (NaCl, K_2HPO_4, NH_4Cl), trace salts ($MgSO_4$, $MnCl_2$, $FeCl_3$), a metal chelator (tricine), and four amino acids (alanine, serine, aspartic acid, proline) (1). The medium for growth in the fermentor was prepared by autoclaving everything except the amino acids in the fermentor vessel. The amino acids were dissolved in about 3 liters of distilled water, adjusted to pH 7.5 with 5 N NaOH, and sterilized separately by autoclaving. Prior to inoculation, the amino acids were added to the fermentor containing about 27 liters of salts and distilled water followed by addition of 6 liters of cells grown in Fernbach flasks. The initial absorbance at 620 nm ranged from 0.20 to 0.30, and growth after 8 h of incubation reached an optical density at 620 nm of 3.0 to 3.5.

Growth conditions. Starter cultures were grown aerobically in 250-ml Erlenmeyer flasks containing 50 ml of minimal salts-tricine medium with 1.0% glucose as described previously (1, 2). After 8 h of incubation at 37°C with shaking (300 rpm), the starter culture was used to inoculate Fernbach flasks each containing 1 liter of defined medium (1). The volume of inoculum was sufficient to adjust the initial absorbance at 620 nm to 0.05. After 8 h of incubation with shaking, six Fernbach flasks were used to inoculate 30 liters of defined medium in an F-50 New Brunswick fermentor. Growth was for 8 h at 37°C with vigorous aeration and stirring. Foaming was controlled by use of a 10% silicone antifoam solution (Dow Corning B; Dow Chemical Co., Midland, Mich.). The antifoam concentration never exceeded 2 μl/liter. The initial pH was 7.5, and samples were withdrawn at hourly intervals to monitor growth and pH. Cells were removed by centrifugation with a Sorvall RC-5 refrigerated centrifuge and an SZ-14 rotor at 18,000 rpm. The culture supernatant was stored at 4°C for the next step in the purification.

Toxin assay and calculation of effective dose. The assay for ST has been previously described (1, 2). Relative toxin levels in the culture supernatant and at each step throughout the purification were determined by use of serial twofold dilutions of the toxin solution, and the last dilution which gave a gut weight to body weight (G/B) ratio greater than or equal to 0.090 was considered positive. Each experimental sample was assayed with three mice, and each experiment was done in triplicate. Nonenterotoxigenic *E. coli* strain O111B4 grown under the same conditions, as well as other appropriate controls for each experiment described in this report, consistently gave a G/B ratio of 0.060 ± 0.005.

The amount of protein necessary for fluid accumulation was defined as the effective dose. Using the amount of protein in each fraction (micrograms per milliliter), the last dilution which gave a positive response, and the volume injected into each suckling mouse (0.1 ml), we were able to calculate the effective dose. The total number of effective doses could then be obtained from the protein recovery in each step.

Purification of ST. After removal of cells, the culture supernatant was fractionated and concentrated 30-fold by ultrafiltration through a PM-10 Diaflo membrane (Amicon). The PM-10 ultrafiltrate was again fractionated and concentrated 20-fold by passage through a UM-2 membrane. The UM-2 ultrafiltrate was discarded, and the material retained on both filters was lyophilized. The lyophilized material was resuspended in distilled water at a protein concentration of 5 to 10 mg/ml and was cooled to 4°C before addition of 1 volume of acetone to 5 volumes of sample (1:5, vol/vol). The suspension was stirred for 30 min at −5°C and then was centrifuged at 12,000 × *g* for 30 min at −5°C; the precipitate was discarded. The supernatant was further fractionated twice as just described with the use of acetone to sample ratios of 1:1 and 9:1 (vol/vol), respectively; the precipitate was discarded in each step. The acetone was removed by flash evaporation, and the sample volume was reduced to about 50 ml. Six volumes of chloroform-methanol (1:2, vol/vol) were added to extract lipid-like material present after evaporation of the acetone. Phase separation was achieved by addition of 0.2 volume of distilled water, followed by vigorous shaking. After several minutes at room temperature, the organic phase was discarded, and the aqueous phase was concentrated by flash evaporation to approximately 50 ml to remove residual chloroform-methanol. The solution was dialyzed against 5 liters of distilled water by use of Spectrapor 6 dialysis tubing and was concentrated to 10 to 20 mg/ml for preparative gel electrophoresis.

Preparative gel electrophoresis. Preparative gel electrophoresis was performed at 4°C with the use of an 8 by 2 cm gel and tris(hydroxymethyl)aminomethane (Tris)-glycine buffer, pH 8.9 (8). The gel was subjected to pre-electrophoresis for 30 min at 20 mA before addition of the sample. The sample (approximately 10 to 15 mg) was applied to the column with electrophoresis at 15 mA until the material penetrated the gel. The current was then increased to 25 mA for the duration of the run. After separation of the bands, the gel was removed by rimming with distilled water injected with a syringe and was cut just in front of the band containing ST activity. The gel was reinserted into the glass tube, and the ST was eluted by electrophoresis into Spectrapor 6 dialysis tubing having a molecular weight cutoff of 2,000. The preparation was concentrated to about 2 ml by flash evaporation for ion-exchange chromatography.

Ion-exchange and gel filtration chromatography. The single band containing ST from preparative gel electrophoresis was loaded on a 1- by 30-cm diethylaminoethyl (DEAE) Bio-Gel A column with 1 mM Tris-hydrochloride, pH 8.3, as the run buffer. A stepwise salt gradient was employed with 25 mM, 50 mM, and 2.0 M NaCl prepared in the run buffer. The flow rate of the column was maintained at 6.0 ml/h by use of a Gilson Minipuls II peristatic pump. The 1 mM

Tris-hydrochloride run buffer was prepared by diluting a 1 M stock solution of Trizma base buffer and adjusting to the desired pH.

The fraction which eluted at 50 mM NaCl contained most of the ST activity and was concentrated to about 2 ml by flash evaporation. The solution was loaded on a Bio-Gel P-10 column (1 by 100 cm) with the same run buffer employed with DEAE Bio-Gel A. The flow rate of the P-10 column was approximately 8.0 ml/h. All chromatography steps were done at 4°C.

Molecular weight determinations. The molecular weight of ST was determined by sodium dodecyl sulfate (SDS)-gel electrophoresis by the procedure of Weber and Osborn (42) with aldolase, chymotrypsinogen, myoglobin, lysozyme, and ribonuclease as reference standards. The bromophenol blue dye front on 100- by 5-mm gels containing 10% acrylamide was marked by piercing with a 24-gauge needle. The ST was located by its absorbance at 260 nm determined by use of a Gilford spectrophotometer with a scanning assembly, as ST had minimal absorbance at 280 nm and did not react with stains specific for protein and carbohydrate. The protein standards were stained with Coomassie brilliant blue. The relative mobilities of the standards located by absorbance at 280 nm and staining were in good agreement and were used to determine the molecular weight of ST.

The molecular weight of ST was also determined by gel filtration as described by Whitaker (43). Polypeptide markers were prepared by cyanogen bromide digestion of myoglobin (16). Purified ST was co-chromatographed and subjected to chromatography separate from the CNBr digest and was observed to migrate with the same v/v_0 in both instances. The void volume of the column was determined by use of an acid-stable enzyme, bisulfate reductase of 230,000 daltons, kindly provided by James Akagi of this department.

Amino-terminal residue. The procedure for identifying the amino-terminal residue was a minor modification of that described by Gray (17). A 1-mg amount of extensively dialyzed ST was lyophilized and resuspended in 0.5 ml of 0.5 M $NaHCO_3$, and 0.5 ml of dansyl chloride (2.5 mg/ml) was added, followed by incubation at 37°C for 4 h. After acid hydrolysis, the hydrolysate was extracted twice with ethyl acetate to remove dansyl hydroxide. The sample was dried by flash evaporation and resuspended in acetone–glacial acetic acid (3:2, vol/vol) for application to polyamide sheets as described by Woods and Wang (44). Solvents used for thin-layer chromatography were water–90% formic acid (200:3, vol/vol) in the first dimension and *n*-heptane–*n*-butanol–glacial acetic acid (3:3:1, vol/vol) in the second dimension.

Amino acid analysis. All samples were extensively dialyzed before hydrolysis against distilled water by use of Spectrapor 6 dialysis tubing with a molecular cutoff of 2,000. Portions containing 0.3 to 0.4 mg were transferred to hydrolysis vials and lyophilized. After addition of constant boiling HCl, the vials were degassed and sealed. The excess HCl was removed by rotary evaporation after 24 h of hydrolysis, and the hydrolysate was dissolved in citrate buffer, pH 2.2. An amount equivalent to about 37 μg of purified ST was applied to a Beckman 120C amino acid analyzer. The observed threonine and serine values were divided by 0.95 and 0.90, respectively, to correct for destruction during hydrolysis (39).

Protein and carbohydrate determination. Protein was determined by the method of Lowry et al. (29), with crystalline bovine serum albumin as a standard. Readings at 280 and 260 nm were also used for determination of protein concentrations according to the procedure of Warburg and Christian (41) and were compared to protein values obtained by the technique of Ehresmann et al. (11). A ratio of 1.0:1.06:1.06 was obtained with pure ST in the Lowry et al., Warburg and Christian, and Ehresmann et al. procedures, respectively. Total carbohydrate was determined by the phenol-sulfuric technique described by Keleti and Lederer (23), with glucose as a standard.

Periodate oxidation. The procedure of Jackson (22) was used to determine the effect on periodate oxidation of ST activity. The reaction mixture contained 0.1 μmol of ST (440 μg), 40 μmol of acetic acid, and 5 μmol of $NaIO_4$ in a final volume of 5.5 ml. The reaction was stopped with 0.2 ml of sodium arsenate (0.2 M) after 60 min of incubation. The incubation mixture was immediately assayed for biological activity.

Enzyme and pH treatment of ST. Several hydrolytic enzymes were incubated with purified ST at ST to enzyme ratios of 10:1, 1:1, and 1:10. Treatment of ST was done at 37°C with a 60-min incubation at pH 7.0. Hydrochloric acid (1.0 N) or sodium hydroxide (1.0 N) was added dropwise to 1 ml of a ST solution, and the pH was monitored by spotting on litmus paper until pH 1.0 and pH values greater than 9.0 were reached. The acid and alkaline solutions were incubated at 37°C for 30 min followed by readjustment to pH 7.0, and the solution was assayed for biological activity.

KDO and limulus lysate assay for endotoxin. Detection of endotoxin (lipopolysaccharide) throughout the purification procedure was done by use of the colorimetric assay for 2-keto-3-deoxyoctonate (KDO) (23). Purified ST was also assayed for lipopolysaccharide by the limulus lysate assay (40). Purified *Salmonella typhimurium* lipopolysaccharide was used as a positive control for both assays.

Immunological studies. Immune sera were obtained from white albino male rabbits immunized with purified ST and ST coupled to a carrier, crystalline bovine serum albumin, ST:BSA. The ST and ST:BSA solutions were suspended in Freund complete adjuvant for the first immunization followed by a second immunization 21 days later with ST and ST:BSA suspended in Freund incomplete adjuvant. The rabbits were immunized intradermally, intramuscularly, intraperitoneally, and intravenously and received 2 mg of ST at each immunization or its equivalent when ST:BSA was the antigen. Serum was collected after the immunizations at weekly intervals.

Coupling of ST to carrier BSA was accomplished with glutaraldehyde (3, 4). Amounts of 2 mg of extensively dialyzed ST and 2.7 mg of BSA were suspended in 5 ml of 0.1 M Na_2HPO_4, pH 6.8, giving a molecular ratio of ST to carrier of 10:1. A 0.1-ml amount of 1.0% glutaraldehyde was added, and gentle stirring was continued for 3 h. The solution was dialyzed against 5

liters of distilled water with two changes by use of dialysis tubing with an approximate molecular weight cutoff of 10,000 (Arthur H. Thomas Co., Philadelphia, Pa.) to permit removal of unbound ST. Any precipitate formed during the dialysis was removed by centrifugation, and the solution was concentrated to 1.0 ml by flash evaporation at 37°C for suspension in Freund complete or incomplete adjuvant.

Neutralization of ST activity. For the neutralization experiments, 0.5 ml of serum was added to tubes containing 0.5 ml of previously prepared twofold serial dilutions of ST in phosphate-buffered saline. After vigorous blending in a Vortex mixer, the tubes were placed in a water bath at 37°C fo 4 h, with gentle shaking at hourly intervals. After incubation, the dilutions were assayed immediately for neutralization of the toxin activity. The dilution which gave positive fluid accumulation was determined, and a positive control in which ST was incubated with normal rabbit serum was included. Anticholeragenoid serum, at concentrations optimal for neutralization of cholera enterotoxin (15), was used in an attempt to neutralize ST. Further, amounts 10- and 100-fold greater than those necessary for neutralization of cholera enterotoxin were employed to insure optimal levels for the possible neutralization of ST.

Passive hemagglutination and hemolysin titration of sera. The passive hemagglutination assay was done with ST esterified to stearoyl chloride (6, 35). A solution containing 1 mg of ST was taken to dryness by flash evaporation and was resuspended immediately in 4 ml of dimethyl formamide. The solution was transferred to a tube containing 0.6 ml of pyridine followed by addition of 20 mg of stearoyl chloride. The mixture was incubated at room temperature for 3 days. After the incubation, the mixture was diluted with 10 volumes of distilled water to stop the reaction and was dialyzed overnight against distilled water by use of Spectrapor 6 dialysis tubing. After dialysis, the suspension was extracted with 6 volumes of chloroform-methanol to remove unreacted stearic acid. Phase separation was achieved by addition of 0.2 volume of distilled water, and the organic phase was discarded. The aqueous phase containing all the biological activity was evaporated to remove chloroform-methanol and was concentrated to 1.0 ml. The solution containing 30 nmol of esterified ST was added to 10 ml of 0.1% sheep erythrocytes (SRBC), and the mixture was stirred gently at 37°C for 30 min. The SRBC had been previously washed three times in Tris-buffered saline (TBS: 0.025 M Tris-hydrochloride, 0.050 M NaCl, 0.002 M ethylenediaminetetraacetic acid, and 0.02% NaN_3, pH 7.5) and diluted to a 0.1% final suspension. The serum was diluted with TBS, and one drop of the twofold dilutions was added to each well of Cooke microtiter plates. One drop of stearoyl-ST-SRBC was then added to each well, and the agglutination patterns were read after the plates had stood overnight at 4°C. The lowest dilution showing agglutination was taken as the end point.

The hemolysin titration was done by adding one drop of guinea pig complement previously absorbed to SRBC to each well containing one drop of the twofold serially diluted sera. One drop of the stearoyl-ST-SRBC was then added, and the last dilution which gave total hemolysis of the cells was taken as the end point.

Reagents used. All media components and reagents employed throughout this study were purchased from Sigma Chemical Co. The organic solvents used were of reagent grade. The electrophoresis reagents, DEAE Bio-Gel A, and Bio-Gel P-10 were purchased from Bio-Rad Laboratories, Richmond, Calif. Thin-layer chromatography plates (Polyamide 6 and PEI Cellulose F) were purchased from Brinkmann Instruments, Inc., Westbury, N.Y. SRBC were obtained from Brown Laboratories, Topeka, Kan., and guinea pig complement was obtained from Pelfreeze Laboratories, Rodgers, Ark. Monospecific horse serum anticholeragenoid prepared by Richard A. Finkelstein (15) was kindly furnished by Carl Miller, National Institutes of Health, Bethesda, Md. Spectrapor 6 was purchased from Spectrum Medical Industries, Los Angeles, Calif.

RESULTS

Purification of ST. The purification scheme devised for isolation of the ST of *E. coli* is summarized in Fig. 1. Although the purification procedure usually employed 30 to 40 liters of culture supernatant, the process can be scaled up as a result of the fold purification achieved by acetone fractionation. The amount of protein necessary for biological activity was defined as the effective dose and was calculated from the amount of protein (milligrams per milliliter) in each fraction, the last dilution which gave a positive response, and the volume injected into each suckling mouse (0.1 ml). The total number of effective doses was extrapolated from the protein recovery at each step and provided a reproducible and quantitative method of following the purification of ST. The culture supernatant was fractionated and concentrated by use of a series of Amicon ultrafiltration membranes with molecular weight cutoffs of 1,000 and 10,000. Most of the ST activity (80%) was present in the material retained on the UM-2 filter, with 10 to 15% in the PM-10 fraction (Table 1). After lyophilization, material retained by the UM-2 filter was subjected to organic solvent fractionation with acetone, and chloroform-methanol extraction of the aqueous fraction was performed to remove residual lipid-like material. At this point, the amount of protein containing biologically active material had been decreased approximately 100-fold. The partially purified preparation was dialyzed, concentrated, and further fractionated by preparative gel electrophoresis. Three bands were observed, only one of which contained essentially all of the biologically active ST from the previous step (Fig. 2). The band migrating behind the leading band, band II, had a characteristic dark brown color usually associated with ST. Although one band during

gel electrophoresis may be considered a criterion of purity, the material was concentrated and loaded on a DEAE Bio-Gel A column (Fig. 3). Four peaks were observed, three of which were retained by the ion-exchange column. A stepwise gradient resolved the toxin peak from the other contaminating species. Most of the ST was present in the 50 mM NaCl eluate (Table 1). Still further purification was achieved by gel filtration with Bio-Gel P-10 (Fig. 3). Two fractions were usually observed, with only one fraction having ST activity. The amount of protein necessary for fluid accumulation decreased slightly after gel filtration, from 1.13 ng to 0.83 ng, resulting in a further increase in fold purification. The protein recovery starting with 40 liters of culture supernatant after Bio-Gel P-10 filtration was 2 to 3 mg, with a 50 to 60% recovery of effective doses.

The ST activity in the material retained by the PM-10 filter, comprising 15% of the total number of effective doses, was purified by the same steps used with the UM-2 fraction and usually yielded about 0.4 mg of material. The chemical characteristics of ST obtained from both fractions were similar (data not shown).

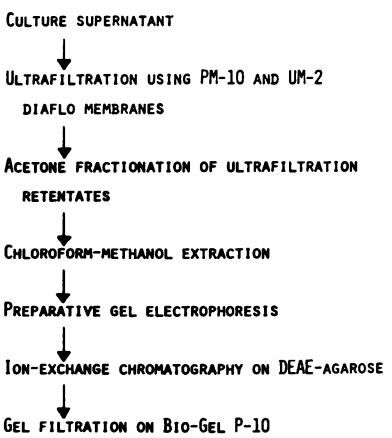

FIG. 1. *Purification scheme for heat-stable porcine enterotoxin.*

Molecular weight of ST. Determination of the molecular weight of ST by sodium dodecyl sulfate (SDS)-gel electrophoresis was complicated by the fact that ST did not stain with Coomassie brilliant R, amido black, hydrazinoacridine (7), or dansyl hydrazine (10); therefore, the gels were scanned after electrophoresis at 280 and 260 nm, and the resulting patterns observed for ST are shown in Fig. 4. The absorbance of ST at 280 nm was minimal; however, there was a 10- to 20-fold greater absorbance at 260 nm. Since the relative mobility of the standards obtained from the scans agreed with those obtained after staining, the relative mobility of ST was calculated from the migration distance located by its absorbance at 260 nm (Fig. 4). The molecular weight of ST was found to be 4,425 ± 300 based on five determinations (Fig. 5).

The results of molecular weight analysis of ST based on gel filtration are shown in Fig. 6. Five peaks were observed when the CNBr digest of myoglobin was used which corresponded to molecular weights of 17,000, 14,450, 10,700, 8,700, and 6,050. The molecular weight of ST was observed to be 4,420 ± 34.

Amino-terminal residue. Only one amino-terminal residue, glycine, was found after reaction of ST with dansyl chloride (Table 2). Glycine comigrated with the amino-terminal residue of ST in both dimensions, unlike the other amino acids. One other amino acid, alanine, migrated with the R_f value in the first dimension corresponding to the amino-terminal residue of ST. Although alanine comigrated with the unknown

TABLE 1. *Purification summary for heat-stable enterotoxin*

Step	Protein (mg)	ED[a] (ng)	Total no. of EDs	Recovery (%)	Fold purification
Supernatant (30 liters)	6,812.0	1,410.0	4.80×10^6	100.0	—
Ultrafiltration[b]					
PM-10	606.5	606.6	0.75×10^6	15.6	1.8
UM-2	1,551.0	404.0	3.84×10^6	80.0	3.5
Purification of UM-2 fraction					
Organic solvent fractionation[c]	16.45	6.1	2.70×10^6	56.0	232.6
Preparative gel electrophoresis	7.34	4.1	2.70×10^6	56.0	346.0
DEAE Bio-Gel A	2.71	1.13	2.40×10^6	50.0	1,255.7
Bio-Gel P-10	2.10	0.85	2.46×10^6	51.2	1,710.0

[a] Effective dose: the amount of protein necessary in the suckling mouse assay to give a gut weight to body weight ratio greater than or equal to 0.090.

[b] Material retained on Diaflo membranes after filtration as described in Materials and Methods. PM-10, molecular weight cutoff of 10,000; UM-2, molecular weight cutoff of 1,000.

[c] Fractionation with acetone and extraction with chloroform-methanol as described in Materials and Methods.

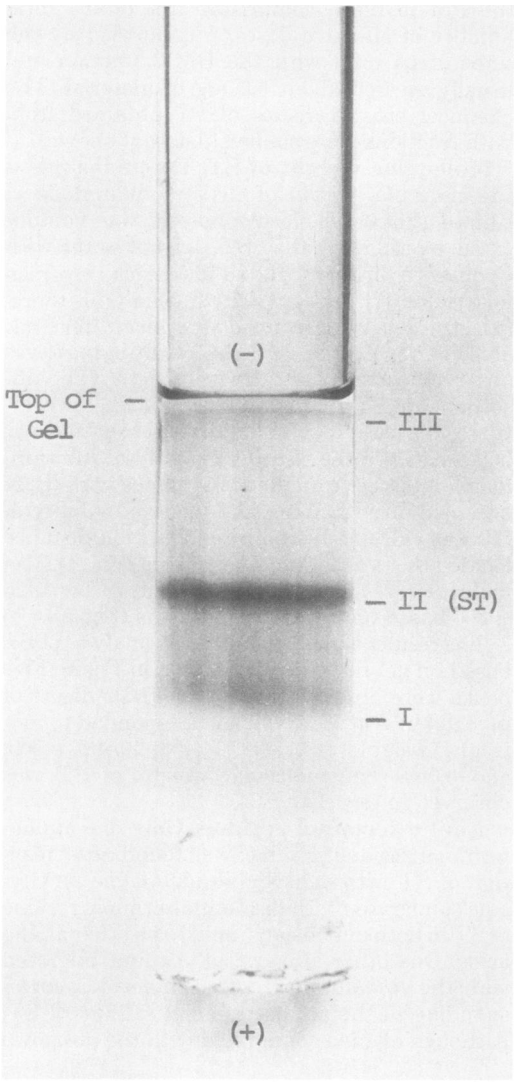

FIG. 2. *Preparative gel electrophoresis of partially purified heat-stable enterotoxin.*

dansylated amino acid in the first dimension, resolution into two distinct derivatives was accomplished in the second dimension.

Amino acid analysis. The amino acid analysis of 37 μg of purified porcine ST is shown in Table 3. The values are expressed per two phenylalanine residues; however, several other residues could have been used for calculation purposes. Isoleucine was not used as the limiting residue since it is known that leucine, isoleucine, and valine are released at slow rates from peptides when these amino acids are in consecutive sequence (39). The recovery of 36.05 μg of the 37 μg applied to the column strongly suggests that purified porcine ST contains negligible carbohydrate or other ninhydrin-negative material. The 6 half-cystines were determined as cystine and may represent a limiting value as a result of decomposition during acid hydrolysis. Additional experiments are in progress to determine

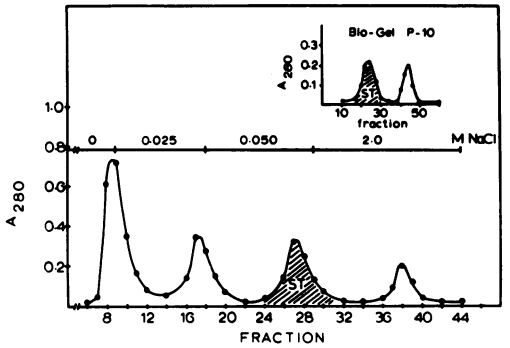

FIG. 3. *DEAE Bio-Gel A ion exchange and Bio-Gel P-10 gel filtration chromatography of ST following preparative gel electrophoresis. A_{280}, Absorbance at 280 nm.*

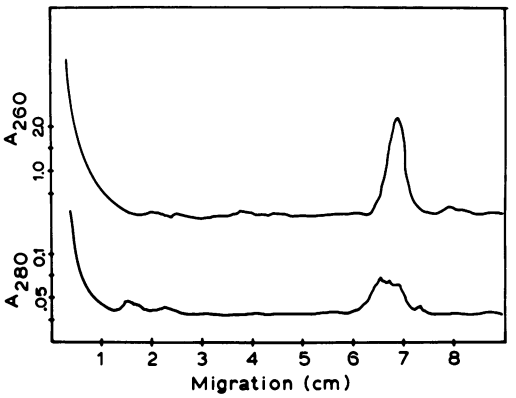

FIG. 4. *Ultraviolet scan of the gel after SDS-gel electrophoresis of purified heat-stable enterotoxin. A_{280}, Absorbance at 280 nm; A_{260}, absorbance at 260 nm.*

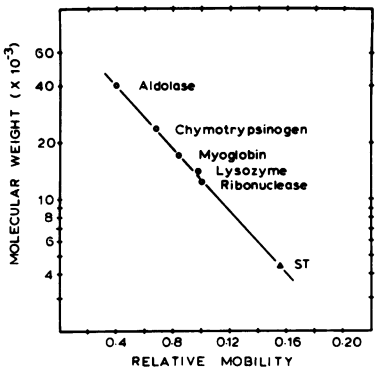

FIG. 5. *Molecular weight determination of ST by SDS-gel electrophoresis.*

half-cystines as cysteic acid following performic acid oxidation and acid hydrolysis. A molecular weight of 5,100 was calculated based on 47 residues and is in good agreement with that determined by other methods.

Chemical characteristics. The purified ST retained the characteristics previously described (20, 21, 36, 38) with partially purified preparations. The toxin was stable to heating at 100°C for 30 min and retained activity at pH 1.0, but alkaline pH values greater than 9.0 destroyed biological activity. Toxin activity was not destroyed by the enzymes trypsin, Pronase, protease K, deoxyribonuclease I, ribonuclease A, or phospholipase C, or by the organic solvents acetone, chloroform-methanol, or phenol.

Purified ST gave a positive carbohydrate reaction; however, attempts to stain the carbohydrate moiety after gel electrophoresis were unsuccessful. Further, because of the positive carbohydrate reaction, it was of interest to determine the effect of periodate oxidation on ST activity. All of the fluid-accumulating ability in the ST preparation was still present after treatment with periodate. Fatty acids were not detected by gas-liquid chromatography after acid hydrolysis and conversion to methyl ester derivatives (27). The purified preparation of ST was not contaminated with lipopolysaccharide as shown by a negative chemical test for KDO, a common lipopolysaccharide marker, and a negative limulus lysate gelation assay, which requires the lipid A portion of lipopolysaccharide for gelation.

ST had a characteristic ultraviolet spectrum, with the purified enterotoxin exhibiting a maximum absorption at 270 nm. A shift from a maximum at 260 nm to 270 nm was observed during the course of purification; however, no absorption at 280 nm, characteristic of aromatic amino acids, was observed. Because of this strong absorbance at 260 and 270 nm, we looked for the presence of nucleic acid material after perchloric acid oxidation and alkaline hydrolysis (5, 18). Neither nucleotides nor free bases could be detected in the hydrolysates by thin-layer chro-

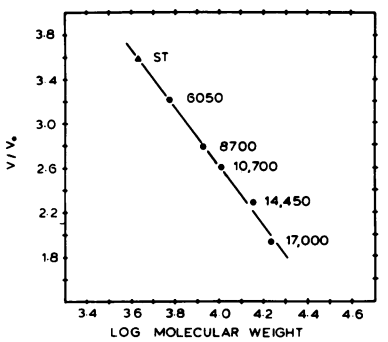

FIG. 6. *Molecular weight determination of ST by use of Bio-Gel P-10 gel filtration and a cyanogen bromide digest of myoglobin as molecular weight markers. The molecular weights of the fragments are indicated.*

TABLE 3. *Amino acid analysis of purified porcine ST*

Amino acid	Residues/two phenylalanine residues	Residues/mol	Wt at 24 h (μg)
Lysine	1.79	2	1.90
Histidine	0.90	1	1.01
Arginine	0.90	1	1.12
Aspartic acid	3.93	4	3.79
Threonine	2.76[a]	3	2.26
Serine	2.15[b]	2	1.47
Glutamic acid	4.69	5	5.00
Proline	2.00	2	1.67
Half-cystine	6.07	6	5.33
Glycine	4.40	4	2.40
Alanine	5.17	5	3.34
Valine	1.72	2	1.46
Methionine	0.76	1	0.82
Isoleucine	1.03	1	0.98
Leucine	3.31	3	3.15
Tyrosine	3.03	3	3.99
Phenylalanine	2.00	2	2.40
Tryptophan	ND[c]	—	—
Sum		47	42.09
Correction for water			−6.04
Corrected sum			36.05

[a] Observed value divided by 0.95.
[b] Observed value divided by 0.90.
[c] Not determined.

TABLE 2. *Identification of amino-terminal residue of porcine heat-stable enterotoxin (ST)*

Solvent	R_f[a]			
	Unknown[b]	Dansyl glycine	Dansyl alanine	Dansyl amine
First dimension: water–90% formic acid	0.49	0.49	0.49	0.84
Second dimension: *n*-heptane–*n*-butanol–glacial acetic acid	0.72	0.72	0.65	0.85

[a] Distance of center of spot relative to the front.
[b] Unknown amino-terminal residue of purified ST detected after dansylation and hydrolysis.

matography, suggesting that the absorption at 270 nm is characteristic of the toxin.

Immunological studies. Neutralization of ST activity was observed with serum from rabbits immunized with ST (anti-ST) or with ST coupled to BSA (anti-ST:BSA). When ST was incubated for 4 h at 37°C with normal rabbit serum, no neutralization of toxin activity was observed. However, incubation with anti-ST or anti-ST:BSA resulted in neutralization of 640 and 1,250 effective doses respectively, and 0.5 ml of antiserum raised against ST or ST:BSA neutralized 1.09 and 2.20 μg of purified ST, respectively. No neutralization of ST activity was observed under the same conditions when various concentrations of cholera antitoxin were used.

Because of the lack of a precipitin reaction of ST with immune sera in Ouchterlony double diffusion, we employed the passive hemagglutination technique to increase the sensitivity for detection of anti-ST antibodies. A low titer, 1:4, of hemagglutination was found with the different sera, whereas no hemagglutination was found with the appropriate controls (Table 4). To increase the sensitivity of the passive hemagglutination assay, we determined the hemolysis titer by adding previously absorbed guinea pig serum to each well of the reaction mixture used for passive hemagglutination. The presence of the specified antibodies would result in lysis of the SRBC coated with esterified stearoyl-ST and increase the sensitivity of passive hemagglutination. As can be seen in Table 4, a titer of 1:32 was found for each serum whereas no lysis was observed for all the appropriate controls. Since the titer for both hemagglutination and the hemolysin titration was low compared to experiments in which streptococcal polysaccharide antigens were used (6, 35), it appeared that ST was not very immunogenic but did elicit a mild humoral response.

TABLE 4. *Passive hemagglutination and hemolysis titration of ST-antisera*

Serum	Antigen	Titer
Passive hemagglutination		
Normal rabbit serum	Stearoyl-ST-SRBC[a]	0
Anti-ST	Stearoyl-ST-SRBC	1:4[b]
Anti-ST:BSA	Stearoyl-ST-SRBC	1:4
Hemolysis titration		
Normal rabbit serum	Stearoyl-ST-SRBC	0
Anti-ST	Stearoyl-ST-SRBC	1:32[c]
Anti-ST:BSA	Stearoyl-ST-SRBC	1:32
Anti-ST	SRBC	0
Anti-ST:BSA	SRBC	0

[a] Sheep erythrocytes coated with ST esterified with stearoyl chloride.

[b] The last dilution which gave hemagglutination.

[c] The last dilution with total complement-dependent hemolysis.

DISCUSSION

This study describes the purification and characterization of ST produced by a porcine strain of ENT+ *E. coli*. Although ENT+ *E. coli* strain 431 produces only ST, the purification has been used to isolate ST produced by ST-LT strains of porcine origin. The purification was based on solubility in the presence of organic solvents, charge, ion-exchange properties, and gel filtration. The toxin appeared to be homogeneous, as indicated by the observation of one peak with intense absorbance at 260 nm by SDS-gel electrophoresis, by the appearance of a symmetrical peak during Bio-Gel P-10 gel filtration, by the presence of one amino-terminal residue, glycine, and by amino acid analysis data.

After Amicon ultrafiltration with filters which retained molecular species of greater than 10,000 daltons, and a range of 1,000 to 10,000 daltons, a batchwise purification step was desired to decrease volumes of crude material to manageable amounts while retaining the ability to scale up the purification if necessary. Based on the known stability properties of ST to organic solvents, we employed acetone fractionation since $(NH_4)_2SO_4$ would not precipitate porcine ST. A small amount of ST precipitated with 9 volumes of acetone; however, most of the ST remained soluble in the acetone throughout the fractionation (Fig. 1). The acetone solubility suggested that ST is somewhat hydrophobic in nature; however, it retained its water solubility upon further purification. These results were somewhat surprising since Smith and Halls (37) have used acetone to precipitate a heat-stable fluid-accumulating substance from concentrated culture supernatants. Further, Klipstein and Engert (25, 26) partially purified heat-stable enterotoxins from *Klebsiella pneumoniae* and *Enterobacter cloacae* by acetone precipitation. We have observed in preliminary experiments that ST produced by both human and bovine strains is precipitated by 9 volumes of acetone; however, parameters such as pH and ionic strength influence the acetone solubility of ST. Although the data suggest that chemical differences exist between STs produced by ENT+ strains of *E. coli* isolated from different animal species, a limited number of amino acid substitutions would drastically alter the net charge of the molecule and its solubility in organic solvents.

Preparative gel electrophoresis of partially purified ST yielded three bands, with a dark brown band characteristic of ST migrating behind the leading band. The electrophoretic mobility suggested that ST was charged and might be further purified by ion-exchange chromatography with DEAE Bio-Gel A. In our early ex-

periments, we observed elution at about 300 mM NaCl and a low recovery of biological activity when porcine ST was applied to DEAE-Sephadex. Similar losses were also observed with Sephadex G-25; thus, we suspected that ST was irreversibly adsorbing to the backbone of the Sephadex derivatives. The ST activity eluted from DEAE Bio-Gel A at 50 mM NaCl with a minor contaminant; thus, Bio-Gel P-10 gel filtration was necessary as a final purification step. The molecular weight of purified ST was 4,400, as determined by both SDS-gel electrophoresis and gel filtration.

It is important to note that ST did not react with several stains, and this made it necessary to scan gels at 260 and 280 nm after SDS-gel electrophoresis. Stains employed for detection of proteins and carbohydrate did not react with ST under denaturing conditions. Further, an ultraviolet scan from 210 to 340 nm showed that purified ST strongly absorbed at 270 nm but exhibited little adsorption at 280 nm, an absorption maximum characteristic of aromatic amino acids. The inability of ST to stain with conventional stains, the strong absorption at 260 nm, and the effect of alkaline pH on ST activity suggested the presence of nucleic acid material in ST. However, no bases or nucleotides were detected after perchloric acid oxidation or alkaline hydrolysis of purified ST (5, 18). The absorbance properties of ST, therefore, seem to be characteristic of the enterotoxin, and the inability to stain may be due to the resistance of ST to denaturation by the fixing reagents.

Purified ST retained many of the chemical characteristics of ST in complex media (20). Biological activity was not affected by treatment with various proteases, nucleases, or phospholipase C, but studies are in progress to determine whether enzymatic cleavage of ST into smaller fragments does occur. The purified preparation did not contain either hemolytic activity or lipase activity.

Porcine ST appears to be a polypeptide of 47 residues, as determined by amino acid analysis, with a calculated molecular weight of 5,133 (Table 2), in contrast to a molecular weight of 4,400 obtained by both SDS-gel electrophoresis and gel filtration (Fig. 4 and 5). The stability properties of ST may be explained in part by the presence of six residues of half-cystine, suggesting the presence of disulfide bonds. Only one methionine residue was observed, along with a minimal number of hydrophobic amino acids.

The apparent nonantigenic nature of ST has been of interest for many years. Smith and Gyles (36) found that the LT produced in vitro by porcine strains of ENT+ *E. coli* was neutralized by antisera whereas ST was not. Smith and Linggood (38) observed that the protection of pigs against diarrhea was due to bactericidal activity of the antiserum rather than antitoxic activity. Similarly, Evans, et al. (13) found that antiserum against the enterotoxins of strain H-10407, a human strain which produces ST and LT, neutralized LT activity but not ST activity. However, we observed that serum raised in rabbits against either ST or ST coupled to BSA did decrease toxin activity, presumably by direct neutralization. The amount of ST neutralized was minimal, 1.1 μg for anti-ST serum and 2.2 μg for anti-ST:BSA serum, when compared to the neutralizing ability of anticholeragenoid on cholera enterotoxin (15). The low titers obtained by passive hemagglutination and hemolysis titration assays (Table 4) suggested that ST was a very poor immunogen, and coupling to BSA did not increase the immunogenicity. Therefore, it may be necessary to chemically modify ST to enhance its immunogenicity. It is also possible that we induced tolerance to ST, making it necessary to examine a range of dosage levels.

The greater than 1,500-fold purification of porcine ST described in this paper substantiated what had been suspected; that is, ENT+ strains of *E. coli* produce low levels of ST. One of the objectives of our early nutrition studies (1) was to increase the yield of ST; however, we were only able to equal that produced in complex media. Nevertheless, the defined medium did decrease levels of ultraviolet-absorbing substances in the culture supernatants 10- to 15-fold and greatly facilitated purification. Considerable biological activity can be isolated from 40 liters of growth medium in a relatively short period of time, and the purification can be scaled up if desired. The availability of pure ST will enhance studies on the mechanism of action and should stimulate new approaches to diagnosis and therapy. The structure-function relationships of this low-molecular-weight polypeptide which causes fluid and electrolyte loss in the small intestine of humans and neonatal animals should be very interesting.

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