Purification and Some Physicochemical Properties of Staphylococcal Enterotoxin E*

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CONCORDIA R. BORJA, ELLEN FANNING, I-YIH HUANG, AND MERLIN S. BERGDOLL

From the Food Research Institute and Department of Food Science, University of Wisconsin, Madison, Wisconsin

SUMMARY

Enterotoxin E produced by Staphylococcus aureus strain FRI (Food Research Institute)-326 was purified by cation exchange chromatography on carboxymethylcellulose, gel filtration through superfine Sephadex G-75, and gel filtration in 6 M urea with superfine Sephadex G-75. The purified toxin appears to be nearly homogeneous by paper, starch gel, and polyacrylamide gel electrophoresis and double gel diffusion tests. It is a simple, colorless, antigenic protein. Its molecular weight was determined to be 29,600 ± 500 by sucrose density gradient centrifugation, molecular sieve chromatography on Sephadex G-100, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Its toxicity and reaction with its specific antibody are destroyed by extreme acidic (pH 2) and basic (pH 12) conditions. Urea-treated enterotoxin E retains its toxic activity and reaction with its specific antibody after removal of the denaturing agent. The enterotoxin consists of 259 amino acid residues and contains no free sulfhydryl groups. End group analysis showed serine to be the NH2-terminal amino acid and threonine to be the COOH-terminal amino acid.

The pioneering investigations of Dack et al. (1) in 1930 on the correlation of vomiting and diarrhea to the ingestion of food contaminated with staphylococci disproved the existence of "ptomaine poisoning" and resulted in the naming of this type of foodborne disease as staphylococcal food poisoning. Little progress was made in the identification of the toxic agent until an intensive research program was undertaken at the Food Research Institute in the late 1940's. This work culminated in the purification and partial characterization of one enterotoxin protein that at least one other antigenically distinct enterotoxin protein was produced by the staphylococci, in particular by strain FRI-196E (6). In 1960 the specific antibody to the enterotoxin elaborated by strain FRI-196E was identified by Casman (7). When a nomenclature for the enterotoxins was devised in 1963 (8), the toxic substance produced by strain FRI-196E was named "enterotoxin A" and the first one purified, "enterotoxin B." Continued world-wide problems with staphylococcal food poisoning and the fact that specific antibodies to the enterotoxins are essential for their detection served as incentives for extensive investigations of new immunological types. As a result, enterotoxins C (9) and D (10) were identified in the 1960's.

This communication reports the purification and some physicochemical properties of enterotoxin E, produced by Staphylococcus aureus strain FRI-326, which originally was isolated from food implicated in staphylococcal food poisoning. The identification of enterotoxin E as being a specific chemical entity, immunologically distinct and different from enterotoxins A, B, C, and D, was reported by Bergdoll et al. (11). A comparison of the properties of enterotoxin E with those of the other purified enterotoxins is included in this paper.

EXPERIMENTAL PROCEDURE

Materials—Reagents included urea (J. T. Baker Chemical Co.), recrystallized twice from ethanol and freshly dissolved prior to use, 2-mercaptoethanol (Calbiochem), acrylamide, N,N'-methylenebisacrylamide, N,N',N'-tetramethylenediamine and 1-fluoro-2,4-dinitrobenzene (Eastman), Coomassie brilliant blue R-250 (Colab), 5,5'-dithiobis (2-nitrobenzoic acid) and iodoacetamide (Aldrich), hydrolyzed starch (Connaught Laboratories), riboflavin (Nutritional Biochemicals), blue dextran 2000 (Pharmacia), and anhydrous hydrazine (Matheson Coleman and Bell). The CM-cellulose* was obtained from Carl Schleicher and Schuell, and the Sephadex G-75 (superfine) and Sephadex G-100 (40 to 120 μ) from Pharmacia. The polyethylene glycol, 20 M, (Carbowax) was from Union Carbide Corp. The proteins used were bovine serum albumin and pepsin (Nutritional Biochemicals), and ovalbumin, trypsin, lysozyme, and yeast alcohol dehydrogenase (Worthington).

Estimation of Protein and Ultraviolet Absorption—Protein concentration of enterotoxin E during its purification was based on

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* The abbreviations used are: CM cellulose, carboxymethylcellulose; SDS, sodium dodecyl sulfate.
the absorbance at 280 nm with an $E_{1\text{cm}}^{1\%}$ of 11.3, the value of enterotoxin C (12). The highly purified sample of enterotoxin E has an $E_{1\text{cm}}^{1\%}$ of 11.9 at 280 nm and 12.5 at 277 nm (maximum absorption). The amount of protein eluted from polyacrylamide gels was determined by the procedure of Lowry et al. (13) with crystalline egg albumin as the standard. Absorbance values in the ultraviolet and visible regions were obtained with the use of quartz cuvettes (1-cm cell path) in a Beckman model DB-G spectrophotometer.

Sucrose Density Gradient Ultracentrifugation—A Spincor model L ultracentrifuge with a swinging bucket rotor (Spinco SW 39) was employed for the determination of the molecular weight of enterotoxin E by sucrose gradient ultracentrifugation. Sucrose gradients in 0.05 M Tris buffer, pH 7.5, were prepared by a gradient former patterned after the design of Britten and Roberts (14) and tested for linearity with dichroeholinodilophenol, according to the method described by Martin and Ames (15). The gradients were stored for 30 to 40 hours at 2-4°C, after which 100 µl of 0.05 M Tris buffer, pH 7.5, containing 200 µg of enterotoxin E, were layered on each gradient and centrifuged at 39,000 rpm for 20 hours at 4°C. Yeast alcohol dehydrogenase and pepticin were used as standards (300 µg each in 100 µl of 0.05 M Tris buffer, pH 7.5) and centrifuged along with enterotoxin E. After centrifugation, the gradient tubes were punctured and the contents fractionated in a modified sample fractionator of Martin and Ames (15). A total of 38 fractions was collected, each containing 7 drops. Enterotoxin E in each fraction was determined by Borja and Bergdoll (12). Yeast alcohol dehydrogenase was assayed as reported by Martin and Ames (15) and pepticin was determined by the method of Anson (16). The sedimentation coefficient of enterotoxin E, corrected to the standard state of water at 20°C, was also determined from the sucrose gradient ultracentrifugation studies (15).

Molecular Sieve Chromatography with Sephadex G-100 to Determine Molecular Weight—A Kontes glass column, 2 cm diameter, was packed with Sephadex G-100 equilibrated with 0.1 M sodium phosphate buffer, pH 8.5, to a height of 144.8 cm. The samples applied to the column contained 4 to 5 mg of solute in 1 ml of the phosphate buffer. The eluate was collected in 2.6 ml fractions. The concentration of eluted protein was determined by measurement of absorbance at 280 nm. Blue dextran 2000 was employed to determine void volume, $v_0$. The proteins used as standards, with their molecular weights, were: bovine serum albumin, 67,000 (17); ovalbumin, 45,000 (18); pepticin, 35,000 (19); trypsin, 24,500 (20); and lysozyme, 14,499 (21).

Disc Gel Electrophoresis in Polyacrylamide Gel—The cationic system of Reisfeld et al. (22), as modified by Denny et al. (23), was employed with some additional modifications. In place of ammonium persulfate as catalyst, riboflavin was mixed in the running gel to a final concentration of 0.0005%. Since riboflavin was used, polymerization of the small pore solution was accomplished by exposing the sides and tops of the glass columns (88 mm long and 5 mm internal diameter) to daylight fluorescent lamps (50 watts) for about 30 min. The β-alanine-acetate buffer was diluted with 3 parts of distilled water before placing in the buffer chambers of a Metalloglass apparatus. Electrophoresis was performed at room temperature (around 22°C) with a current of 1 ma per gel column for approximately 24 hours. More rapid staining and easier destaining were obtained when Coomassie brilliant blue R-250 (24) was used instead of Amido schwarz stain. The protein bands from unstained gel columns, located by comparison with stained gel columns, were cut out with a razor blade. The gel containing the proteins was macerated with a glass mortar and pestle and transferred quantitatively with a small volume of 0.02 M sodium phosphate buffer, pH 7.5, to polyethylene centrifuge tubes. The samples were left overnight in the cold (3-5°C) before centrifuging at 0-2°C for 20 min at 15,000 rpm to sediment the gel. The gel was washed twice with the eluting buffer. Fractions were analyzed for protein, fed to monkeys to locate the toxic fraction, and placed in Ouchterlony double gel diffusion plates to identify the enterotoxin-antienterotoxin precipitin line.

Determination of Molecular Weight by Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—A modification of the method described by Shapiro et al. (25) was employed to estimate the molecular weight of enterotoxin E by electrophoresis in SDS-polyacrylamide. The running gel had a final concentration of 5% acrylamide, 0.13% N,N'-methylenebisacrylamide, 0.05% N,N',N'-tetramethylethylenediamine, and 0.075% ammonium persulfate in 0.1 M sodium phosphate buffer, pH 7.1, containing 0.1% SDS. Glass columns (88 mm long and 5 mm internal diameter) were sealed at one end with moist dialysis membranes. Small pore solution (1.4 ml) was introduced into each column, and immediately about 0.05 ml of distilled water was layered carefully on top of the running gel solution. The columns were kept in the dark for about 1 hour at room temperature. After polymerization, the water layer was drained and any water remaining was blotted with lint-free disposable wipes. Prior to the preparation of the gel columns, samples (enterotoxin E and standards) were dissolved at a concentration of about 1.5 mg per ml in 0.1 M sodium phosphate buffer, pH 7.1, containing 1% SDS and 1% 2-mercaptoethanol. About 0.3 ml of each was pipetted into two sets of small test tubes and incubated for about 3 hours at 37°C. After reduction, solid iodoacetamide (2.5 to 3.0 mg) was added to one set of tubes. The protein samples not treated with iodoacetamide were dialyzed at room temperature for about 16 hours against 0.01 M sodium phosphate buffer, pH 7.1, containing 0.1% SDS and 0.1% 2-mercaptoethanol, whereas the samples treated with iodoacetamide were dialyzed against the same buffer with the omission of 2-mercaptoethanol. After dialysis, about 70 µl of each protein solution were mixed with 30 µl of 40% sucrose and the mixture layered on top of the running gel. Immediately the electrophoresis buffer (0.1 M sodium phosphate buffer, pH 7.1, with 0.1% SDS) was layered carefully on top of the sample. Bromphenol blue was added to the buffer in the upper vessel of the Metalloglass apparatus as a tracking dye to ascertain the completion of the electrophoresis, which took approximately 2 hours at 4 ma per gel column. To stain and destain the gels, the procedure described by Weber and Osborn (26) was followed. All proteins were run in triplicate. The standard proteins used to prepare the calibration curve for determination of the molecular weight of enterotoxin E were bovine serum albumin, pepticin, trypsin, ovalbumin, and lysozyme. Since the gels swelled in the acidic solution used for staining and destaining, mobilities were calculated as:

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\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length after staining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}
\]
Effects of pH and heat on the serological activity of enterotoxin E. RPM for 15 min at O-2°. The precipitate was washed once with filter paper in t-amyl alcohol saturated with 0.05 dinitrophenyl amino acids were examined by paper chromatography. The color intensity was measured at 360 nm. The water-soluble (determined by single gel diffusion), recovery of the enterotoxin was 40 to 50%. The percentage of enterotoxin in Fraction C was calculated from the data obtained.

Enterotoxin Production—Enterotoxin E was produced by growing S. aureus strain FRI-326 as described in the literature (11).

Antisera to Staphylococcus aureus Strain FRI-326 Antigens—Antisera to crude enterotoxin E and to the highly purified toxin were prepared in rabbits by following the procedure reported by Bergdoll et al. (11).

Detection and Assay of Enterotoxin E—Fractions from the different purification steps were tested for toxicity by intragastric administration of 50 ml of the aqueous test solution to groups of six young rhesus monkeys (2 to 3 kg). Highly purified enterotoxin E was assayed for biological activity in monkeys by the intragastric method and by intravenous injection of the toxin into the saphenous vein in 2 ml of 0.85% pyrogen-free NaCl solution (groups of three monkeys). Emesis within 5 hours after administration indicated a positive reaction.

Identification of the specific antibody to enterotoxin E was by Ouchterlony plate gel diffusion method as modified by Bergdoll et al. (9). When the purified enterotoxin became available, specific assays were carried out by the single gel diffusion tube method (12). This procedure was employed to obtain a standard curve, to determine the enterotoxin E concentration in the different steps of purification, and in the experiments on the effects of pH and heat on the serological activity of enterotoxin E.

Amino Acid Analysis—A Spinco model 120 B amino acid analyzer remodeled to 120 C was used to determine the amino acid composition of enterotoxin E according to the procedure of Benson and Patterson (28). Protein was hydrolyzed in 6 M HCl at 110° for 24 and 72 hours in evacuated sealed tubes. Cystine was determined with the amino acid analyzer after converting cystine to cysteic acid by performic acid oxidation (29), followed by hydrolysis of the oxidized enterotoxin at 110° for 24 hours. Tryptophan was determined by the method of Goodwin and Morton (30).

Detection of Sulfhydryl Group—Sulfhydryl groups were detected by the technique of Glaser et al. (31) with the use of 5,5'-dithiobis(2-nitrobenzoic acid).

Amino-terminal Amino Acid Analysis—The NH₂-terminal amino acid of enterotoxin E was determined by the L-fluoro-2,4-dinitrobenzene method of Sanger (32), as modified by Fraenkel-Conrat et al. (33). The ether-soluble dinithiophenyl amino acids were identified after paper chromatography on Whatman No. 4 filter paper in t-amyl alcohol saturated with 0.05 M phthalate, pH 5.0 and pH 6.0, and on Whatman No. 1 filter paper in 1.5 M phosphate buffer, pH 5.0. The ether-soluble dinithiophenyl amino acids separated on the chromatograms were cut out and eluted with 1% sodium bicarbonate solution in test tubes at 55° for 15 min. The color intensity was measured at 360 nm. The water-soluble dinithiophenyl amino acids were examined by paper chromatography on Whatman No. 4 paper in t-amyl alcohol saturated with 0.05 m phthalate, pH 6.0.

Carboxyl-terminal Amino Acid Analysis—The COOH-terminal amino acid of enterotoxin E was determined by the procedure described previously for determination of the COOH-terminal amino acid of enterotoxin C (34).

pH Measurements—All pH measurements were performed with a Radiometer titration assembly, type TTA31, with type electrodes G2222C and K4112 (35).

**RESULTS**

**Purification Procedure**

All purification steps were performed in the cold (3–5°).

**STEP I: Concentration of Bacterial Culture Supernatant Fluid**—After sedimentation of the cells in a Sorvall centrifuge operated at 10,000 rpm for 10 min at O-2°, the culture supernatant fluid was concentrated by dialysis for about 24 hours against polyethylene glycol, 20 M, followed by dialysis against distilled water. The mixture was lyophilized and kept in the dry form at room temperature until used for subsequent purification. The enterotoxin content of the bacterial culture supernatant fluid (3 to 5 mg per ml) represented about 0.01% of the total protein in the supernatant fluid. After concentration with polyethylene glycol, recovery of enterotoxin was 60 to 70%. In order to minimize denaturation of the proteins, the culture supernatant fluid was not removed completely from the dialysis tubing by the polyethylene glycol-water mixture. When 50 ml of the supernatant fluid (30 mg of protein per ml) were fed intragastrically to monkeys, 9 of 12 animals vomited.

**STEP II: Ion Exchange Chromatography on CM-cellulose**—The dry toxic material from Step I, obtained from 6.0 to 6.5 liters of original culture, was redissolved in about 200 ml of 0.02 M sodium phosphate buffer, pH 5.6. The components in Fraction C were identified after paper chromatography on Whatman No. 4 paper in t-amyl alcohol saturated with 0.05 M phthalate, pH 6.0.

**Identification of the Specific Antibody to Enterotoxin E**—Specific antisera to enterotoxin C caused vomiting in the animals (10 of 12 vomited). On Ouchterlony double gel diffusion plates, Fraction C gave two major precipitin lines and two minor ones with the antiserum obtained from rabbits injected with crude preparations of the enterotoxin. The toxin content of each tube in Fraction C (Fig. 1) was determined with the amino acid analyzer after converting (2.2 equal parts and applied separately to two CM-cellulose columns (2.2 × 60 cm), previously equilibrated with 0.02 M sodium phosphate buffer, pH 5.6. Elution of the adsorbed proteins was carried out by a stepwise increase in concentration and pH of the sodium phosphate buffer. A typical elution pattern is shown in Fig. 1. Fraction A represents unadsorbed material and contained most of the yellowish-brown substances that contaminated the enterotoxin. When Fractions A, B, C, and D were administered to monkeys (1 mg of protein per animal), only Fraction C caused vomiting in the animals (10 of 12 vomited). On Ouchterlony double gel diffusion plates, Fraction C gave two major precipitin lines and two minor ones with the antiserum obtained from rabbits injected with crude preparations of the enterotoxin. The toxin content of each tube in Fraction C (Fig. 1) was determined after highly purified enterotoxin E was obtained (at a later stage in the work). The components in Fraction C were pooled, dialyzed against distilled water to remove most of the salts, and lyophilized. Based on the amount of enterotoxin E in Fraction C and in the original culture supernatant fluid (determined by single gel diffusion), recovery of the enterotoxin was 40 to 50%. The percentage of enterotoxin in Fraction C
about 8%. One-half of the lyophilized toxic fraction from Step II was redisolved in 0.02 M phosphate buffer, pH 6.8 (about 1% of gel volume), and centrifuged to clarify the solution. The clear sample was layered carefully on a column of superfine Sephadex G-75 (2.0 × 146 cm), previously equilibrated with the buffer used for elution. A typical elution profile is illustrated in Fig. 2. Only Fraction C evoked vomiting in monkeys (7 of 12 vomited) when fed at a level of 100 μg of protein per animal. The hatched area represents the enterotoxin content which was determined after preparation of a homogeneous sample of the enterotoxin. Two major antigen-antibody precipitin lines were given by Fraction C on Ouchterlony double gel diffusion plates. The toxic fraction was lyophilized and kept dry until the next step. The percentage of enterotoxin in the preparation was increased from 0.01% in the culture supernatant fluid to about 8%.

**STEP III: Gel Filtration Through Superfine Sephadex G-75**

One-half of the lyophilized toxic fraction from Step II was redisolved in 0.02 M phosphate buffer, pH 6.8 (about 1% of gel volume), and centrifuged to clarify the solution. The clear sample was layered carefully on a column of superfine Sephadex G-75 (2.0 × 146 cm), previously equilibrated with the buffer used for elution. A typical elution profile is illustrated in Fig. 2. Only Fraction C evoked vomiting in monkeys (7 of 12 vomited) when fed at a level of 100 μg of protein per animal. The hatched area represents the enterotoxin content which was determined after preparation of a homogeneous sample of the enterotoxin. Two major antigen-antibody precipitin lines were given by Fraction C on Ouchterlony double gel diffusion plates. The toxic fraction was lyophilized and kept dry until the next step. The percentage of enterotoxin in the preparation was increased from about 8% in Step II to approximately 60% in Step III. The recovery at this stage of purification was 25%.

**STEP IV: Refiltration Through Superfine Sephadex G-75**

The dry, toxic samples collected at Step III in four separate preparations were combined, redissolved in 0.02 M phosphate buffer, pH 6.8, dialyzed against distilled water to remove most of the salts, and lyophilized. The dry sample was redissolved in the same buffer, centrifuged to clarify, and refiltered through a column of superfine Sephadex G-75 (2.0 × 146 cm). The major component contained the bulk of enterotoxin E, but it also contained a second antigenic protein. To determine which antigen was the enterotoxin, this fraction was subjected to trypsin digestion (1:20, 37°, 15 hours) since it had previously been observed that enterotoxins A (36) and B (4, 37) resisted tryptic digestion. The digest was toxic in monkeys (90 μg of protein, 3 of 6 vomited) and gave only one precipitin line in Ouchterlony gel diffusion plates (Fig. 3, Well 1).

**STEP VA: Polyacrylamide Gel Electrophoresis**

The results of disc electrophoresis of the sample from Step IV in 5% and 10% polyacrylamide gels without urea are shown in Fig. 4 (gel columns 1 and 2). The material composing the heavy band in gel column 1 (5% gel) was toxic when fed to monkeys (30 μg of protein, 3 of 6 vomited). Two heavy precipitin lines (Fig. 3, Well 2) (the lines appear to overlap because of the limitations of photography) and a very faint hazy line which is not readily visible in the picture were observed on Ouchterlony double gel diffusion plates. Even in 10% gel (Fig. 4, gel column 2), the separation was not sharply defined and the heavy band gave two precipitin lines against antiserum to crude preparation of enterotoxin E. The toxic sample from refiltration through superfine Sephadex G-75 (Step IV) was subjected, therefore, to polyacrylamide gel (5%) electrophoresis with 8 M urea. The results of this experiment are shown in Fig. 4 (gel column 5). The band that moved most rapidly towards the cathode was found to be the enterotoxin by monkey feeding experiments (25 to 30 μg of protein, 3 of 6 vomited). The antigen-antibody line given by this protein (Fig. 3, Well 8) joined with the line formed by the toxic antigen from the trypsin experiments (Fig. 3, Well 1). A faint protein band in gel column 3 (Fig. 4) followed the toxic protein band and in turn was followed by a heavy and distinct third protein band, both proteins being nontoxic.

The enterotoxin from stained gels containing urea was eluted with 0.02 M phosphate buffer, pH 7.5, and dialyzed exhaustively in 0.005 M phosphate buffer, pH 6.8. This material was toxic in monkeys after elimination of the urea. This toxic material was also reduced with 2-mercaptoethanol and part of the reduced sample was alkylated with iodoacetamide. Both the reduced and reduced alkylated preparations were subjected to polyacrylamide gel (5%) electrophoresis in the presence of sodium dodecyl sulfate. Only one band was observed in the gels (gel columns 4 and 5, Fig. 4).

In the polyacrylamide gel electrophoresis experiments, riboflavin was used in place of ammonium persulfate as catalyst, in order to eliminate the possibility of persulfate oxidizing effect on the enterotoxin. A small amount of current (1 ma per gel column) was applied during electrophoresis; since enterotoxin E was observed to be sensitive to the combined effects of low pH (4.5) during electrophoresis and heating effects in the gel caused
Fig. 3 (left). Immunodiffusion tests of different samples of enterotoxin and contaminating impurities with antisem prepared against impure toxin, obtained from an Ouchterlony double-gel diffusion plate. Conditions are described under “Experimental Procedure.” Well 1, sample from superfine Sephadex G-75 after digestion with trypsin (96 μg per ml); Well 2, sample from 5% polyacrylamide gel without urea (120 μg per ml); Well 3, fastest moving protein (toxic) from polyacrylamide gel with urea, as shown in Fig. 4, gel column 3 (70 μg per ml); Well 4, nontoxic Fraction C in Fig. 5 (120 μg per ml); Well 5, nontoxic Fraction A in Fig. 5 (80 μg per ml); Well 6, slowest moving heavy fraction (nontoxic) from polyacrylamide gel with urea, as shown in Fig. 4, gel column 3 (70 μg per ml); Well 7, toxic Fraction B in Fig. 5 (70 μg per ml); Well 8, fastest moving protein (toxic) from polyacrylamide gel with urea, as shown in Fig. 4, gel column 3 (60 μg per ml); center well, antiserum against crude enterotoxin E.

Fig. 4 (right). Polyacrylamide gel electrophoretic patterns. Conditions are described under “Experimental Procedure.” The cathode for gel columns 1, 2, and 3 is at the bottom and at the top for gel columns 4 and 5. Gel columns 1 and 2, toxic samples applied to 5% gel without urea and 15% gel without urea, respectively, were from the major component in Step IV; gel column 3, toxic sample applied to 5% gel with 8 M urea was from Step IV; gel column 4, highly purified enterotoxin E applied to 5% gel with 0.1% SDS was reduced with 2-mercaptoethanol; gel column 5, highly purified enterotoxin E applied to 5% gel with 0.1% SDS was reduced with 2-mercaptoethanol and alkylated with iodoacetic acid.

The component in Fraction A (nontoxic) gave a precipitin line in double gel diffusion plates (Fig. 3, Well 5) that joined with the line formed by the slowest moving protein observed in polyacrylamide gel-urea electrophoresis (Fig. 3, Well 6) whereas Fraction B (toxic) formed a common precipitin line (Fig. 3, Well 7) with the one given by the fastest moving band (Fig. 3, Well 8). Fraction C (nontoxic) contained two components, indicated by two precipitin lines (Fig. 3, Well 4), one of which joined with the line formed by the component of Fraction A (Fig. 3, Well 5). Neither component reacted with the enterotoxin antibody (Fig. 3, Well 3). Reduced and alkylated Fraction B gave only one band in polyacrylamide gel electrophoresis with sodium dodecyl sulfate. It also reacted with antibodies against native enterotoxin E.

Step VII: Gel Filtration Through Superfine Sephadex G-75 in 6 M Urea—Only very small amounts of enterotoxin E could be prepared from polyacrylamide gels with the analytical apparatus being used in our laboratory. It was necessary, therefore, to devise another technique for separation of the enterotoxin from the impurities that remain after filtration through superfine Sephadex G-75. For this purpose, gel filtration in the presence of 6 M urea was explored. The dry toxic sample from Step IV was dissolved in 0.02 M sodium phosphate buffer, pH 6.8, with 6 M urea and allowed to stand in the cold for about 45 hours before applying to the column of superfine Sephadex G-75 which had been equilibrated with the same buffer. The result of a typical experiment is shown in Fig. 5. The three fractions were exhaustively dialyzed to remove the urea and fed to monkeys (20 μg of protein per animal). Only Fraction B elicited vomiting in the animals (11 of 12 reacted). The immunological reactions of the antigens in Fractions A, B, and C toward their specific antibodies were retained.

The component in Fraction A (nontoxic) gave a precipitin line in double gel diffusion plates (Fig. 3, Well 5) that joined with the line formed by the slowest moving protein observed in polyacrylamide gel electrophoresis (Fig. 3, Well 6) whereas Fraction B (toxic) formed a common precipitin line (Fig. 3, Well 7) with the one given by the fastest moving band (Fig. 3, Well 8). Fraction C (nontoxic) contained two components, indicated by two precipitin lines (Fig. 3, Well 4), one of which joined with the line formed by the component of Fraction A (Fig. 3, Well 5). Neither component reacted with the enterotoxin antibody (Fig. 3, Well 3). Reduced and alkylated Fraction B gave only one band in polyacrylamide gel electrophoresis with sodium dodecyl sulfate. It also reacted with antibodies against native enterotoxin E.

Since enterotoxin E could be separated from its impurities by gel filtration through superfine Sephadex G-75 in 6 M urea (Step VII), this procedure was used in place of polyacrylamide gel electrophoresis (Step VA) to collect quantities of the toxin for the physicochemical studies.

Molecular Weight
With the use of the calculations of Martin and Ames (15) for the determination of molecular weight by sucrose gradient ultracentrifugation, values of 29,750 and 30,030 were obtained for enterotoxin E when yeast alcohol dehydrogenase and pepsin, respectively, were used as standard proteins. The molecular weight of enterotoxin E was determined also by measuring the elution volume on a column of Sephadex G-100 calibrated with
several macromolecules of known molecular weight (Fig. 6). Based on the elution volumes of these standards and that of enterotoxin E, a molecular weight of 29,000 was obtained. A molecular weight of 30,000 was calculated for enterotoxin E (mobility of 0.089) from a standard curve obtained by plotting the mobilities of marker proteins determined by SDS-polyacrylamide gel electrophoresis against the log of their molecular weights (Fig. 7). The average of the molecular weights determined by the three techniques is 29,600 ± 500.

Fig. 5. Elution pattern from a column of superfine Sephadex G-75. The enterotoxin-containing fraction from Step IV (10 to 15 mg) was placed on the Sephadex column (2.0 × 94 cm). The eluting solution was 0.02 M sodium phosphate buffer, pH 6.8, with 6 M urea. The flow rate was 2.6 ml (one fraction) per 46 min. ○—○, absorbance at 280 nm in buffer with 6 M urea.

Fig. 6. Semi-log plot of the ratios of elution volume ($V_e$) and void volume ($V_0$) of five marker proteins obtained from a Sephadex G-100 column (2.0 × 144.8 cm) against their molecular weights, for determination of the molecular weight of enterotoxin E. The points represent the average of three trials. The arrow indicates the $V_e/V_0$ of enterotoxin E from three trials.

Fig. 7. Semi-log plot of the mobilities (average of duplicate runs) of five standard proteins obtained by SDS-polyacrylamide gel electrophoresis against their molecular weights for determination of the molecular weight of enterotoxin E. The arrow indicates the mobility of enterotoxin E from two gel columns. See under "Experimental Procedure" for details.

Isoelectric Point

Paper electrophoresis experiments indicated an isoelectric point of 7.0 ± 0.05 in sodium phosphate buffer of 0.1 ionic strength. Confirmation of this value was obtained by starch gel electrophoresis. Only one protein spot was detected in any of the electrophoresis experiments.

Sedimentation Coefficient

The sedimentation constant of enterotoxin E calculated from sucrose gradient ultracentrifugation and corrected to the standard state of water at 20° (n_20,w) (15) is 2.6.

Amino Acid Composition

The amino acid composition of purified enterotoxin E is presented in Table I. Each value is an average of four independent experiments except for half-cystine in which only two trials (24-hour hydrolysis) were performed. The values for serine, threonine, and amide nitrogen were obtained by extrapolating to zero hydrolysis time. The tyrosine content estimated by measuring the absorbance at 294.4 nm and 280 nm in 0.1 M NaOH with corrected for spurious absorption (30) is 9.65%, which agrees with the value of 9.74% obtained from the amino acid analysis. Since the estimation of tryptophan in proteins is generally unreliable, the result of 1.5 (2 tryptophanyl residues) obtained from spectral analysis can be taken as the best present estimate. No detectable free sulfhydryl groups were found in the purified enterotoxin. The results of amino acid analysis indicate that the toxin is composed of 259 amino acid residues.

Terminal Amino Acids

The results obtained from the 1-fluoro-2,4-dinitrobenzene method showed the presence of dinitrophenyl serine, trace amounts of dinitrophenyl glutamic acid, usual by-products of dinitroaniline, and trace amounts of dinitrophenol. Dinitrophenol was almost completely removed by sublimation. The recovery of dinitrophenyl serine was calculated to be 0.53 mole per mole of enterotoxin E after corrected for destruction during the hydrolysis and for loss in paper chromatography. The
TABLE I

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Protein*</th>
<th>Calculated residues*</th>
<th>Nearest integral residues</th>
</tr>
</thead>
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<tr>
<td>Lysine</td>
<td>0.845</td>
<td>24.8</td>
<td>25</td>
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<tr>
<td>Histidine</td>
<td>0.222</td>
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<td>7</td>
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<tr>
<td>Arginine</td>
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<td>8</td>
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<tr>
<td>Aspartic acid</td>
<td>1.312</td>
<td>38.4</td>
<td>38</td>
</tr>
<tr>
<td>Threonine*</td>
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<tr>
<td>Serine</td>
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<td>Glycine</td>
<td>0.719</td>
<td>21.1</td>
<td>21</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.335</td>
<td>9.8</td>
<td>10</td>
</tr>
<tr>
<td>Half-cystine*</td>
<td>0.075</td>
<td>2.2</td>
<td>2</td>
</tr>
<tr>
<td>Valine*</td>
<td>0.440</td>
<td>12.9</td>
<td>13</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.034</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>Isoleucine*</td>
<td>0.360</td>
<td>11.1</td>
<td>11</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.891</td>
<td>26.2</td>
<td>26</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.600</td>
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<td>18</td>
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<tr>
<td>Phenylalanine</td>
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<td>8.9</td>
<td>9</td>
</tr>
<tr>
<td>Tryptophan*</td>
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<td>2.3</td>
<td>2</td>
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<tr>
<td>Amide nitrogen</td>
<td>1.038</td>
<td>20.1</td>
<td>20</td>
</tr>
</tbody>
</table>

* Average of four separate analyses (two analyses each at 24-hour and 72-hour hydrolysates).

a Calculated on 29,600 molecular weight and corrected to 100% recovery.
b Values for threonine and serine extrapolated to zero hydrolysis time.
c Determined as cysteic acid, corrected according to Hirs (29).
d Average value for 72-hour hydrolysis.
e Determined spectrophotometrically.

Hydrazinolysis followed by amino acid analysis revealed that threonine is the COOH-terminal amino acid of enterotoxin E. The recovery of threonine was calculated to be 0.91 mole per mole of enterotoxin E. The recovery of threonine was calculated to be 0.91 mole per mole of enterotoxin E after correcting for destruction during the hydrazinolysis process. Insignificant amounts (<0.1 mole per mole of protein) of serine and glycine, and an unknown by-product which emerged at the lysine position in a 5-cm column, were also detected. Serine and glycine were considered to be by-products formed during hydrazinolysis because they are often found after hydrazinolysis of proteins which have no COOH-terminal serine or glycine (38). An unknown by-product which emerged at the lysine position (5-cm column) was also detected in the COOH-terminal amino acid determination of enterotoxin C (34).

Effect of Heat on Serological Activity

When enterotoxin E was heated at 45° for 2 hours in a water bath, there was essentially no loss of immunological reaction with its specific antibody. There was about a 5% reduction in the enterotoxin E-antiterotxin precipitin reaction when the toxin was heated at 50° for 1 hour, 10% reduction at 60° for 1 hour, 40% reduction at 70° for 1 hour, 55% reduction at 80° for 20 min, 85% reduction at 80° for 10 min, and 95% reduction at 100° for 5 min.

Toxicity of Purified Enterotoxin E

Intragastric administration of 10 to 20 μg of enterotoxin E per monkey evoked vomiting in 60% of the animals, whereas intravenous injection of 0.5 μg of toxin per animal resulted in 50% of the animals vomiting. Enterotoxin E that was neutralized with its specific antibody failed to induce vomiting in monkeys.

Stability and Other Characteristics of Enterotoxin E

Enterotoxin E containing a small amount of sodium phosphate is relatively stable for long periods (up to 6 months) when kept in the dry form at room temperature. There was no significant reduction in the reaction of enterotoxin with its specific antibody when solutions of the toxin in dilute phosphate buffer, pH 7.0, were kept in the cold (2-5°C) for about 6 months or when it was kept at pH 11.0 at 25°C up to 2 weeks. However, when the enterotoxin was kept at pH 12 for about 24 hours, it lost its ability to react with its homologous antibody and did not evoke vomiting in monkeys. Holding the enterotoxin at pH 4.5 for 24 hours did not appreciably affect the antigen-antibody reaction as observed in single gel diffusion tubes. At pH of about 2.0, the precipitin reaction and toxic activity were completely lost. The toxic, immunological, and antigenic properties were not affected by urea solutions. After removal of the urea by exhaustive dialysis, the enterotoxin was still able to evoke vomiting in monkeys, still reacted with its specific antibody, and was still antigenic. Native enterotoxin E is resistant to the proteolytic action of trypsin.

Dry enterotoxin E is a fluffy, white material which is very soluble in aqueous salt solutions. The test for carbohydrate of Dubois et al. (39) on the highly purified enterotoxin was negative. No lipid material was detected in the chloroform-methanol extract.

The ratio of the absorbance at 260 nm and 280 nm is about 1.5, a value characteristic of a simple protein lacking bound nucleotides or other substances absorbing in the 260 nm region.

DISCUSSION

With the purification procedures described in this communication, it is possible to purify enterotoxin E in a few steps: (a) ion exchange chromatography on CM-cellulose columns of the concentrated culture supernatant fluid; (b) gel filtration (twice) with superfine Sephadex G-75 in 0.02 M phosphate buffer, pH 6.8; and (c) superfine Sephadex G-75 gel filtration with 6 M urea in 0.02 M phosphate buffer, pH 6.8. By the use of 6 M urea in at least one step of the purification, it was possible to purify this enterotoxin. Urea was utilized by Reichert (40) as an effective extraction agent to separate protease from pituitary tissue proteins. It was deduced that urea reduces protein-protein interaction or minimizes intercomponent attraction.

Enterotoxin E preparation obtained by the procedures described here appears to be homogeneous by the following criteria: (a) migration as a single component during electrophoresis on paper and starch gel over a wide range of pH values (4.5 to 11.0); (b) a single band in SDS-polyacrylamide gel electrophoresis; and (c) the presence of only one single antigenic component over a wide range of concentration with the use of antisera prepared against a crude preparation of the enterotoxin.

The reliability of the method of determining molecular weight by dodecyl sulfate-polyacrylamide gel electrophoresis has been...
discussed in detail by Weber and Osborn (26). They reported that the accuracy of this technique is better than ±10% for polypeptide chains with molecular weights between 15,000 and 100,000. This method should be at least as reliable as the one involving sedimentation equilibrium since in the latter method a deviation of 0.02 in the partial specific volume results in a 10% deviation of the molecular weight (26). Therefore, a molecular weight of 30,000 for enterotoxin E obtained from SDS-polyacrylamide gel electrophoresis is adequate within the limits of error of the method. Furthermore, the molecular weight value of 29,800 determined from sucrose gradient ultracentrifugation confirms the molecular weight obtained by SDS-polyacrylamide gel electrophoresis. Gel filtration with the use of standardized conditions also has been used to determine molecular weights of a wide variety of proteins (41-43). A value of 29,000 for enterotoxin E obtained by this technique is in accord with that obtained from the other physical methods.

Enterotoxin E is a simple protein, consisting of a single polypeptide chain. This was confirmed when the reduced and alkylated toxin gave a single component in SDS-polyacrylamide gel electrophoresis. Additional evidence for this conclusion is the fact that only one NH2-terminal amino acid (serine) and one COOH-terminal amino acid (threonine) were found per molecule. Enterotoxins A, B, C1, and C2 (45) are also simple proteins consisting of one polypeptide chain. However, purification of enterotoxin D was claimed (10), physical and chemical studies of the molecule were not reported. The enterotoxins are relatively small proteins with molecular weights around 30,000 and an absorption maximum at 277 nm. They are potent, toxic substances; as low as 10 pg per monkey evoke vomiting in 30,000 and an absorption maximum at 277 nm. They are potent, toxic substances; as low as 10 pg per monkey evoke vomiting in alcoholic or non-alcoholic conditions.

Enterotoxins including enterotoxin E are resistant to the action of trypsin. It is conceivable that the points of trypsin attack on the arginyl and lysyl residues are not readily available for proteolytic action. The enterotoxins have a high content of lysine, aspartic acid, or asparagine (or both), glutamic acid or glutamine (or both), and tyrosine, but have only one disulfide bridge and 1 or 2 tryptophanyl residues.

Antibodies are usually sensitive indicators of conformational change. Since reduced and alkylated enterotoxin E still reacts with antibodies directed to native enterotoxin E, the disulfide bonds, therefore, are not essential for immunological reactivity. This conclusion has also been reported for enterotoxin B (46). Conformational changes of enterotoxin E induced by extreme acidic and basic conditions and heating relate to the loss of its emetic and serological activities.

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Purification and Some Physicochemical Properties of Staphylococcal Enterotoxin E
Concordia R. Borja, Ellen Fanning, I-Yih Huang and Merlin S. Bergdoll


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