Purification and Some Physicochemical Properties of Staphylococcal Enterotoxin D†

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ABSTRACT: A method was developed for the isolation of staphylococcal enterotoxin D in highly purified form from cultures of *Staphylococcus aureus* strain 1151m. The method involves removal of the toxin from the culture supernatant fluid with the ion-exchange resin CG-50 followed by chromatography on carboxymethylcellulose (twice) and by gel filtration on Sephadex G-75 (twice). The purified toxin is homogeneous by polyacrylamide gel and sodium dodecyl sulfate–polyacrylamide gel electrophoresis and double gel diffusion tests. It is a simple, colorless, antigenic protein with an isoelectric point of 7.4 as determined by isoelectric focusing. Its molecular weight was determined to be 27 300 ± 700 by molecular sieve chromatography on Sephadex G-100 and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Its serological activity is stable over a wide range of pH values (1.2–10.7). The enterotoxin consists of 236 amino acid residues and contains no free sulfhydryl groups. End-group analysis showed serine to be the NH_2-terminal amino acid and lysine to be the COOH-terminal amino acid.

Staphylococcus aureus produces a group of toxic substances which are called enterotoxins because of their effects on the gastrointestinal tract. Vomiting and diarrhea occur 2–5 h after ingestion of food contaminated with these substances. In 1963 a systematic nomenclature using sequential lettering was established to classify them as immunologically distinct entities (Casman et al., 1963). The purification and physicochemical properties of enterotoxins A (Chu et al., 1966; Schantz et al., 1972), B (Bergdoll et al., 1959, 1965b; Schantz et al., 1965; Spero et al., 1965; Wagman et al., 1965), C (Avena & Bergdoll, 1967; Borja, 1969; Borja & Bergdoll, 1967, 1969; Huang et al., 1967), and E (Borja et al., 1972) have been reported. Enterotoxin D was identified by Casman et al. (1967), but purification was not achieved.

In this communication, we report the purification of enterotoxin D and describe some of its properties.

Materials and Methods

Materials. Reagents included 2-mercaptoethanol (Calbiochem), sodium dodecyl sulfate (Sigma), *N,N'*-methylenebisacrylamide, acrylamide, *N,N,N',N'*-tetramethylenediamine, 1-fluoro-2,4-dinitrobenzene, and anhydrous hydrazine (Eastman), Coomassie brilliant blue R-250 (Colab), riboflavin (Nutritional Biochemicals), 5,5'-dithiobis(2-nitrobenzoic acid) (Aldrich), and Blue dextran 2000 (Pharmacia). The CM-cellulose[1] was obtained from Carl Schleicher and Schuell and Amberlite CG-50 (100–200 mesh) from Mallinckrodt Chemical Works. Sephadex G-75 (40–120 μm) and Sephadex G-100 (40–120 μm) were purchased from Pharmacia and poly(ethylene glycol), 20 M (Carbowax), was purchased from Union Carbide Corp. The proteins used were bovine serum albumin, ovalbumin, trypsin, and lysozyme (Sigma).

Estimation of Protein and Ultraviolet Absorption. Protein concentration of enterotoxin D during its purification was based on the absorbance at 278 nm with an $E_{1cm}^{1\%}$ of 14.0, the value of enterotoxin B (Schantz et al., 1965). The highly purified enterotoxin D has an $E_{1cm}^{1\%}$ of 10.6 at 280 nm and 10.8 at 278 nm (maximum absorption). Absorbance values in the ultraviolet and visible regions were obtained with use of quartz cuvettes (1-cm cell path) in a Beckman Model DB-G spectrophotometer.

Molecular Sieve Chromatography with Sephadex G-100 for Determination of Molecular Weight. An Ace glass column, 2.5 cm in diameter, was packed with Sephadex G-100 which had been equilibrated with 0.1 M sodium phosphate buffer, pH 8.5, to a height of 182 cm. The sample applied to the column contained 10–15 mg of solute in 1 mL of solution. The eluate was collected in 2.9-mL fractions. Blue dextran 2000 was employed to determine the void volume. The proteins used as standards were bovine serum albumin, 67 000 (Schachman & Edelstein, 1966); ovalbumin, 45 000 (Gutfreund, 1944); enterotoxin B, 28 366 (Dayhoff, 1972); trypsin, 24 500 (Kay et al., 1961); and lysozyme, 14 499 (Sophianopoulos & Van Holde, 1964).

Disc Gel Electrophoresis in Polyacrylamide Gel. The cationic system of Reisfeld et al. (1962) was employed with some modifications. Fifty microliters of sample (10 μg of enterotoxin D) was applied to a glass tube (8.8 cm long with a 0.5-cm internal diameter) containing 0.15 mL of a large pore solution and 1.3 mL of a small pore solution of the gel. Electrophoresis was performed at room temperature (around 20 °C) with a current of 1 mA/gel column for 15 min and then 3 mA/gel column for 1.5 h. Gels were stained with 0.25% Coomassie brilliant blue R-250 in 20% Cl_3CCOOH for 1 h and then destained with 7% acetic acid.

Determination of Molecular Weight by Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. The method described by Shapiro et al. (1967) as modified by Borja et al. (1972) was employed to estimate the molecular weight of enterotoxin D by electrophoresis in $NaDodSO_4$–polyacrylamide gel. Samples (enterotoxin D and standards) at a concentration of about 0.3 mg/mL in 0.1 M sodium phosphate buffer, pH 7.1, containing 0.1% $NaDodSO_4$ and 1% 2-mercaptoethanol were incubated for 3 h at 37 °C. One milliliter of each protein solution was mixed with 1 mL of 40% sucrose solution containing 0.1% bromophenol blue as tracking dye. About 100 μL of each protein solution was layered on the top of the running gel. Immediately the electrophoresis buffer (0.1 M sodium phosphate buffer, pH 7.1, with 1%

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[1] Abbreviations used: CM-cellulose, carboxymethylcellulose; $NaDodSO_4$, sodium dodecyl sulfate; TLC, thin-layer chromatography.

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$NaDodSO_4$) was layered carefully on top of the sample. Electrophoresis was performed at room temperature with a current of 6 mA/gel column for approximately 4.5 h. All proteins were run in triplicate. The standard proteins used to prepare the calibration curve for determination of the molecular weight of enterotoxin D were serum albumin, ovalbumin, enterotoxin B, and lysozyme. Mobilities were calculated according to Weber & Osborn (1969).

Isoelectric Focusing. Isoelectric focusing of enterotoxin D preparations was performed in an LKB 2117 preparative flat bed isoelectric focusing apparatus (LKB-Produkter) at a constant current of 8 W for 18–20 h at 10 °C by using Servalytes (Serva), 2.5 mL each of pH ranges 6–8 and 7–9 to give a working range of 6–9.

Enterotoxin Production. The *Staphylococcus aureus* strain 1151m used for the production of enterotoxin D was obtained from the Department of Microbiology, Tokyo Metropolitan Research Laboratory of Public Health, Tokyo. The culture was preserved on porcelain beads (Hunt et al., 1958). One bead was placed in each of four 250-mL Erlenmeyer flasks containing 50 mL of sterile medium, composed of 3% N-Z Amine NAK (Humko Sheffield Chemical), 3% Protein Hydrolysate Powder (Mead Johnson & Co.), 10 mg/L niacin, and 0.5 mg/L thiamin and adjusted to pH 6.8. Cultures were incubated at 37 °C without shaking for 18 h and aseptically inoculated into a Microferm Fermenter Model 128S (New Brunswick Scientific Co.) containing 20 L of sterilized medium. The culture was agitated at 500 rpm with an air flow of 20 L/min. It was incubated at 37 °C for 12–18 h. Antifoam (Dow Corning Silicone B diluted 1 to 10) was added automatically by peristaltic pump to control foaming. The culture was centrifuged in a continuous flow centrifuge (Model A-2 Beta-Fuge, Vernitron Medical Products, Inc.), at a rate of approximately 200 mL/min.

Antisera to Staphylococcus aureus Strain 1151m Antigens. Antisera to the highly purified toxin were prepared in rabbits by injecting the toxin with complete Freund's adjuvant subcutaneously at the rabbits' shoulders. The amounts injected were 7, 13, 39, 117, and 260 μg at weeks 0, 1, 3, 4, and 5, respectively. After 6 weeks, 50-mL bleedings were taken from an ear artery by syringe weekly for 5 weeks. Antisera to crude enterotoxin D were prepared in rabbits by following the procedure reported by Bergdoll et al. (1971).

Detection and Assay of Enterotoxin D. Fractions from the different purification steps were tested for serological activity by the Ouchterlony plate gel diffusion method as modified by Bergdoll et al. (1965a). When the purified enterotoxin became available, specific assays were carried out by the single gel diffusion tube method (Borja & Bergdoll, 1967). The procedure was employed (1) to obtain a standard curve; (2) to determine the concentration of enterotoxin D in different steps of the purification; and (3) to determine in the experiments the effects of pH and heat on the serological activity of the enterotoxin.

Amino Acid Analysis. A Spinco Model 120B amino acid analyzer remodeled to 120C was used to determine the amino acid composition of enterotoxin D according to the procedure of Benson & Patterson (1965) as applied by Borja et al. (1972).

Detection of Sulfhydryl Groups. Sulfhydryl groups were detected by the technique of Glaser et al. (1970) with the use of 5,5′-dithiobis(2-nitrobenzoic acid).

Amino-Terminal Amino Acid Analysis. The NH_2-terminal amino acid of enterotoxin D was determined by the 1-fluoro-2,4-dinitrobenzene method of Sanger (1945) as modified by Fraenkel-Conrat et al. (1955). The ether-soluble dinitrophenyl amino acids were identified after one-dimensional ascending TLC on silica gel G in benzene–pyridine–acetic acid (70:5:25), chloroform–1-propanol–acetic acid (20:80:1), and chloroform–methanol–acetic acid (60:40:1) (Brenner et al., 1969). The ether-soluble dinitrophenyl amino acids separated on the silica gel G chromatograms were eluted with 1% sodium bicarbonate solution. The color intensity was measured at 360 nm. The water-soluble dinitrophenyl amino acids were examined with one-dimensional ascending TLC on silica gel G in benzene–pyridine–acetic acid (70:5:25).

Carboxy-Terminal Amino Acid Analysis. The COOH-terminal amino acid of enterotoxin D was determined by a slight modification of the procedure used for the determination of the COOH-terminal amino acid of enterotoxin C (Huang et al., 1967). The enterotoxin (0.1 μmol) was hydrazinolyzed at 100 °C for 7.5 h.

Toxicity of Enterotoxin D in Monkeys. The toxicity of enterotoxin D in the various samples was tested by the intragastric administration of 50 mL of enterotoxin solutions to each of six rhesus monkeys. The neutralizing effect of anti-enterotoxin D sera was ascertained by mixing the enterotoxin solution (2 μg/mL) with a slight excess of the antiserum and allowing it to stand overnight before feeding to the monkeys. Emesis within a 5-h period was accepted as a positive reaction for enterotoxin.

Results

Purification Procedure. Step I. Approximately 20 L of culture supernatant fluid (0.5–1.0 μg of toxin/mL) was adjusted to pH 5.6 with 6 N HCl and 10 L was transferred to a 50-L container. About 250 mL of CG-50 resin in 0.02 M sodium phosphate buffer at pH 5.6 was added and the final volume made to about 45 L with distilled water. The mixture was stirred for 1 h at room temperature after which the liquid was separated from the resin by decanting. The second half of the culture supernatant fluid was treated in the same manner. The resin from the two extractions was packed into a chromatographic tube and washed with 1 L of distilled water. The toxin was eluted with 0.5 M sodium phosphate at pH 6.2 in 0.5 M sodium chloride at a flow rate of 15 mL/5 min (one fraction). The fractions containing the toxin were combined and concentrated by dialysis for 24 h against 5 kg of 20% poly(ethylene glycol) solution (w/w) to reduce the volume to 200–250 mL. Recovery of the enterotoxin was about 90%. The concentrated toxin was frozen.

Step II: Ion-Exchange Chromatography on CM-cellulose. The frozen toxin solutions from three separate preparations were thawed, pooled, and dialyzed against 0.02 M sodium phosphate buffer, pH 5.6, to lower the pH and reduce the salt content. Any precipitate was removed by centrifuging at 16000*g* for 20 min at room temperature. The pH of the supernatant was readjusted to 5.6 with 0.1 M H_3PO_4, if necessary. The toxin solution (containing 840 mg of protein) was applied to a CM-cellulose column (4.0 × 48 cm) previously equilibrated with 0.02 M sodium phosphate buffer at pH 5.6. A typical elution pattern is shown in Figure 1. The column was first washed with 0.02 M sodium phosphate buffer at pH 5.6 and then with 0.03 M sodium phosphate buffer at pH 6.1. The toxin was eluted with an increasing linear gradient of sodium phosphate buffer using 900 mL of 0.03 M at pH 6.1 and 900 mL of 0.06 M at pH 6.8. The flow rate was 11 mL/10 min (one fraction). Fraction A represents unadsorbed protein and contained most of the yellowish brown substances that contaminated the enterotoxin. Fractions C and F contained one antigenic substance each which, if not

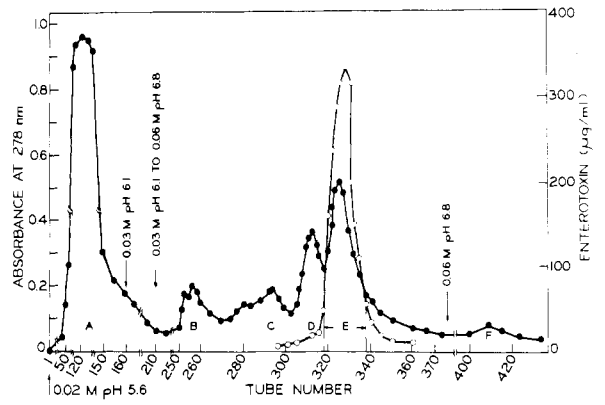

FIGURE 1: Elution pattern of enterotoxin D from a CM-cellulose column. Crude enterotoxin from 60 L of culture supernatant fluid from step I was applied to the column (4.0 × 48 cm) and eluted with an increasing linear gradient of sodium phosphate buffer by using 900 mL of 0.03 M, pH 6.1, and 900 mL of 0.06 M, pH 6.8. The flow rate was 11 mL/10 min (one fraction). Absorbance at 278 nm (●—●); enterotoxin content (μg/mL) (O—O).

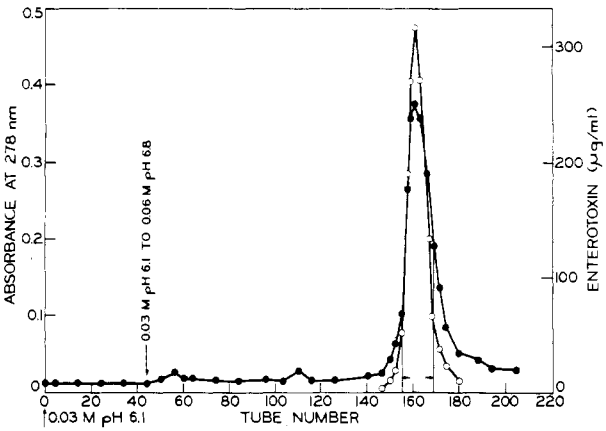

FIGURE 2: Elution pattern of enterotoxin D from rechromatographing on CM-cellulose. Partially purified enterotoxin D from step II (74 mg of protein) was applied to the column (4.0 × 48 cm) and eluted with an increasing linear gradient of sodium phosphate buffer by using 900 mL of 0.03 M, pH 6.1, and 900 mL of 0.06 M, pH 6.8. The flow rate was 11 mL/10 min (one fraction). Absorbance at 278 nm (●—●); enterotoxin content (μg/mL) (O—O).

removed at this stage, could not be separated from the enterotoxin by gel filtration. Fraction E gave two major and two minor precipitin lines with antiserum prepared against crude enterotoxin D. The percentage of enterotoxin increased from about 2% in step I to approximately 32% in step II with a recovery of 65%.

Step III: Rechromatography on CM-cellulose. Fraction E from step II was dialyzed against 0.02 M sodium phosphate buffer at pH 5.6. The dialyzed toxin solution (245 mL containing 74 mg of protein) was rechromatographed on a CM-cellulose column (4.0 × 48 cm) in 0.02 M sodium phosphate buffer at pH 5.6. The adsorbed toxin was eluted with a linear gradient of sodium phosphate buffers as in step II (Figure 2). Two major antigen–antibody precipitin lines were formed with the toxin fractions on double gel diffusion plates. The fractions containing toxin were pooled, concentrated, and lyophilized. The purity of the enterotoxin was about 40% with a recovery of about 50%.

Step IV: Gel Filtration through Sephadex G-75. The dried samples from two columns from step III were combined and redissolved in 5 mL of distilled water. The toxin solution (66 mg of protein) was layered carefully on a column of Sephadex G-75 (4.0 × 95 cm) which had been equilibrated with 0.02

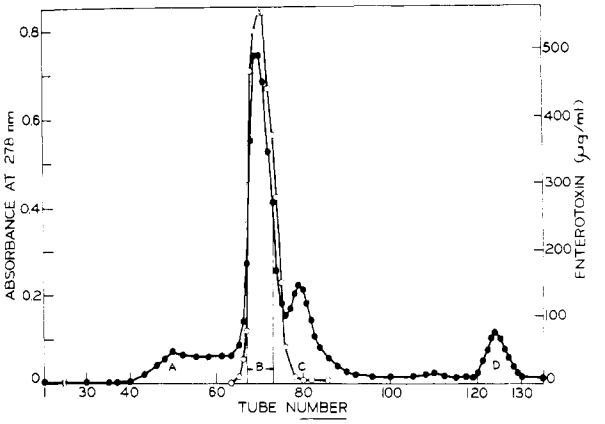

FIGURE 3: Elution pattern of enterotoxin D from a column of Sephadex G-75 (40–120 μm). The enterotoxin preparation from step III (66 mg of protein) was placed on the Sephadex column (4.0 × 95 cm) and eluted with 0.02 M sodium phosphate buffer, pH 6.8. The flow rate was 11 mL/12.7 min (one fraction). Absorbance at 278 nm (●—●); enterotoxin content (μg/mL) (O—O).

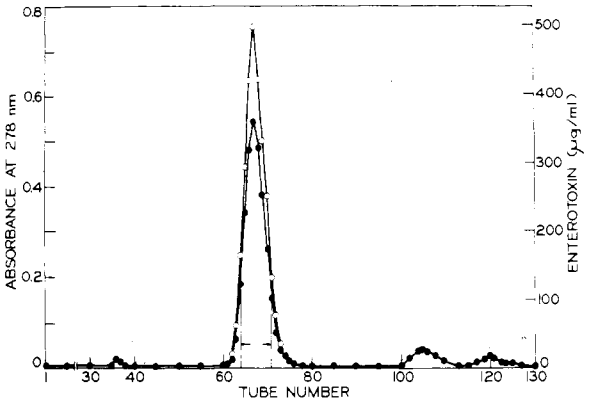

FIGURE 4: Elution pattern of enterotoxin D from a second column of Sephadex G-75 (40–120 μm). The enterotoxin preparation from step IV was placed on the Sephadex column and eluted with 0.02 M sodium phosphate buffer, pH 6.8. The flow rate was 11 mL/17.4 min (one fraction). Absorbance at 278 nm (●—●); enterotoxin content (μg/mL) (O—O).

M sodium phosphate buffer at pH 6.8. The same buffer was used for elution of the toxin from the column. The flow rate was 11 mL/12.7 min (one fraction). A typical elution profile is shown in Figure 3. The major portion of the enterotoxin D appeared in fraction B and was separated from the other major antigen in the material from step III. This antigen was in fraction C. Fraction B was concentrated and lyophilized. The purity of the enterotoxin in fraction B was about 80% with a recovery of 28%.

Step V: Refiltration through Sephadex G-75. The dried toxin from step IV was dissolved in 5 mL of distilled water and refiltered through a column of Sephadex G-75 (4.0 × 93 cm). The flow rate was 11 mL/17.4 min (one fraction). The elution pattern is shown in Figure 4. The recovery of enterotoxin at this stage was about 20%.

Test for Purity. The results of the immunological tests in double gel diffusion plates with antiserum against the crude toxin showed a high degree of purity for the enterotoxin from step V. The appearance of one faint precipitin line in addition to the enterotoxin line at an enterotoxin D concentration of 0.3 mg/mL indicated the presence of a small amount of impurity. The faint line disappeared at an 8-fold dilution of the enterotoxin D solution, while the enterotoxin line disappeared only after more than a 1000-fold dilution. This indicated the impurity was less than 1.0%. The purified en-

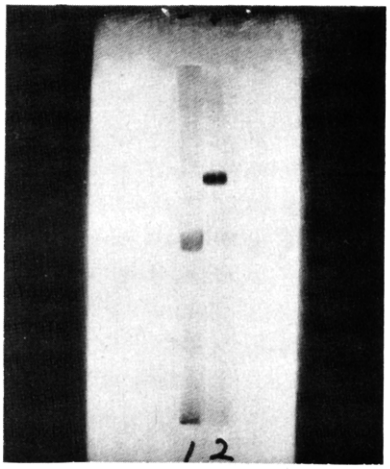

FIGURE 5: Polyacrylamide gel electrophoretic patterns. The cathode for gel column 1 is at the top and for column 2 is at the bottom. (Column 1) Purified enterotoxin from step V was applied to 5% gel with 0.1% $NaDodSO_4$ reduced with 2-mercaptoethanol, pH 7.1; (column 2) purified enterotoxin from step V was applied to 7.5% gel, pH 4.5.

terotoxin D gave a single band in disc polyacrylamide gel and $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 5).

Molecular Weight. The ratio of elution volume to void volume of enterotoxin D was determined on a Sephadex G-100 column that had been calibrated with bovine serum albumin, ovalbumin, enterotoxin B, and lysozyme. On the basis of this calibration curve, the molecular weight of enterotoxin D was calculated to be approximately 28 000. A molecular weight of 26 600 for enterotoxin D was calculated from its mobility on $NaDodSO_4$–polyacrylamide gel as compared with the mobilities of bovine serum albumin, ovalbumin, enterotoxin B, trypsin, and lysozyme. The average of the molecular weights determined by the two methods is 27 300 ± 700.

Isoelectric Point. Isoelectric focusing of pure enterotoxin D and of crude enterotoxin produced in a fermenter, in shake flasks, by the sac culture, and cellophane-over-agar methods gave an isoelectric point of 7.40 ± 0.05 for the major peak.

Amino Acid Composition. The amino acid composition of enterotoxin D is given in Table I. The results represent the average of four separate analyses (two each for the 48-h and 72-h hydrolysis). The values for serine, threonine, and amide nitrogen were obtained by extrapolating to zero hydrolysis time. The values for valine and isoleucine are from the 72-h hydrolysis. No detectable free sulfhydryl groups were found in the purified enterotoxin. Calculations from the amino acid composition showed the toxin to contain a total of 236 amino acid residues.

Terminal Amino Acids. The results obtained from 1-fluoro-2,4-dinitrobenzene showed the presence of dinitrophenylserine and the usual byproducts of dinitroaniline and dinitrophenol. The recovery of dinitrophenylserine was calculated to be 0.83 mol/mol of enterotoxin D after correction for destruction during the hydrolysis and loss in thin layer chromatography. The aqueous phases of dinitrophenylenterotoxin D hydrolysates contained no α-dinitrophenyl amino acids.

Hydrazinolysis followed by amino acid analysis revealed that lysine is the COOH-terminal amino acid of enterotoxin D. The recovery of lysine was calculated to be 1.02 mol/mol of enterotoxin D with the use of enterotoxin B for correcting the losses during the hydrazinolysis and extraction processes. Insignificant amounts (<0.2 mol/mol of protein) of serine and glycine were detected but these are often found as byproducts of hydrazinolysis of proteins which have no COOH-terminal serine or glycine (Okude & Iwanaga, 1970).

Table I: Amino Acid Composition of Enterotoxin D

amino acid	mol/mg of protein[a]	calcd residues[b]	nearest integral residues	integral no. of residues × respective residue mol wt
Lys	1.028	28.06	28	3584
His	0.198	5.40	5	685
Arg	0.221	6.03	6	936
Asp	1.476	40.29	40	4600
Thr[c]	0.455	12.41	12	1212
Ser[c]	0.596	16.28	16	1392
Glu	1.038	28.33	28	3612
Pro	0.148	4.05	4	388
Gly	0.481	13.13	13	741
Ala	0.287	7.82	8	568
1/2-cystine[d]	0.078	2.13	2	204
Val[e]	0.425	11.60	12	1188
Met	0.078	2.12	2	262
Ile[e]	0.544	14.84	15	1695
Leu	0.833	22.75	23	2599
Tyr	0.451	12.32	12	1956
Phe	0.329	8.99	9	1323
Trp[f]	0.034	0.93	1	186
amide nitrogen	1.087	29.68	30[g]	(−30 + 18)
total			236	27179

[a] Average of four separate analyses (two analyses each at 48- and 72-h hydrolysis). [b] Based on 27 300 molecular weight. [c] Values for threonine and serine extrapolated to zero hydrolysis time. [d] Determined as cysteic acid. [e] Average value for 72-h hydrolysis. [f] Determined spectrophotometrically. [g] Excluded from total.

Table II: Toxicity of Enterotoxin D in Monkeys

enterotoxin sample	amt[a] given	results (no. positive/ no. fed)
culture supernatant	20[b]	1/6
culture supernatant	100	5/6
purified	25[b]	2/6
culture supernatant plus antiserum D	100	0/6

[a] In units of μg per 50 mL per monkey. [b] Diluted or dissolved in sterile medium.

Effect of Heat on Serological Activity. About 50% of the serological activity was lost rapidly during the first few minutes of heating at 90 and 100 °C with the remainder inactivated at a slow rate. There was an approximate reduction in enterotoxin D–anti-enterotoxin D precipitin reaction of 65 and 85% when the toxin was heated at 90 °C for 1 and 2 h, respectively, and a reduction of 85 and 95% when heated at 100 °C for 1 and 2 h, respectively.

Enterotoxicity of Enterotoxin D. The results of toxicity tests in monkeys showed that the antigen purified is the enterotoxin and that the antibody identified with the toxin does neutralize it (Table II).

Stability and Other Characteristics of Enterotoxin D. Enterotoxin D is relatively stable for up to 1 year when kept in the dry form, even at room temperature, or in the frozen state. The toxin molecule is stable at pHs ranging from 1.2 to 10.7. Denaturation of the toxin occurs at or above pH 11.2. The antigen–antibody activity was lost completely after incubation for 3 h at pH 12.8. Native enterotoxin D is resistant to the proteolytic action of trypsin.

Enterotoxin D is a fluffy, white material which is very soluble in aqueous salt solutions. The test for carbohydrate

in the highly purified enterotoxin was negative (Dubois et al., 1956). No lipid material was detected in the chloroform–methanol extract. The ratio of the absorbance at 278 and 260 nm is about 1.9, indicating the toxin contains no bound nucleotides.

Discussion

Three reports on the purification of enterotoxin D have been published (Igarashi, 1972; Langner & Kloss, 1974; Yamada, 1977). Thorough examination in the Food Research Institute of "purified" materials supplied to us by Drs. Igarashi and Langner indicated them to have a purity of less than 20%. Yamada (1977) reported that one impurity was not separated from the toxin by his procedure.

It is possible with the purification procedure described here to purify enterotoxin D in a few steps: (a) adsorption of enterotoxin D from culture supernatant fluids with CG-50 resin; (b) ion-exchange chromatography (twice) on CM-cellulose columns with a gradient phosphate buffer from 0.03 M at pH 6.1 to 0.06 M at pH 6.8; (c) gel filtration (twice) with Sephadex G-75 in 0.02 M phosphate buffer, pH 6.8. Gradient elution with phosphate buffer from the CM-cellulose columns was a key step in the purification. The enterotoxin D appears to be homogeneous by the following criteria: (a) a single band in disc polyacrylamide gel electrophoresis (pH 4.5); (b) a single band in $NaDodSO_4$–polyacrylamide gel electrophoresis; and (c) the presence of a single antigenic component over a wide range of concentrations with the use of antiserum prepared against a crude preparation of the enterotoxin.

The reliability of the sodium dodecyl sulfate–polyacrylamide gel electrophoresis method for determining molecular weight has been discussed in detail by Weber & Osborn (1969). They reported the accuracy of this technique is better than 10% for polypeptide chains with molecular weights between 15 000 and 100 000. The molecular weight of 26 000 obtained for enterotoxin D by $NaDodSO_4$–polyacrylamide gel electrophoresis is within the limits of error of the method. Gel filtration under standardized conditions also has been used to determine molecular weights of a wide variety of proteins (Andrews, 1965; Siegel & Monty, 1966; Whitaker, 1963). A value of 28 000 for enterotoxin D obtained by this technique is in accord with that from $NaDodSO_4$–polyacrylamide gel electrophoresis.

Enterotoxin D is a simple protein, consisting of a single polypeptide chain. This was confirmed when the reduced toxin gave a single component in $NaDodSO_4$–polyacrylamide gel electrophoresis. Additional evidence for this conclusion is the fact that only one NH_2-terminal amino acid (serine) and one COOH-terminal amino acid (lysine) were found per molecule. Enterotoxins A (Schantz et al., 1972), B (Spero et al., 1965), C_1 (Borja & Bergdoll, 1967), C_2 (Avena & Bergdoll, 1967), and E (Borja et al., 1972) are also simple proteins consisting of one polypeptide chain. The enterotoxins are relatively small proteins with molecular weights of around 26 000 to 30 000 and an absorbance maximum at 277 or 278 nm.

The enterotoxins are relatively potent, toxic substances in rhesus monkeys as reported by Schantz et al. (1965) for enterotoxin B (0.9 μg/kg, oral dose) and Schantz et al. (1972) for enterotoxin A (1 μg/kg, oral dose). In their experiments over 100 monkeys were used for each toxin. It has been impossible to repeat these studies in the Food Research Institute, because only small numbers of animals were available at any one time and considerable variation in their response to enterotoxin was observed in different lots. In general, the results which we have obtained indicated more toxin was required (3–5 μg/kg) than that reported by Schantz and his co-workers. At times, at least twice as much (5–10 μg/kg) was required to cause emesis in the monkeys. This was the case when the enterotoxin D was tested. No attempt was made to determine the specific toxicity even though it was realized that this would be useful information. Under the circumstances, it was impossible to obtain this information. The main purpose of the monkey feedings with enterotoxin D was to show that the purified antigen was the toxin. This we accomplished. It is difficult to compare its toxicity with that of the other enterotoxins but from the results obtained with the others at the time the testing of enterotoxin D was done it would appear that it is only slightly less toxic than the others.

The antigenicity of the enterotoxins provides a means for detection of the different types by immunological techniques. All of the enterotoxins including enterotoxin D are resistant to the action of trypsin except for the nicking of enterotoxins B and C_1 in the cystine loop (Spero et al., 1973, 1976), but this has no effect on their toxicity or reaction with the specific antibodies. It is conceivable that the points of 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) and glutamic acid or glutamine (or both) but have only one disulfide bridge and 1 or 2 tryptophanyl residues. Enterotoxin D has 2 methionine residues which is similar to enterotoxins A and E but different from enterotoxins B and the C's which have 8 or 9 residues. It has been proposed that at least one methionine is essential for the toxic action (Huang et al., 1976). The tyrosine content of enterotoxin D is lower than that for the other enterotoxins (12 vs. 19–21) which might account in part for its apparent lower toxicity.

Acknowledgments

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Identification of Interacting Amino Acids at the Histone 2A–2B Binding Site†

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ABSTRACT: Histones 2A and 2B of calf thymus were cross-linked within intact nuclei by UV irradiation. This procedure induces the formation of covalent cross-links between non-covalently interacting residues in the histones of native chromatin. Tryptic peptide and partial sequence analysis of the cross-linked product has shown that the covalent linkage is between tyrosine-37, -40, or -42 (we have not yet determined which) of H2B and proline-26 of H2A. We conclude that these residues constitute part of the hydrophobic H2A–H2B binding domain within the nucleosomes of native chromatin.

The DNA of eukaryotes is packaged tightly within chromatin together with histones to form compact subunits called nucleosomes (Felsenfeld, 1978). Nucleosomes contain two each of the so-called core histones (2A, 2B, 3, and 4) around which are wrapped 144 base pairs of the core DNA. Some additional DNA (often called spacer) as well as histone 1 is usually associated with the core in a complete nucleosome.

We are engaged in studying the structure of the nucleosome histone core by means of analysis of the products of histone–histone cross-linking. Zero-length cross-links are induced at sites of noncovalent histone–histone association within intact nuclei. This yields cross-linked histone products which contain within their covalent structure information about the noncovalent arrangement of histones in the nucleosome core of native chromatin. Sequence analysis identifies the sites of cross-linking and thus permits precise identification of sites of noncovalent histone–histone associations within the native structure.

UV irradiation of whole cells induces the nearly quantitative conversion of monomer H2A and H2B into an H2A–H2B dimer (Martinson et al., 1976). The nature of the UV cross-linking reaction is such that the cross-link can be presumed to represent the covalent joining of the two histones within their mutual binding sites (Martinson et al., 1976; Martinson & McCarthy, 1976). This expectation arises because UV is known to induce cross-links by activation of tyrosine. Subsequent attack by this tyrosine on a precisely adjacent neighbor then yields a zero-length cross-link. Preliminary CNBr peptide mapping results (Martinson & McCarthy, 1976) have shown that the H2A–H2B link is

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