Purification and Biochemical Properties of \textit{Clostridium perfringens} Type A Enterotoxin

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The sporation-specific enterotoxin of \textit{Clostridium perfringens} type A, which is the toxin active in human food poisoning, has been purified from extracts of sporingating cells. Highly purified enterotoxin was obtained by treatment of crude cell extract with ribonuclease for 30 min, followed by sequential chromatography on Sephadex G-100, Cellex T cellulose, and hydroxyapatite. Recovery was 65 to 75\% of the initial activity. Enterotoxin purity was > 99\% as indicated by sedimentation velocity, sedimentation equilibrium, disc electrophoresis, and serological methods. Purified enterotoxin focused at pH 4.3 during isoelectric focusing. Molecular weights of 34,000 and 35,000 were obtained by Sephadex G-100 chromatography and sedimentation equilibrium, respectively. An $S_{20,w}$ of 3.08 was obtained for the purified enterotoxin. The enterotoxin precipitated heavily at its isoelectric point and at concentrations greater than 4 mg/ml.

The diarrhea-producing ability of \textit{Clostridium perfringens} type A enterotoxin in humans and monkeys (5, 13, 18) and the possible mechanisms involved have been investigated (12, 15, 17). The enterotoxin is found in cell extracts and culture filtrates of certain strains of \textit{C. perfringens} type A sporulating cells. Enterotoxin production has been shown to be directly related to the ability of the organism to sporulate (6). This correlates well with the fact that the organism is known to sporulate readily in the intestine, and under these conditions would synthesize and release the biologically active toxin. The function of the enterotoxin in sporulation is unknown.

The enterotoxin was recently purified to greater than 97\% pure with 25 to 30\% recovery of original activity (11). The enterotoxin molecular weight as determined by Sephadex G-100 chromatography was 36,000 ± 4,000. The purified enterotoxin was shown to have an isoelectric point of pH 4.3, and to be free from nucleic acids, fatty acids, and reducing carbohydrates.

This report describes purification of the enterotoxin by two different procedures, with an increase in recovery and purity above that previously accomplished. Characteristics of the purified enterotoxin are also reported.

**MATERIALS AND METHODS**

\textbf{Enterotoxin production.} \textit{C. perfringens} type A strain NCTC 8239 was used throughout the study. Enterotoxin-containing cell extracts were used for all purification procedures. Cell extracts were prepared as previously described by employing DS sporulation medium (3) with a modification of the inoculation sequence. An active culture was obtained by transfer from a cooked-meat stock culture to 10 ml of fluid thioglycolate medium with subsequent incubation for 10 to 15 hr. The culture was then inoculated into a second volume of fluid thioglycolate medium and incubated for 15 hr. This culture was transferred (2\%, v/v) to DS sporulation medium and incubated for 7 to 8 hr. Cells were harvested at 4°C by centrifugation. All cultures were incubated at 37°C.

\textbf{Immunodiffusion and immunoelectrophoresis.} Immunodiffusion and immunoelectrophoresis were carried out as previously described (16), except that the slides were incubated at room temperature for 12 to 24 hr. Antiserum was prepared against enterotoxin (>98\% pure) as previously described (16).

\textbf{Animal assay.} The rabbit ligated intestinal loop technique was performed as previously described (4, 7). Erythemal activity in terms of erythematous units was assayed in the skin of guinea pigs as previously described (16). The mouse minimal lethal dose (MLD) was determined by intravenous injection of 0.3-ml portions of samples undiluted or appropriately diluted with 0.9\% saline. A total of three mice was used per sample. Control samples contained heat-inactivated enterotoxin or enterotoxin neutralized with specific antiserum. Animals were observed for 24 hr, with most mice being dead within 20 to 30 min of injection.

Protein was determined by the method of Lowry et al. (14) with bovine serum albumin as a standard. Total nitrogen was calculated from the protein determinations by assuming a nitrogen to protein ratio of 1:6.25.

\textbf{Chromatography beds.} Sephadex G-100 was swollen for 5 days in 0.05 M tris(hydroxymethyl)aminomethane
(Tris) buffer, pH 7.6, containing 0.1 M KCl and was poured into a column 2.6 by 70 cm. The packed column was equilibrated with the same buffer.

The anion exchanger diethylaminoethyl (DEAE) Sephadex A 50 was swollen and equilibrated with 0.05 M Tris buffer, pH 7.6, and was poured into a column (1.5 by 30 cm) with the aid of an extension tube. The column was equilibrated by eluting with the buffer for 24 hr at a flow rate of 0.15 ml/min.

The anion-exchange Cellex T (Bio-Rad Laboratories) cellulose was suspended in 10 volumes of 0.001 M sodium phosphate buffer, pH 8.0, and equilibrated for 24 hr with stirring and two changes of buffer. The slurry was then poured into a column (0.9 by 30 cm) and equilibrated with the same buffer at an elution flow rate of 0.5 ml/min for 24 hr.

Hydroxyapatite (Bio-Gel HTP, Bio-Rad Laboratories) was prepared by suspension in 0.001 M sodium phosphate buffer, pH 6.0, with occasional stirring for 1 hr. The suspension was allowed to settle and the fines were decanted. The column (0.9 by 15 cm) was then poured and equilibrated by elution with 0.01 M sodium phosphate buffer for 24 hr. A flow rate of 0.1 ml/min was maintained with the aid of a peristaltic pump.

Concentration and dialysis. Concentration and dialysis of chromatography fractions was by ultrafiltration through PM-10 membranes (Amicon Corp., Lexington, Mass.) under nitrogen pressure. In sequential column chromatography, all enterotoxin-containing fractions from preceding columns were dialyzed against the buffer of the subsequent column and concentrated before application to the column.

Disc electrophoresis. Disc electrophoresis was performed as previously described (16). The gels were stained with Coomassie brilliant blue and scanned at a wavelength of 535 nm in a Gilford no. 2400 spectrophotometer (Gilford Co., Oberlin, Ohio) fitted with a linear transport.

Nuclease treatment. Crude cell extract was treated with ribonuclease or deoxyribonuclease before application to a Sephadex G-100 column. Crude cell extract at pH 7.0 was treated with ribonuclease A (Worthington Biochemical Corp.), 1 mg/100 mg of cell extract protein, and deoxyribonuclease (Sigma Chemical Co.), 1 mg/100 mg of cell extract protein, for 30 min at room temperature.

Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) in chromatography fractions were determined by the orcinol method (19) and the diphenylamine method (19), respectively.

Sephadex molecular weight. The method of Andrews (1, 2) was used, except that Kav values were used in comparing enterotoxin with known proteins. Known proteins were bovine albumin, ovalbumin, chymotrypsinogen (Calbiochem), yeast alcohol dehydrogenase, and pyruvate kinase (Sigma Chemical Co.). Descending chromatography was performed on a column (2.6 by 61 cm) of Sephadex G-100 with 0.05 M Tris buffer, pH 7.6, containing 0.1 M KCl. A 13-cm pressure head was used for elution.

Isoelectric focusing. An LKB isoelectric focusing column (110 ml) (LKB, Stockholm, Sweden) was used. The sucrose gradient (0 to 50%) was prepared manually and contained ampholytes of a pH 3 to 5 range. Enterotoxin (2 to 10 mg) was equilibrated against 1% glycine on a Sephadex G-25 column and then mixed with the light sucrose solution. The column was maintained at 4 C with the voltage being increased from 300 to 1,000 V during the run, which was complete in 36 to 48 hr. The column was eluted with the aid of a peristaltic pump at a flow rate of 100 ml/hr with collection of 2-ml fractions. The absorbance was measured at 280 nm, and the pH at 4 C. The fractions were then dialyzed against 0.05 M Tris buffer, pH 7.6, containing 0.1 M KCl, and assayed for erythemal activity. Ampholytes were removed from the pooled fractions having erythemal activity by chromatography on a Sephadex G-100 column (2.5 by 30 cm) with the use of 0.05 M Tris buffer, pH 7.6, containing 0.1 M KCl.

Ultracentrifugation. Sedimentation equilibrium experiments were performed at 20 C in a Spinco model E analytical ultracentrifuge, with the use of a double-sector cell and interference optics. The molecular weight was determined at 24,000 rev/min in 0.01 M sodium phosphate buffer, pH 6.0, containing 0.5 M NaCl. Calculations were made according to Yphantis (20).

A partial specific volume (μ) of 0.720 g/ml was used for the enterotoxin.

The sedimentation coefficient was determined at 20 C by use of a single-sector Vinograd cell at 59,780 rev/min with ultraviolet optics at 276 nm. The protein was run in 0.01 M sodium phosphate, pH 6.0, buffer containing 0.5 M NaCl. The enterotoxin concentration was adjusted to an optical density of 1.6 at 276 nm in 0.01 M sodium phosphate buffer, pH 6.0, before application of a 20-pliter sample to the cell.

RESULTS

Purification. (Procedure 1) Initial purification involved elution of the crude cell extract from a Sephadex G-100 column (Fig. 1). Approximately 110 mg of protein was initially applied to the column. Enterotoxin activity was eluted on the frontal side of the second major absorbancy peak, with a 1.5-fold purification and a 90 to 95% recovery of activity. Fractions having more than 50 erythemal units/ml were dialyzed and concentrated by ultrafiltration.

This concentrated partially purified enterotoxin was then subjected to ion-exchange chromatography on DEAE Sephadex (Fig. 2). The majority of the enterotoxin was eluted between 0.185 and 0.240 M NaCl by a linear NaCl gradient from 0 to 0.4 M. A minor peak of activity preceded the major fraction of activity in the elution pattern. The minor peak had a very low 280 to 260 nm absorbancy ratio of approximately 0.34 as compared to a 280 to 260 nm ratio of 2.28 for the enterotoxin peak. Protein from this minor activity peak cross-reacted serologically with the enterotoxin from the major activity peak. Enteroto-
Fig. 1. Column chromatography of C. perfringens NCTC 8239 crude cell extract on a column (2.6 by 61 cm) of Sephadex G-100. Symbols: ○, \( A_{280} \); ▲, erythemal units/ml.

Fig. 2. Chromatography of partially purified enterotoxin from a Sephadex G-100 column (Fig. 1) on a column (1.5 by 25 cm) of DEAE Sephadex. A linear gradient of 0.0 to 0.4 M NaCl was used for elution. Symbols: O, erythemal units/ml; ▲, \( A_{260} \); ○, \( A_{380} \).
toxin proteins from the two activity peaks could not be distinguished from each other on disc acrylamide gels. The protein from these fractions was also lethal to mice.

The low 280 to 260 nm ratio indicated the presence of contaminating nucleic acid associated with a portion of the enterotoxin protein. In an attempt to remove what seemed to be contaminating nucleic acid, the crude cell extract was pretreated with ribonuclease or deoxyribonuclease before chromatography on Sephadex G-100.

Deoxyribonuclease was found to have no effect on the behavior of the cell extract preparations. Pretreatment of the crude cell extract with ribonuclease gave an obvious change in the elution pattern obtained on Sephadex G-100 (Fig. 3), and gave a 3.6-fold purification as compared to 1.5-fold without ribonuclease treatment. Ribonuclease treatment did decrease the recovery from 90 to 80% of the original activity. When the ribonuclease-treated material from the Sephadex G-100 column was subjected to chromatography on DEAE Sephadex (Fig. 4), the elution pattern remained essentially the same as with untreated material. The peak with the low 280 to 260 nm absorbancy ratio still appeared, and contained 4 to 5% of the total activity. These fractions were assayed for DNA and RNA with no detectable amounts of either nucleic acid being present. The peak with a low 280 to 260 nm ratio did not appear in all cell extract preparations that were tested. The significance of this minor peak of activity has not been determined. The purification procedure (procedure 1) described above is very reproducible and results in enterotoxin which is 95 to 97% pure with a 7.0-fold purification. However, only 30 to 40% recovery of original activity is obtained. Disc acrylamide electrophoresis of the purified protein indicated a major protein peak which corresponded to the enterotoxin activity and a small area of protein toward the anode which constituted 3 to 5% contaminating protein (Fig. 5).

**Biological activity.** The biological activities of the purified (procedure 1) enterotoxin are presented in Table 1. The enterotoxin had a total of 28,688 erythemal units/mg of N and 5,000 units/mg of protein. A total of 14 erythemal units was equivalent to one mouse MLD. A minimal loop response in ligated intestinal loops of rabbits was obtained with 140 to 200 erythemal units (0.028 to 0.040 mg of protein) of enterotoxin. The specific toxicity was 356 mouse MLD/mg of protein and 2,232 MLD/mg of N.

![Figure 3](http://iai.asm.org/)
In an attempt to increase the recovery of enterotoxin activity above the 30 to 40% recovery obtained on DEAE Sephadex, the use of different ion exchangers was investigated (procedure 2). Cellex T, a Bio-Rad anion-exchange cellulose, was found to increase the recovery to 65 to 75% of the original activity, and resulted in an enterotoxin preparation 97 to 98% pure.

The majority of the enterotoxin activity was eluted with 0.025 M sodium phosphate buffer, pH 8.0 (Fig. 6). Four to five minor peaks of activity were eluted with increased molarity of ions and decreased pH of the buffer. These minor peaks constituted 4 to 5% of the enterotoxin activity. Enterotoxin from the major peak had only 2 to 3% contaminating protein remaining in the preparation, as indicated by disc acrylamide electrophoresis (Fig. 7). One major and five to six minor protein peaks were present on the acrylamide gels.

The Cellex T-purified enterotoxin was applied to a hydroxylapatite column in an attempt to remove the minor protein contamination.

### Table 1. Biological characteristics of purified (procedure 1) enterotoxin (minimum 98% pure)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>ET/mg of N</td>
<td>28,688</td>
</tr>
<tr>
<td>ET/mg of protein</td>
<td>5,000</td>
</tr>
<tr>
<td>ET/MLD*</td>
<td>14</td>
</tr>
<tr>
<td>ET/loop response</td>
<td>140–200</td>
</tr>
<tr>
<td>MLD/mg of protein</td>
<td>356</td>
</tr>
<tr>
<td>MLD/mg of N</td>
<td>2,232</td>
</tr>
</tbody>
</table>

* Erythemal unit (that amount of enterotoxin producing an 8-mm erythema response in the skin of guinea pigs).

* Based on a nitrogen to protein ratio of 1:6.25.

* Mouse MLD as determined from intravenous injection.
enterotoxin (Fig. 8) activity was recovered in a single protein peak by elution with 0.03 M sodium phosphate buffer, pH 6.0. Hydroxylapatite chromatography not only gave a preparation >99% pure but also gave a significant concentration of the enterotoxin applied. Recovery was greater than 95% of the activity applied. A comparison of the two purification procedures is presented in Table 2.

The purified enterotoxin obtained from hydroxylapatite showed only one band of protein after electrophoresis on disc acrylamide gels (Fig. 9). A very small shoulder appeared on the cathode side of the protein band. This shoulder and an additional one on the anode side of the protein band were also reported by Hauschild and Hilsheimer (11) with enterotoxin purified by a different procedure.

Further evidence for purity of the enterotoxin protein was obtained by serological methods (Fig. 10). Crude cell extract, DEAE Sephacel-purified enterotoxin, and hydroxylapatite-purified enterotoxin were subjected to immunoelectrophoresis against antiserum prepared with crude cell extract. Several precipitin bands appeared with the crude cell extract. A major precipitin band and one minor precipitin band that migrated toward the anode appeared with the enterotoxin from the DEAE Sephacel
purification. Only one precipitin band appeared with enterotoxin from the hydroxylapatite purification.

Characteristics of the purified toxin. The freeze-dried toxin obtained from hydroxylapatite was a snow-white powder which was soluble in water and salt solutions. Solubility was limited at concentrations greater than 4 mg/ml, with visible

<p>| Table 2. Comparison of two purification procedures for Clostridium perfringens type A enterotoxin* |
|-------------------------------------------------|---------------------------------|----------------|-----------------|-----------------|----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Purification</th>
<th>Vol (ml)</th>
<th>ET/ml</th>
<th>Total ET</th>
<th>Percent recovery</th>
<th>Protein (mg/ml)</th>
<th>Specific activity (ET/mg)</th>
<th>Fold purification</th>
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<tbody>
<tr>
<td>Procedure 1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Crude CE</td>
<td>3</td>
<td>26,880</td>
<td>80,640</td>
<td>100</td>
<td>36.32</td>
<td>740</td>
<td>1.0</td>
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<td>Crude CE</td>
<td>6</td>
<td>10,322</td>
<td>61,931</td>
<td>76.8</td>
<td>3.97</td>
<td>2,660</td>
<td>3.59</td>
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<tr>
<td>DEAE Sephadex</td>
<td>6</td>
<td>4,800</td>
<td>28,800</td>
<td>36.0</td>
<td>0.954</td>
<td>5,047</td>
<td>6.82</td>
</tr>
<tr>
<td>Procedure 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude CE</td>
<td>6</td>
<td>36,240</td>
<td>217,440</td>
<td>100</td>
<td>46.0</td>
<td>780</td>
<td>1.0</td>
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<tr>
<td>Crude CE</td>
<td>6</td>
<td>17,120</td>
<td>171,200</td>
<td>79.5</td>
<td>6.0</td>
<td>2,853</td>
<td>3.66</td>
</tr>
<tr>
<td>Cellex T</td>
<td>10</td>
<td>15,000</td>
<td>150,000</td>
<td>69.2</td>
<td>2.13</td>
<td>7,050</td>
<td>9.05</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>10</td>
<td>14,500</td>
<td>145,000</td>
<td>67.0</td>
<td>1.97</td>
<td>7,400</td>
<td>9.5</td>
</tr>
</tbody>
</table>

* CE, cell extract from sporulating cells; ET, erythemal units; RNase, ribonuclease.
precipitation or aggregation of the enterotoxin occurring. It had a maximal absorption at 276 nm with a shoulder at 282 nm. The ratio of the absorption at 260 nm to that at 276 nm was 1.8 to 2.3, indicating an absence of nucleic acid material in the preparation.

**Molecular weight and sedimentation coefficient.**
The Kav value obtained for the purified enterotoxin corresponded to an apparent molecular weight of 34,000 ± 3,000 (Fig. 11). The enterotoxin was eluted as one symmetrical peak of protein and activity from the column.

The molecular weight was also determined from sedimentation equilibrium experiments with purified enterotoxin at a concentration of 0.7 mg/ml (Fig. 12). The curve resulting from a plot of fringe displacement against radial distance had a slope of 0.550. The molecular weight calculated from these data, assuming a partial specific volume for the protein of 0.720, was 35,000.

Sedimentation velocity experiments indicated a sedimentation coefficient of $S_{20,w}$ 3.08, with one symmetrical peak for the purified enterotoxin.

The Vinograd cell was used to determine the sedimentation coefficient, because with the double-sector cell one symmetrical peak was obtained but the peak underwent considerable spreading as the run progressed, making it difficult to determine its center. This spreading may indicate a slow aggregation of the enterotoxin, although only one peak was apparent.

**Isoelectric focusing.** The purified enterotoxin was found to have only one major peak of protein and activity at a pH of 4.3 (Fig. 13). Approximately 10 mg of enterotoxin was used in these experiments because of the heavy precipitation which occurred at the isoelectric point. The incorporation of detergent (Brij 35, Nutritional Biochemicals Corp.; 8) did not enhance solubility of the enterotoxin at its isoelectric point.

**DISCUSSION**
The enterotoxin of *C. perfringens* type A was recently purified by use of Sephadex G-100 and DEAE cellulose (11). A 97% pure enterotoxin preparation was obtained, with 25 to 30% of the original activity being recovered.
Enterotoxin purification by two different procedures and characteristics of the purified enterotoxin are presented in this report. The purification procedure of choice results in a protein of higher purity and 30% higher recovery than that reported previously (11). Purification of the enterotoxin with a Sephadex G-100 to DEAE Sephadex sequence resulted in a 95 to 97% pure protein with 30 to 40% recovery of activity. These values are similar to those obtained with a Sephadex G-100 to DEAE cellulose sequence by Hauschild and Hilsheimer (11). Chromatography on DEAE Sephadex revealed a major peak of enterotoxin activity and an additional minor peak of activity that had a very low 280 to 260 nm absorbancy ratio. Although the low ratio indicated the presence of nucleic acid contamination, treatment of the crude cell extract with ribonuclease or deoxyribonuclease prior to Sephadex G-100 chromatography did not eliminate the minor peak on DEAE Sephadex. However, ribonuclease treatment did significantly alter the elution pattern of the crude cell extract on Sephadex G-100. The treatment removed contaminating material present in the second absorbancy peak and resulted in the elution of the enterotoxin as a distinct protein peak. The ribonuclease treatment produced a 3.6-fold purification as compared to a 1.5-fold purification without ribonuclease treatment. The low 280 to 260 nm absorbancy ratio peak containing erythemal activity did not appear with all cell extract preparations, and its significance has not been determined. Assays for DNA and RNA in this peak were negative. A second minor peak of erythemal activity also was previously reported (11) when partially purified enterotoxin was eluted from a DEAE cellulose column.

The enterotoxin was purified (>9.0 fold) to greater than 99% pure by eluting partially purified enterotoxin from Cellex T cellulose followed by elution from a hydroxylapatite column. This procedure was found to give up to 75% recovery of original enterotoxin activity as compared to approximately 30% with DEAE cellulose or DEAE Sephadex. The minor peaks of activity

Fig. 11. Molecular weight determination of purified enterotoxin from hydroxylapatite on a column (2.6 by 61 cm) of Sephadex G-100.
C. PERFRINGENS TYPE A ENTEROTOXIN

M = 35,000
0.7 mg/ml

Slope \( \frac{2.20}{4.00} = .550 \)

24,000 rpm

FIG. 12. Sedimentation equilibrium of purified enterotoxin from hydroxylapatite in a model E ultracentrifuge equipped with a double-sector cell and interference optics.

obtained with both of the DEAE exchangers and Cellex T cellulose may indicate multiple forms of the enterotoxin in cell extract preparations, similar to those found with the epsilon protoxin of C. perfringens type D (9).

Cellex T chromatography did not yield a minor peak having a low 280 to 260 nm absorbancy ratio and erythemal activity. Our results showed the formation on disc acrylamide gels of an anodal shoulder on the protein band of purified enterotoxin. Both an anodal and a cathodal shoulder were reported with the protein purified by Hauschild and Hilscheimer (11). This may be a further indication of heterogeneity of the enterotoxin protein. The enterotoxin behavior during sedimentation velocity experiments would indicate that the protein is possibly aggregating.

The molecular weight determined by Sephadex chromatography and sedimentation equilibrium indicated an apparent molecular weight of 34,000 to 35,000. A sedimentation coefficient of \( S_{20,w} \) 3.08 was obtained. This molecular weight is in close agreement with the 36,000 ± 4,000 value previously determined with only Sephadex chromatography (11).

The enterotoxin is relatively insoluble at its isoelectric point of pH 4.3. Solubility of enterotoxin was also found to be limited, with concentrations greater than 4 mg/ml at pH 6.0 resulting in visible precipitation or aggregation.

The purified enterotoxin has a specific toxicity of 2,232 mouse MLD/mg of N, which is slightly greater than the 2,100 MLD/mg of N previously reported (11). Biological activity in terms of erythemal units was 28,688 units/mg of N and 14 units/mouse MLD. These values differ by a factor of 10 from those previously reported (11). This difference is reflected by the difference in the definition of an erythemal unit (10, 16).

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FIG. 13. Isoelectric focusing of purified enterotoxin in pH range 3 to 5 at 2 w of power. Approximately 10 mg of protein was used.

LITERATURE CITED


