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THE APPLICATION OF QAE-SEPHADEX FOR THE PURIFICATION OF TWO STAPHYLOCOCCAL ENTEROTOXINS

I. PURIFICATION OF ENTEROTOXIN C_2

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UMMARY

A new method developed for purification of enterotoxin C_2 from *Staphylococcus aureus* strain 361 consisted of four steps: batchwise adsorption from culture supernatant on QAE-Sephadex; gel filtration on Sephadex G-100; chromatography on QAE-Sephadex using a buffer of constant pH and molarity; and gel filtration using a volatile buffer as the eluting solvent. The purified enterotoxin appeared homogeneous by gel immunodiffusion, gel chromatography and in the analytical ultracentrifuge, although an apparent heterogeneity was noted on QAE-Sephadex chromatography and polyacrylamide disc electrophoresis at pH 4.5. The emetic dose, ED_{50}, by intravenous route in cynomolgus monkeys was 0.04 μg/kg of animal weight.

Upon treatment with sodium dodecylsulfate, β-mercaptoethanol and urea, enterotoxin C_2 separated into 3 bands in sodium dodecylsulfate-electrophoresis. One band mol. wt 29 000, and two bands of lower molecular weight were so close that they moved as a single zone. After elution from gels, the zone of lower molecular weight oligopeptides emerged as a single peak at the same position as untreated enterotoxin C_2 during gel filtration with buffer lacking thiol and denaturant, and gave a reaction of complete identity to enterotoxin C_2 in Ouchterlony immunodiffusion. The results suggest that enterotoxin C_2 is a mixture composed of intact polypeptide chains, mol. wt 29 000, and two fragments cleaved in the disulfide region of molecular weight of approx. 15 400 and 12 800 linked by the single disulfide bond in the toxin molecule.

Amino acid analysis indicates that enterotoxin C_2 consists of 255 amino acid residues.

INTRODUCTION

In connection with our work on the determination of staphylococcal enterotoxins by radioimmunoassay [1], attempts have been made to handle large volumes of *Staphylococcus aureus* strain 361 cultures for isolation of relatively large quantities of enterotoxin C_2. In the initial stages of this study crude toxin was obtained by concentration of the supernatant culture fluid 10–20 fold by dialysis against carbowax,

according to the procedure of Avena and Bergdoll [2]. The experiments reported here were carried out with the quaternized exchanger QAE-Sephadex. Under appropriate conditions, the concentration of toxin from culture fluids by adsorption to QAE-Sephadex has been found to be as effective as, and simpler than concentration by dialysis against carbowax.

The method of purification consists of gel filt ration of the concentrated toxin through Sephadex G-100, chromatography of the active fractions on QAE-Sephadex and gel filtration of the eluted material giving highly purified enterotoxin C_2. This method of purification is simpler than the procedure described by Avena and Bergdoll [2] who used stepwise and gradient elution from carboxymethylcellulose columns and gel filtration twice.

Additional experiments were made following the same principle in the purification of staphylococcal enterotoxin A (see accompanying paper). The results indicate that highly purified preparations of the antigenically-distinct enterotoxin types A and C_2 can be obtained by the use of a common purification procedure; some properties of the purified toxins are discussed.

MATERIALS AND METHODS

Microorganism. The strain used for the production of enterotoxin C_2 was *Staphylococcus aureus* 361, which was very kindly provided by Dr M. S. Bergdoll of the Food Research Institute, University of Wisconsin at Madison. The organism was kept on porcelain beads at 5 °C until used [3].

Culture medium. The culture medium used for production of enterotoxin consisted of 3% protein hydrolysate powder (Mead Johnson), 3% NZ-amine, type NAK (Sheffield), 0.00005% thiamine and 0.001% niacin and was adjusted to pH 6.7. The reverse modification of the sac-culture method [4] was used. 500 ml-portions of the culture medium were placed into 4.3 cm flat-width dialysis bags. Each bag was suspended in a 2-l Erlenmeyer flask containing 90 ml of $3 \cdot 10^{-4}$ M phosphate buffer (pH 7.2) and autoclaved. A solution of thiamine and niacin was sterilized by filtration and added to the phosphate-buffered water.

Preparation of crude enterotoxin. A subculture of strain 361 was inoculated into the culture medium, and incubated for 24 h at 35 °C. Then 10 ml of the seed culture, harvested and resuspended in 10 ml of phosphate-buffered water, was added as inoculum to the liquid surrounding the sac. The inoculated sac culture was incubated for 48 h at 35 °C on a rotary shaker. The cells from the fluid surrounding the sac were removed by centrifugation and the supernatant obtained served as a source of crude enterotoxin.

Materials. Reagent grade urea obtained from British Drug Houses, Ltd., Toronto, was recrystallized twice from ethanol before use. β-Mercaptoethanol was obtained from Eastman Kodak Co., Rochester, New York and was used without further purification. Sodium dodecylsulfate was purchased from Fisher Scientific Co., Montreal, acrylamide and *N,N'*-methylene-bis-acrylamide from Canalco, Rockville, Md., and Coomassie brilliant blue R from Sigma Chemical Co., St Louis, Mo. Reference proteins used as molecular weight markers were purchased from Mann Research Laboratories. The quarternary aminoethyl exchanger QAE-Sephadex A-50 (Pharmacia) and Sephadex G-100 were prepared and used as described below.

Immunodiffusion. Fractions containing enterotoxin were located by gel immunodiffusion [5] employing reference toxin and anti-enterotoxin serum very kindly made available to us by Dr M. S. Bergdoll. Purification of the enterotoxin was monitored by the double-gel diffusion tube test [6] using crude antisera.

Immunization. Antigen for immunization of rabbits was prepared by mixing equal volumes of Freund's complete adjuvant with material obtained after step 2 of the purification procedure or with the final purified product obtained after step 4 (vide infra). Five injections of step 2 material were given at 0.1, 0.1, 0.2, 0.4, 2.0 and 8.0 mg of total protein in that order at one-week intervals and animals were bled at 7 days and at 14 days after the final injection. Seven days after the final bleeding two additional injections each consisting of 8 mg of protein of the step 4 material were injected one week apart into the same animals. The animals were bled over a period of four weeks beginning one week after the last injection of the step 4 material.

Quantitation of toxins. Recovery of enterotoxin during purification was quantitated by the method of Weirether et al. [7]. The concentration of the final product was also determined from the absorbance at 277 nm using $E_{1\,cm}^{1\,\%} = 12.1$ for enterotoxin C_2 [2].

Gel electrophoresis. Polyacrylamide disc electrophoresis was performed at pH 4.5 as described by Reisfield et al. [8] with the large pore solution made up according to Davis [9]. The sample was applied on top of the spacer gel after the anode compartment was filled with buffer. Electrophoresis was performed at room temperature with a constant current of 5 mA per tube for about 1.3 h using methylene green as a marker dye. The gels were stained with amido black for 1 h and destained by diffusion. For the estimation of molecular weight the procedure of Weber and Osborne [10] was followed using gel which contained 10% acrylamide. Samples (0.6 mg/ml in 0.1 M phosphate buffer, pH 7.0) were combined with an equal volume of solution containing 2% sodium dodecylsulfate, 2% β-mercaptoethanol and 8 M urea in the same buffer and incubated for 2–4 h at 37 °C. When elution of the bands from the gels was intended (vide infra) the concentration of enterotoxin was increased 2-fold. The preparations were dialyzed for 17 h at room temperature against 0.01 M phosphate buffer (pH 7.0) containing 0.1% sodium dodecylsulfate and 0.1% β-mercaptoethanol. In some instances the dialysis step was omitted and samples were applied on the gels immediately after incubation. In both cases samples were mixed with tracking dye and 0.1 ml was applied per gel. Electrophoresis was performed at room temperature with a constant current of 8 mA per gel column for approx. 3 h until the band of the tracking dye moved 75% of the distance down the gel. The gels were stained with Coomassie brilliant blue for 1–2 h and destained over a 48 h period with several changes of destaining solution.

Analytical ultracentrifuge studies. The substance from the last purification step was dissolved in 0.05 M phosphate buffer (pH 6.8) to a concentration of 10 mg/ml. Sedimentation experiments were performed in a Spinco Model E instrument equipped with a rotor temperature indicating control unit, at 20 °C and maximum speed (57 000 rev./min) for 2 h and 26 min.

Protein hydrolysis. Samples (2 mg each) of enterotoxin C_2 were hydrolyzed according to the method of Liu and Chang [11]. The amino acid composition of the hydrolysate was determined with a Beckman 116 amino acid analyzer, modified for multiple sample injection and single column operation.

RESULTS

Purification of enterotoxin C_2

Step 1. Concentration of toxin from culture supernatant with QAE-Sephadex. 20 l of culture supernatant, containing 100–150 μg of toxin/ml, was diluted with 4 volumes of water and the pH adjusted to 9.6. The toxin was removed by adsorption to QAE-Sephadex, equilibrated in 0.033 M ethylenediamine/HCl buffer (pH 9.6) containing 0.02% sodium azide. The ion-exchanger was stirred in the diluted supernatant for about 1 h at room temperature and recovered by filtration through a sintered glass filter. Ouchterlony tests [5] for the presence of toxin in the filtrate were negative. Approx. 2 g of QAE-Sephadex was sufficient to remove the toxin from 1.0 l of original culture supernatant. The ion-exchanger containing adsorbed toxin was resuspended in 1.0 l of 0.033 M ethylenediamine buffer at pH 6.4, stirred for 0.5 h and the mixture was filtered. The resuspension and filtration steps were repeated until the pH of the filtrate was between 6.4 and 6.7 (about 3 times). The filtrates were combined and concentrated by ultrafiltration vs a UM-2 membrane (Amicon) to approx. 1/200 of the original culture volume. Recovery of toxin at this step was 90%. All subsequent chromatographic steps utilized 5 × 80 cm columns fitted with flow through adaptors and cooling jackets. All ethylenediamine buffers contained 0.02% sodium azide. Column temperature was maintained at 5 °C and sample application was to the bottom of the column.

Step 2. Sephadex G-100 gel filtration. After centrifugation of the material from step 1 the supernatant fluid (75 ml) was applied to a Sephadex G-100 column, previously equilibrated with 0.033 M ethylenediamine buffer at pH 6.4. The column was eluted with the same buffer at a flow rate of 45 ml/h and resolved two peaks at 277 nm

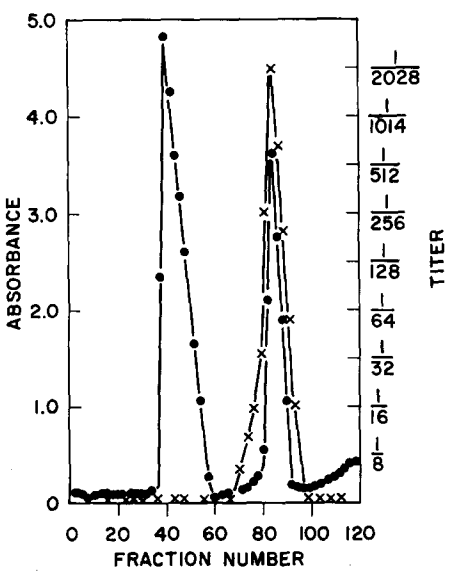

Fig. 1. Gel filtration of crude enterotoxin C_2 on Sephadex G-100. The concentrated toxin (75 ml) from step 1 was chromatographed on a column (5 × 80 cm) of Sephadex G-100, equilibrated with 0.033 M ethylenediamine buffer (pH 6.4) containing 0.02% sodium azide. The flow rate was 45 ml/h (at 5 °C). (●), absorbance of fractions (12 ml each) at 277 nm; (×), doubling dilution titer.

as shown in Fig. 1. Ouchterlony tests [5], using reference antiserum, showed that the toxin was associated with the second peak. After removal of the buffer by dialysis, intravenous administration of this fraction to cynomolgus monkeys at the level of 20 μg protein per animal evoked emesis. A serum obtained after immunizing rabbits with a mixture of the Fractions 75–90 of Fig. 1 revealed at least 3 antigens by the Oakley test [6]. The recovery of toxin after this step was about 80–90%.

Step 3. QAE-Sephadex chromatography. After concentration to about 50 ml by ultrafiltration the preparation from step 2 was applied to a QAE-Sephadex A-50 column, previously equilibrated with 0.033 M ethylenediamine buffer at pH 6.4. The column was eluted with the same buffer at a flow rate of 40 ml/h (Fig. 2). Three peaks at 277 nm were obtained, two of which contained toxin activity (Fractions 104–180). A single precipitation line of identity developed when fractions from second and third peaks were tested against reference antiserum by immunodiffusion. Recovery of enterotoxin C_2 after this step was about 40–50%.

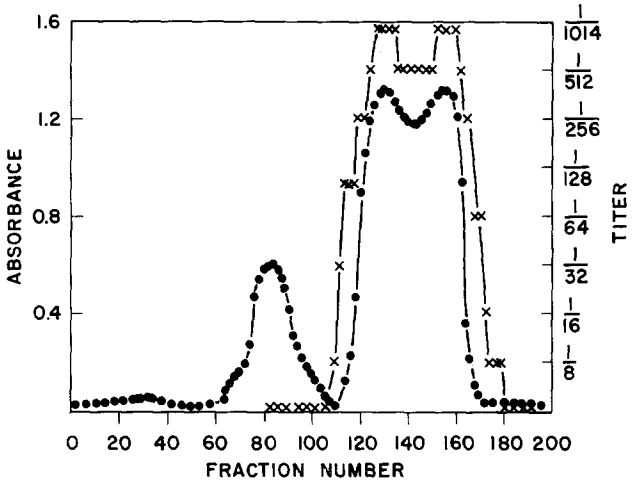

Fig. 2. QAE-Sephadex chromatography of enterotoxin C_2 purified by gel filtration on Sephadex G-100 (Fig. 1). Active fractions from step 2 were pooled, concentrated to 50 ml and applied to a column (5 × 80 cm) of QAE-Sephadex A-50 equilibrated with the buffer described in Fig. 1. Elution was performed with the same buffer at a flow rate of 45 ml/h (at 5 °C). (●), absorbance of fractions (12 ml each) at 277 nm; (×), doubling dilution titer.

Step 4. Sephadex G-100 gel filtration. After concentration to approx. 75 ml on a UM-2 membrane filter (Amicon), the material from step 3 (e.g., pooled Fractions 110–170 of Fig. 2) was applied to a Sephadex G-100 (5 × 80 cm) column, previously equilibrated with 0.1 M ammonium bicarbonate buffer (pH 8.1). The toxin was eluted with the same buffer at a flow rate of 45 ml/h, as indicated in Fig. 3. Fractions of the main peak (Fig. 3) which revealed a single precipitin band when analysed against antiserum to step 2 material in the Oakley test [6] were pooled and freeze-dried. Recovery of toxin after this step was about 85–90%. The overall yield of purified enterotoxin C_2 was approx. 30%.

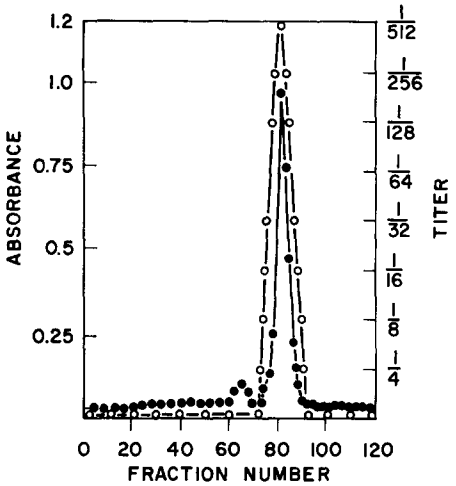

Fig. 3. Gel filtration of enterotoxin C_2 on Sephadex G-100. Active fractions from step 3 (fraction numbers 110–170 of Fig. 2) were pooled, concentrated to 75 ml and applied to a column (5 × 80 cm) of Sephadex G-100 equilibrated with 0.1 M ammonium bicarbonate buffer (pH 8.1). Elution was performed with the same buffer at a flow rate of 45 ml/h (at 5 °C). (●), absorbance of fractions (12 ml each) at 277 nm; (○), doubling dilution titer.

Biological activity

Administration of the purified toxin to monkeys by the intravenous route caused illness characterized by vomiting or diarrhea or both in 50% of the animals at 0.04 μg/kg body weight. When 10 ml of the toxin (10 μg/ml) were mixed with an equal volume of specific antiserum (original antiserum diluted 1:10 with saline) and held at room temperature for 30 min prior to injection, the animals did not become ill. Tests for α- and β-hemolysins were negative.

Ultracentrifuge studies

A sedimentation experiment in the analytical ultracentrifuge indicated enterotoxin C_2 to be homogenous (Fig. 4).

Molecular weight estimation

A molecular weight of 29 500 was estimated for enterotoxin C_2 by gel filtration, by comparison with a plot of log mol. wt vs V_e/V_0 (elution volume/void volume) for a number of proteins with known molecular weight.

Gel electrophoresis

Polyacrylamide disc electrophoresis at pH 4.5 of the final product revealed a comparatively strong band and an indistinct band with lower mobility toward the cathode (Fig. 5). In experiments (not shown), the gels were washed with water immediately after electrophoresis and inserted into larger tubes. The gels were overlayered with agar (at 45°) containing specific antiserum and incubated at room temperature in a moist chamber overnight. The tubes were compared with stained gels and revealed that an immunoprecipitate was obtained with both bands.

Samples of the above material were treated and untreated with 1% sodium

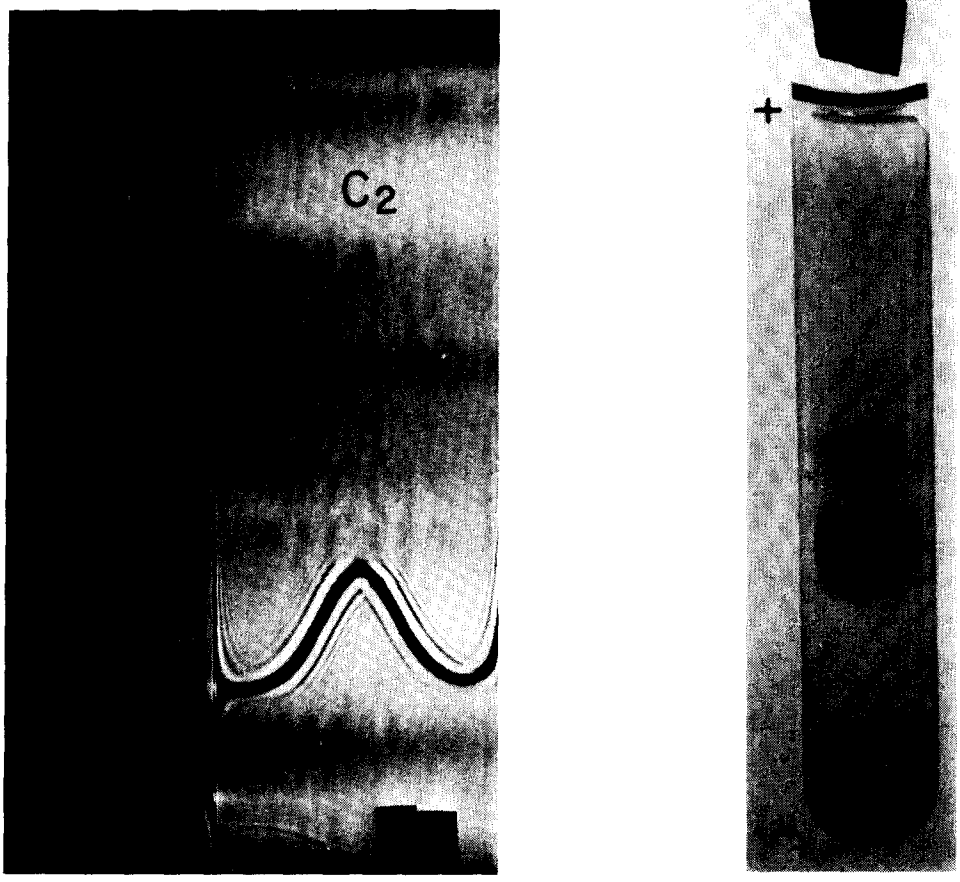

Fig. 4. Sedimentation of enterotoxin C_2 in a Spinco Model E analytical ultracentrifuge at 10.0 mg/ml 0.05 M sodium phosphate buffer pH 6.8. A synthetic boundary cell was used and pictures were taken at a bar angle of 65° after reaching full speed of 56 000 rev./min. The temperature was 20 °C. The picture shown was taken 48 min after reaching full speed.

Fig. 5. Polyacrylamide gel electrophoresis of enterotoxin C_2. About 40 μg of toxin was applied on 7.5% acrylamide in β-alanine-acetic acid buffer pH 4.5.

dodecylsulfate, 1% β-mercaptoethanol and 4 M urea were subjected to sodium dodecylsulfate gel electrophoresis [10]. One band was observed with the untreated sample (Fig. 6, I) but 3 bands, designated a, b, c, were observed (Fig. 6, II) when the sample was previously incubated with thiol and denaturant.

In further experiments these bands were eluted from the gel (component a separately and components b and c together) by the method described for enterotoxin E [12]. The bands were incubated in thiol and denaturant, applied separately to identical gels, and subjected to electrophoresis as before. Results of this experiment are shown in Fig. 7. Band a chromatographed as a single band corresponding to a mol. wt of 29 000 and bands b and c ran as a single zone corresponding to a mol. wt of 15 400 and 12 800 respectively. Additional bands were not observed on either gel.

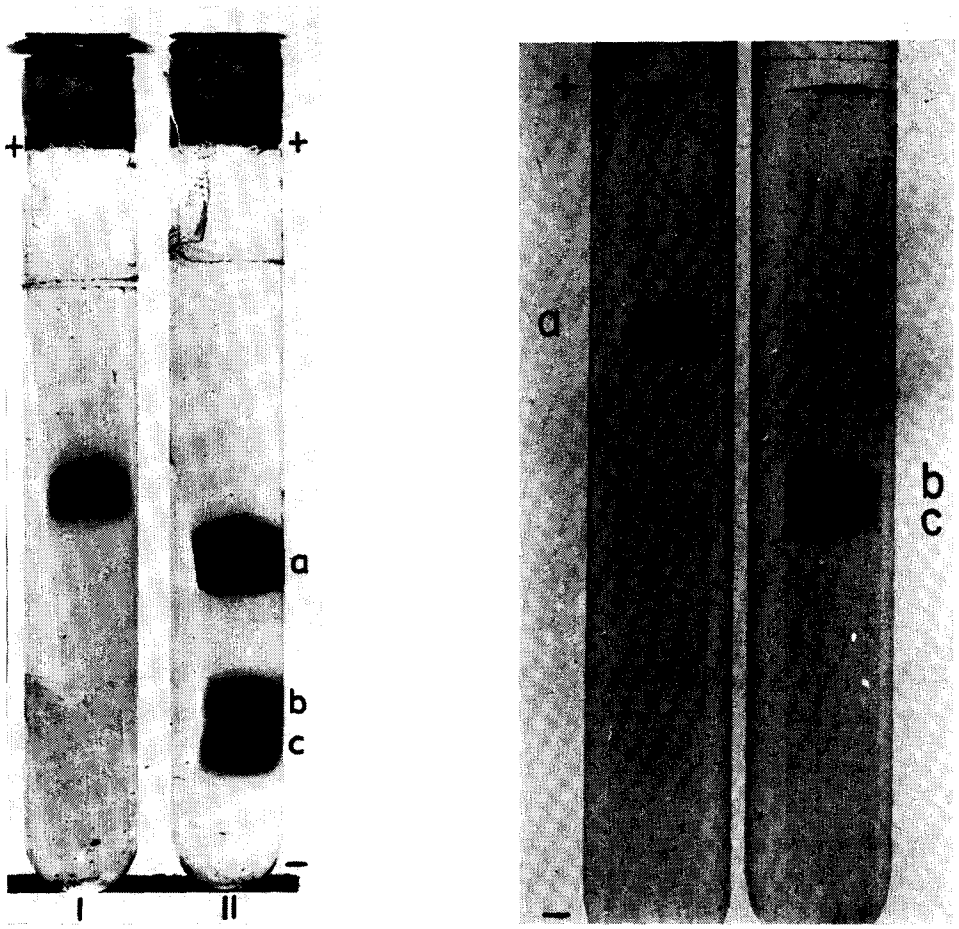

Fig. 6. Polyacrylamide gel electrophoresis of enterotoxin C_2. About 80 μg of toxin was applied on gels containing 10% acrylamide, 0.27% cross linker and 0.1% sodium dodecylsulfate. Gel 1, untreated enterotoxin C_2. Gel 2, enterotoxin C_2 with β-mercaptoethanol and urea.

Fig. 6. Polyacrylamide gel electrophoresis of enterotoxin C_2. The gels contained 10% acrylamide, 0.27% cross linker and 0.1% sodium dodecylsulfate. Components a, b and c obtained from gel 2, Fig. 6 were incubated at 37 °C for 2 h in 0.01 M phosphate buffer (pH 7.0), 1% sodium dodecylsulfate and 1% in β-mercaptoethanol. Samples are from left to right: component a, components b and c.

In a separate experiment band a and bands b and c were applied separately to identical calibrated columns (1.5 × 30 cm) of Sephadex G-100, operated as described in Fig. 1 except that the volume of each fraction was 1 ml. In this step excess denaturants and β-mercaptoethanol were removed. The separated fractions were immunologically identical (Fig. 8) and in addition had the same retention times ($V_e/V_0 = 1.75$) on the gel column indicating close similarity in molecular weight.

The results of the average of 8 determinations of the molecular weight by gel electrophoresis under the experimental conditions of Weber and Osborne [10] showed that the mol. wt of enterotoxin C_2 (band a) was 29 000 which is in good agreement with the value of 29 500 as determined by gel filtration.

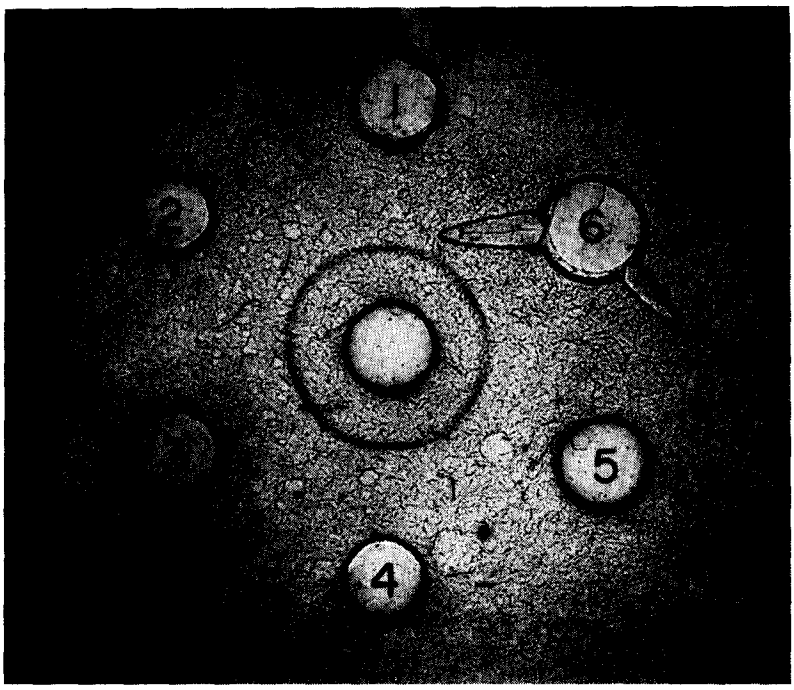

Fig. 8. Gel-immunodiffusion of enterotoxin C_2 components eluted off gel 2, Fig. 7. Wells 1, 3, 5 reference enterotoxin; well 2, component a; well 4, components b and c; well 6, components a, b and c; center well, enterotoxin C_2 antisera raised against crude enterotoxin.

Amino acid composition

Table I shows the amino acid composition of enterotoxin C_2. Each value is an average of three independent experiments. The values for serine, threonine, half-cystine and tryptophan were extrapolated to zero hydrolysis time. The results of amino acid analysis indicate that enterotoxin C_2 is composed of 255 amino acid residues.

DISCUSSION

The present purification scheme gives highly purified preparations of enterotoxin C_2 by a few chromatographic procedures with yields of approx. 30%. The initial adsorption of enterotoxin C_2 to QAE-Sephadex is an important improvement because it has allowed the handling of large volumes of culture necessary for isolation of sufficient quantities of highly purified C_2 toxin for chemical and immunological studies. Procedures based on ion-exchange have already been reported for large scale recovery of enterotoxins A [13] and B [14] from culture fluids.

The final preparation of enterotoxin C_2 appears to be homogeneous because a single symmetrical peak of uniform immunological activity was obtained on gel filtration, sedimentation experiment in the ultracentrifuge gave a single peak, and a single line was obtained in the Ouchterlony and Oakley tests.

However, the partially purified toxin (Fig. 2) was heterogeneous in QAE-

TABLE I

AMINO ACID RESIDUES OF ENTEROTOXIN C_2

Amino acid	Grams/100 g of protein[a]	Calculated residues[b]	Nearest integral residues
Lys	14.51	32.74	33
His	2.92	6.13	6
Arg	1.75	2.21	2
Asp	19.04	47.81	48
Thr[c]	5.58	15.94	16
Ser[c]	5.23	15.57	16
Glu	9.65	21.61	21
Pro	2.35	6.99	7
Gly	3.10	15.68	16
Ala	1.62	6.56	7
Cys[c]	0.72	2.02	2
Val	6.03	17.58	18
Met	3.54	7.79	8
Ile	3.72	9.50	10
Leu	6.32	16.15	16
Tyr	10.25	18.15	18
Phe	5.18	10.17	10
Trp[c]	0.59	0.904	1
Total	102.1		255

[a] Average of 3 analyses.

[b] Based on 29 500 mol. wt of enterotoxin C_2 and corrected to 100% recovery.

[c] The 24, 48 and 72 h values were extrapolated to zero time to correct for decomposition during hydrolysis. Amide nitrogen was not determined.

Sephadex chromatography and in polyacrylamide gel disc electrophoresis of the purified material at pH 4.5 (Fig. 5). These results parallel previous observations that enterotoxin C_2 from strain 361 contains subfractions of different isoelectric points [15], perhaps reflecting small differences in amino acid sequence or different degrees of amidation of asparginyl and/or glutamyl residues. Recently, partial deamidation was shown to be responsible for the multiple form of enterotoxin B observed in isoelectric focusing [16].

The toxin, after treatment with 1% sodium dodecylsulfate-4 M urea migrated as a single band in sodium dodecylsulfate-gel electrophoresis. A mol. wt of 29 000 was estimated by the method of Weber and Osborne [10]. When 1% β-mercaptoethanol was added to the toxin during the treatment with sodium dodecylsulfate-urea, two additional bands appeared at positions corresponding to mol. wt of approx. 15 400 and 12 800. A similar result with enterotoxin B was noted by Spero et al. [17]. These authors suggest that enzymatic action or chemical hydrolysis either during fermentation or subsequent purification can cleave some of the enterotoxin in the disulfide region into a form ("nicked" enterotoxin) which can be dissociated into two oligopeptides by thiol and denaturant. An analogous phenomenon has also been observed with diphtheria toxin [18]. The size of the resulting oligopeptides of enterotoxin C_2 were sufficiently large to retain immunological reactivity. The reaction of immunological identity to enterotoxin C_2 upon removal of excess denaturant and

thiol also appears to be associated with the recovery of a structure similar to the parent molecule as judged by gel filtration. A similar type of behavior has been observed by Spero et al. [17] using enterotoxin B. In the latter case, however, this kind of retention of immunological reactivity was noted using the oligopeptides produced from a trypsin digest of enterotoxin B.

The amino acid composition of enterotoxin C_2 shows differences in almost every residue between our observations and those of Avena and Bergdoll [2]. Their values however, were based on a mol. wt of 34 100 whereas the present values are based on a mol. wt for enterotoxin C_2 of 29 500, which is closer to the molecular weight range of enterotoxin A, B, C_1, and E [12, 13, 19].

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REFERENCES

1 Dickie, N., Yano, Y., Park, C., Robern, H. and Stavric, S. (1973) in Proceedings: Staphylococci in Foods Conference, The Pennsylvania State University, March, pp. 188–204
2 Avena, M. and Bergdoll, M. S. (1967) Biochemistry 6, 1474–1480
3 Hurst, G., Gourevitch, A. and Lein, J. (1958) J. Bacteriol. 76, 453–454
4 Donnelly, C. B., Leslie, J. E., Black, L. A. and Lewis, K. H. (1967) App. Microbiol. 15, 1382–1392
5 Ouchterlony, O. (1949) Ark. Kemi Mineral Geol. B26, 1–9
6 Oakley, C. L. and Fulthrope, A. J. (1953) J. Pathol. and Bacteriol. 65, 49–60
7 Weirether, F. J., Lewis, E. E., Rosenwald, A. J. and Lincold, R. E. (1966) App. Microbiol. 14, 284–291
8 Reisfeld, R. A., Lewis, U. J. and Williams, D. E. (1962) Nature 195, 281–283
9 Davis, B. J. (1964) Ann. N.Y. Acad. Sci. 121, 404–427
10 Weber, K. and Osborne, M. (1969) J. Biol. Chem. 244, 4406–4412
11 Liu, T.-Y. and Chang, Y. H. (1971) J. Biol. Chem. 246, 2842–2848
12 Borja, C. R., Fanning, E., Huang, I.-Y. and Bergdoll, M. S. (1972) J. Biol. Chem. 274, 2456–2463
13 Schantz, E. J., Roessler, W. G., Woodburn, M. J., Lynch, J. M., Jacoby, H. M., Silverman, S. J., Gorman, J. C. and Spero, L. (1972) Biochemistry 11, 360–366
14 Schantz, E. J., Roessler, W. G., Wagman, J., Spero, L., Dunnery, D. A. and Bergdoll, M. S. (1965) Biochemistry 4, 1011–1016
15 Dickie, N., Yano, Y., Robern, H. and Stavric, S. (1972) Can. J. Microbiol. 18, 801–804
16 Spero, L., Warren, J. R. and Metzger, J. F. (1974) Biochim. Biophys. Acta 336, 79–85
17 Spero, L., Warren, J. R. and Metzger, J. F. (1973) J. Biol. Chem. 248, 7289–7294
18 Gill, D. M. and Dinius, L. L. (1971) J. Biol. Chem. 246, 1485–1491
19 Huang, I.-Y. and Bergdoll, M. S. (1970) J. Biol. Chem. 245, 3518–3525