Simplified Method for Purification of Clostridium perfringens Type A Enterotoxin

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Purification of Clostridium perfringens type A enterotoxin from sporulated cells was simplified. The method consisted of precipitation of the enterotoxin from the extract of sonically treated cells at 40% saturation of ammonium sulfate at pH 7, differential solubilization in 0.02 M phosphate buffer, pH 6.7, and repeated gel filtration on Sephadex G-200. The purified enterotoxin was at least 98% pure in ultracentrifugation, polyacrylamide gel electrophoresis, and agar gel double diffusion. Recovery was over 74% from the sporulated cell extract. The toxin had biological activities of at least 4,700 mouse intravenous minimal lethal doses/mg of N, 3,900 capillary permeability-increasing U/mg of N in the guinea pig skin, and 210 rabbit intestinal loop distension U/mg of N. The toxin, containing no hexose, lipid, or nucleic acid, appeared to be identical in sedimentation constant, isoelectric point, and ultraviolet absorption spectrum to the toxin purified previously by different procedures.

Certain strains of Clostridium perfringens type A produce enterotoxin, which is the cause of diarrhea in humans and monkeys (4, 8). The enterotoxin production is closely associated with sporulation of the cells (5). Stark and Duncan (11) attempted purification of the enterotoxin from culture supernatant fluid and extract of the sporulated cells of C. perfringens grown in DS sporulation medium (3) by gel filtration on Sephadex G-200. The highly purified toxin was shown to cause fluid accumulation in ligated intestinal loops of rabbit, erythema in the skin of guinea pig, and death in mouse. These activities were neutralized only with the specific antienterotoxin serum but not with C. perfringens diagnostic serum against type A, B, C, D, or E.

Hauschild and Hilsheimer (7) also purified the enterotoxin from sporulated cells of a different strain by Sephadex G-100 gel filtration followed by successive ion exchange chromatography on diethylaminoethyl (DEAD)-cellulose and carboxymethyl (CM)-cellulose. They found that the enterotoxin is a simple protein with a molecular weight of 36,000 ± 4,000 and an isoelectric point at pH 4.3. It contained 2,100 minimal lethal doses (MLD) and 2,500 erythematous U/mg of N. Eighteen erythematous U or 7.2 μg of N of the enterotoxin caused fluid accumulation in the ligated intestinal loops in the rabbit.

An improved method for purification of the enterotoxin was reported by Stark and Duncan (13). The method involved ribonuclease treatment of the sporulated cell extract and sequential chromatography on Sephadex G-100, Cellex T-cellulose, and hydroxylapatite. The purity of the enterotoxin was higher than 98% and recovery was 65 to 75% from the cell extract.

The present report describes a simplified method for purification of the enterotoxin from the sporulated cell extract by ammonium sulfate precipitation at 40% saturation followed by gel filtration on Sephadex G-200. Some biological activities and physicochemical properties of the purified enterotoxin were compared with those reported previously. The enterotoxin obtained by the simplified method behaved as a homogenous protein; its biological and physicochemical properties were essentially the same as those reported previously by Stark and Duncan (11, 13) and by Hauschild and Hilsheimer (7).

MATERIALS AND METHODS

Strain. C. perfringens type A strain NCTC 8798, isolated by B. C. Hobbs, England, was obtained through A. H. W. Hauschild, Department of National Health and Welfare, Ottawa, Canada. The strain was maintained in cooked meat medium.

Seed cultures. Freshly prepared fluid thioglycollate medium (pH 7.1) was inoculated with a stock culture.
(0.5 ml/10 ml), heated at 75°C for 20 min, and incubated for 18 h. A 5-ml portion of the culture was subcultured in 45 ml of the same medium for 4 h. The whole culture was transferred to 450 ml of the same medium, which was incubated for 4 h.

**Culture for toxin production.** The 500-ml seed culture was transferred to 4.5 liters of DS medium (5) in a 5-liter flask, which was incubated for 8 h. All incubations were made at 37°C.

**Preparation of sporulated cell extract.** The cells containing more than 50% sporangia were collected by continuous-flow centrifugation. The sporulated cells were washed once with cold distilled water and suspended in 200 ml of cold saline. The suspension was treated with a Tomy UR-150P Sonicator (Tomy Seiko, Tokyo) for 20 min and centrifuged to obtain a clear extract.

**Buffer.** Unless otherwise specified, the buffer refers to 0.02 M phosphate buffer, pH 6.7.

**Ammonium sulfate precipitation.** A saturated ammonium sulfate solution in distilled water was diluted so as to make 80% saturation. The pH was adjusted to 7.0 with a 10% ammonium hydroxide solution. An equal volume of the 80% saturated ammonium sulfate solution was added slowly to the cell extract which was kept in an ice bath. The mixture was allowed to stand overnight under refrigeration and centrifuged at 8,600 × g for 30 min.

**Gel filtration on Sephadex G-200.** The ammonium sulfate precipitate, containing most of the enterotoxin, was made into a homogenous slurry in a small volume of the buffer (16 ml per precipitate from a 5-liter culture). The slurry was centrifuged in the cold for 30 min at 8,600 × g. The supernatant fluid contained a large amount of protein, but the precipitated enterotoxin redisolved slowly and less than 15% was lost to the supernatant fluid. The precipitate was dissolved by shaking in a minimum amount of the buffer, usually 16 to 20 ml per precipitate from a 5-liter culture. Most of the precipitate went into solution, and after centrifugation at 8,600 × g for 30 min in the supernatant fluid the enterotoxin was recovered.

An 8-ml portion of the supernatant fluid was applied to a column (2.5 by 95 cm) of Sephadex G-200 medium (Pharmacia Fine Chemicals, Uppsala) equilibrated with the buffer. Up-flow elution was made at room temperature with the buffer at a flow rate of 10 to 20 ml/h. Fractions (10 ml) were collected.

**Mouse test.** Mice weighing approximately 15 g were injected intravenously (i.v.) with 0.5 ml of twofold serial dilutions in saline. Deaths occurring within 30 min were observed and used to calculate minimal lethal dose per milliliter (7).

**Guinea pig skin test.** Capillary permeability-increasing activity was determined in guinea pigs by the method described by Stark and Duncan (12). Serial dilutions of the enterotoxin were injected in 0.05-ml doses into the depilated back skin of guinea pigs. After 20 to 30 min, each animal was injected i.v. with 1 ml of a 2.5% solution of Evans blue. The diameters of the resulting blue spots were measured after 60 min.

**Rabbit intestinal loop test.** Rabbits weighing 2.0 to 2.5 kg were allowed to starve for 2 days. They were anesthetized with halothane and operated on according to De and Chatterje (2). The small intestine, leaving a portion of about 50 cm from the ileocecal valve, was ligated into 10 to 12 segments, each about 8 to 10 cm long. A 3.0-ml portion of an enterotoxin dilution or saline was injected into each segment. The animals were killed in 18 to 24 h by i.v. injection with 3 to 5 ml of a 10% magnesium sulfate solution, and fluid accumulation in each segment was examined by visual inspection.

**Agar gel double diffusion test.** Rabbit antienterotoxin serum was obtained by subcutaneous (s.c.) injections with 20 µg of the purified enterotoxin in two doses, one with the equal volume of Freund complete adjuvant and the other without adjuvant, at a 5-week interval. Serum was taken 14 days after the second injection. The serum gave a positive agar gel double diffusion test at 1:128 dilution and positive passive hemagglutination test with formalinized sheep red blood cells coupled with the purified enterotoxin at 1:64,000 dilution.

On a glass plate (5 by 5 cm), about 4 ml of 1% agar gel (special agar A, Wako Pure Chemical, Tokyo) in 0.05 M acetate buffer, pH 6.0, was spread. Wells of 2 mm in diameter were cut at a distance of 7 mm between the center and each peripheral well. The center well received the antitoxin, and each peripheral well well received the purified enterotoxin (100 µg/ml) or the crude cell extract (500 µg/ml). The plates were incubated in a moist chamber at room temperature for 2 days.

**Neutralization tests.** Equal volumes of an enterotoxin solution (50 µg/ml) and purified antienterotoxin serum or C. perfringens diagnostic serum type A, B, C, D, or E (Welcome Research Laboratories, Beckenhamp, Kent) were mixed. The mixtures were allowed to stand overnight in a refrigerator at 30°C for 30 min and injected i.v. into mice at 0.5-ml doses.

**Analytical ultracentrifugation.** The purified enterotoxin dissolved in the buffer at 3.9 mg/ml was spun in a Beckman model E ultracentrifuge at 59,780 rpm at 20 C. The bar angle was 65°. Photographs were taken every 16 min after attaining the indicated speed of revolution.

**Polyacrylamide gel electrophoresis.** Polyacrylamide gel (7.5%, pH 8.9) and tray buffer (pH 8.3) were prepared by the method of Davis (1). An 80- or 300-µg sample of the purified enterotoxin was applied to each column (0.5 by 7.0 cm). A current of 2 mA per column was applied at room temperature for approximately 3 h. The protein disks were stained with Amido Schartz 10B and scanned with a Fujiox Densitometer, model FD-AIV (Fuji Riki, Tokyo).

**UV absorption spectrum.** Ultraviolet (UV) absorption of the purified enterotoxin dissolved in the buffer at a concentration of 1.3 or 3.9 mg/ml was measured in a Shimazu SV-50V recording photoelectric spectrophotometer (Shimazu Works, Kyoto).

**Isoelectric focusing.** A 110-ml isoelectric focusing column (LKB, Stockholm) and ampholine with the pH range of 3 to 10 were used. A sucrose density gradient of 0 to 50% was used. The enterotoxin, 2.5 mg/0.5 ml, was subjected to electrophoresis at 2 C for
47 h at 410 to 160 V. The column content was withdrawn at a speed of 60 drops/min, and every 60-drop amount (2 ml) was collected manually. Each fraction was measured for pH, absorbancy at 280 nm, and mouse lethality by diluting serially in saline.

**Protein determination.** Protein content was determined by the method of Lowry et al. (10) with crystalline bovine serum albumin (Armour and Co., Chicago, Ill.) as a standard.

**Hexose determination.** Total hexose content was determined by the phenol-sulfuric acid method of Hodge and Hofreiter (9).

**Lipid determination.** To determine lipid content, 3.6 mg of the purified enterotoxin was extracted and washed by the method of Folch et al. (6). After saponification with 0.5 N methanolic potassium hydroxide, the liberated fatty acids were methylated by the method of Stoffel et al. (14). The methylesters were analyzed with a Yanagimoto GCT-550FP gas chromatograph, fitted with hydrogen flame ionization detectors.

**Heat stability of enterotoxin.** A solution of purified enterotoxin in the buffer (50 μg/ml) was introduced into capillaries; the tips were sealed by soldering. The capillaries were heated in a mineral-oil bath at different temperatures for different periods of time. The remaining toxin was determined by the reversed passive hemagglutination test with formalinized sheep red blood cells coupled with a partially purified immunoglobulin fraction of the rabbit antipurified enterotoxin serum.

**RESULTS**

**Preparation of sporulated cell extract.** The whole culture in DS sporulation medium had a toxicity of 10 MLD/ml. Sixty-four percent of the toxicity was recovered in the washed cell suspension. The sonically treated cell extract (200 ml/5-liter culture) contained 2.7 mg of protein per ml and 400 MLD/ml; the recovery in toxicity was 160% of the whole culture. A considerable increase in mouse lethality often resulted from disruption of the cells by sonic treatment; but prolonged sonic treatment resulted in destruction of the toxin.

**Ammonium sulfate precipitation.** To reduce the amount of the material to be subjected to gel filtration, we attempted fractionation of the sporulated cell extract with ammonium sulfate. At 40, 50, and 60% saturation of ammonium sulfate, the yields of the enterotoxin in the precipitate were on the same level; but the higher the concentration of ammonium sulfate, the lower the specific toxicity of the resulting precipitate. Hence, we selected 40% saturation to precipitate the enterotoxin from the cell extract.

The enterotoxin did not go into solution when the precipitate from a 5-liter culture was suspended in a minimum volume of the buffer (16 ml), but a large portion of inactive protein was removed in the centrifugal supernatant fluid. The residual precipitate could be dissolved in buffer by shaking; usually 16 to 20 ml was enough for this purpose. Most material went into solution, which was clarified by centrifugation.

**Gel filtration on Sephadex G-200.** The second extract of the ammonium sulfate precipitate was applied onto a column of Sephadex G-200 and eluted with buffer. The elution patterns are shown in Fig. 1. Fig. 1A shows the pattern of the ammonium sulfate precipitate dissolved in an excess amount of the buffer; Fig. 1B shows the pattern of the second extract, and Fig. 1C shows the pattern of repeated gel filtration of the enterotoxin fraction of Fig. 1B concentrated by filtering through a collodion membrane.

There was an indication that the initial
extraction of the ammonium sulfate precipitate removed almost all of the contaminating protein, leaving only enterotoxin. Purification at different steps are summarized in Table 1. The specific toxicity increased five times, and the recovery in mouse lethality was 74% from the cell extract. Most purification was accomplished by ammonium sulfate precipitation and differential solubilization.

Examinations for purity of the enterotoxin. The purified enterotoxin (3.9 mg/ml) gave a single symmetrical boundary in analytical ultracentrifugation with a sedimentation constant of 2.93S. In polyacrylamide gel electrophoresis, a sample of 80 μg gave a single disk; whereas 300 μg gave another faint disk with a slightly lower mobility, indicating a possible minor contaminant (Fig. 2A and B).

In agar gel double diffusion test, the purified enterotoxin (100 μg/ml) and crude sporulated cell extract (500 μg/ml) gave a single, common precipitation band (Fig. 3). The purified enterotoxin was not neutralized with C. perfringens type A, B, C, D, or E diagnostic serum, but was neutralized only with the purified antitenterotoxin serum. No lipid or hexose was detected. From the absence of absorption at or near 260 nm, it appeared to contain no nucleic acid.

Some physicochemical properties. Isoelectric focusing indicated the isoelectric point of the enterotoxin at pH 4.3. The maximum absorption was found at 277 nm and the minimum was at 250 nm, with shoulders at 282 nm and also at 291 nm (Fig. 4). The enterotoxin was found to be fairly heat labile; one decimal reduction of activity at 60, 57, and 55 C at pH 6.7 required 4, 37, and 38 min, respectively.

Biological activities. Upon i.v. injection, 0.5 ml of a 300-fold dilution of an enterotoxin solution (0.8 mg/ml) killed a mouse, but 600-fold dilution did not. The specific toxicity, therefore, should be about 750 MLD/mg of protein or 4,700 MLD/mg of N. The inoculated mice usually died within 30 min; only a few died later than 30 min.

Marked distension of the intestinal loop from fluid accumulation occurred by injection 30 μg of the toxin; with 15 μg, fluid accumulation was not apparent to the naked eye.

The diameter of the blue spot in the guinea pig skin caused by intracutaneous injection with the toxin followed by i.v. injection with Evans blue appeared to be linearly proportional to the log dose of the toxin within a range from about 0.05 to 5 μg. Injection with 0.05 μg toxin induced a blue spot of about 3 mm in diameter; that with 0.5 μg, about 8 mm; and that with 5 μg, about 13.2 mm. If a capillary permeability-increasing unit is defined as that amount producing a blue spot of 8 mm in diameter (11), 0.5 μg of the purified enterotoxin seemed to correspond to 1 U. Hence, 1 mg of the enterotoxin should contain at least 2,000 capillary permeability-increasing units.

Table 1. Purification of C. perfringens type A enterotoxin

| Sample          | Volume (ml) | Mouse lethality | Protein | MLD/mg of N |
|-----------------|-------------|-----------------|---------|-------------|
| Whole culture   | 10,000      | 10              | 100,000 |             |
| Washed cells    | 400         | 160             | 64,000  |             |
| Cell sonicate   | 400         | 400             | 160,000 |             |
| Cell extract    | 395         | 400             | 158,000 | 2.7         | 1,070 | 925 |
| G-200 filtrate  | 195         | 600             | 117,000 | 0.8         | 156  | 4,680 |
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DISCUSSION

Hauschild and Hilsheimer (7) and Stark and Duncan (11) subjected the crude extract of the sporulated cells directly to gel filtration on Sephadex G-100 or G-200. This resulted in elution of several protein peaks both before and after the elution of the enterotoxin. In contrast with this, gel filtration of the precipitate at 40% saturated ammonium sulfate of the cell extract under similar conditions resulted in only two protein peaks, one at the void volume and the other at a remote position. Only the retarded fraction contained the enterotoxin. Ammonium sulfate precipitation not only removed a large amount of contaminating proteins but also resulted in concentration of the material, thus making it easier to handle a larger amount of the material in a single run of gel filtration. It is likely that the enterotoxin, being unpurifiable once purified at 40% saturation of ammonium sulfate, was co-precipitated with macromolecular protein(s).

Another advantage of the ammonium sulfate precipitation is the fact that the salted-out enterotoxin was more difficult to dissolve in a small amount of the buffer in the presence of residual ammonium sulfate than was the co-precipitated protein(s). A large portion of the macromolecular protein could be removed by discarding the initial extract of the precipitate in a small volume of the buffer, thus affording a considerable degree of further purification.

By twice repeated gel filtration of the second extract on a Sephadex G-200 column, the enterotoxin was obtained in a pure form. The whole purification process was very simple to perform, and the recovery was fairly high.

Homogeneity of the purified enterotoxin was indicated by ultracentrifugation (3.9 mg/ml), polyacrylamide gel electrophoresis (80 µg/ml), and agar gel double diffusion (100 µg/ml). However, polyacrylamide gel electrophoresis with 300 µg of the toxin demonstrated a minor contaminant. It is not known whether this minor component represents a derivative form of the enterotoxin as suggested by Hauschild and Hilsheimer (7) and also by Stark and Duncan (13). The amount of the second component seemed to be significantly smaller than that reported by these authors. From the densitometric determination of the color intensity, the second component was estimated to be no more than 2%.

The purified enterotoxin contained at least

Fig. 3. Agar gel double diffusion tests. Center well received the undiluted antipurified enterotoxin serum; the top and the bottom wells received the purified enterotoxin (100 µg/ml); and both side wells received the crude cell extract (500 µg of protein/ml).

Fig. 4. UV absorption spectrum of the purified enterotoxin.
4,700 mouse i.v. MLD, 3,900 capillary permeability-increasing U, and 210 intestinal loop distension U per mg of N. The toxin we obtained possessed a mouse lethal activity of 229% and a capillary permeability-increasing activity of 13.6% of those of the toxin purified by Stark and Duncan (13). The intestinal loop distending activities, however, agreed well with each other. It is not likely that the discrepancy in the biological activities reflects different purities of the toxin or different substances resulting from different purification procedures, but it may have been due to the different procedures and criteria in determining the activities.

The purified enterotoxin contained no nucleic acid, hexose, or lipid. The UV absorption spectrum sedimentation constant and isoelectric point agreed very well with the values reported previously. The toxin was not neutralized with C. perfringens type A, B, C, D, or E diagnostic antiserum, but neutralized completely only with the purified antienterotoxin serum. Thus the toxin purified by the present method appeared to be identical to that purified previously by different procedures by different authors.

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