APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1980, p. 1120-1122
0099-2240/80/06-1120/03$02.00/0

Vol. 39, No. 6

Improved Method for Purification of Enterotoxin from *Clostridium perfringens* Type A

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The purification procedure of *Clostridium perfringens* type A enterotoxin has been improved. The cell sonic extract was precipitated twice with ammonium sulfate, first 40% saturated to concentrate the enterotoxin and then 15% saturated. The two precipitations were followed by gel filtration on Sephadex G-100. The enterotoxin appeared to be homogeneous on 7% polyacrylamide gel electrophoresis after this three-step purification procedure, with a recovery of 56% and a 12.3-fold purification. The solubility properties at different pH values, temperatures, and ammonium sulfate concentrations are also given as basis for the purification procedure.

Clostridium perfringens type A is one of the major causes of food poisoning in humans (9). A heat-labile enterotoxin, formed during sporulation in certain strains, is known to be responsible for the poisoning (4). The enterotoxin is a protein with a molecular weight of 34,000 and consists of a single polypeptide chain (2).

Hauschild and Hilsheimer (4) and Stark and Duncan (8) have developed purification procedures for the enterotoxin involving several chromatographic steps. In 1973 Sakaguchi et al. (7) published a simplified method for purification of the enterotoxin involving ammonium sulfate precipitation, differential solubilization, and gel filtration on Sephadex G-200. This method, although fast, does not work if the enterotoxin concentration in the crude extract is lower than 8 to 10% of the total protein (20 to 30 mg/ml, unpublished data).

We report here the solubility properties and an improved method for purification of the enterotoxin.

MATERIALS AND METHODS

Production and purification of the enterotoxin. *C. perfringens* type A strain NCTC 8239, maintained in cooked meat medium, was used for enterotoxin production in Duncan and Strong (DS) sporulation medium (1). Cell extracts from cultures grown in DS medium at 37°C for 8 h were prepared by the method of Stark and Duncan (8). The enterotoxin was purified from the centrifuged cell sonic extract by adding an equal volume of 80% saturated (3.26 M; 25°C) ammonium sulfate. After 30 min, the suspension was centrifuged at 10,000 × *g* for 20 min. The precipitated protein was dissolved in 0.02 M phosphate buffer, pH 6.8, to give an enterotoxin concentration of 2 to 4 mg/ml. The dissolved protein was then adjusted to 15% saturation (0.611 M, 25°C) with respect to ammonium sulfate and centrifuged after 30 min as described above. The precipitated protein was then dissolved in 0.02 M phosphate buffer, pH 6.8, and chromatographed on a Sephadex G-100 column in the same buffer. All steps were performed at room temperature (23 to 25°C).

Solubility studies. Solubility studies at different concentrations of ammonium sulfate were performed by stepwise addition of 3.26 M (80% saturated, 25°C) ammonium sulfate, adjusted to pH 6.8 with 1 N NaOH, to a solution of purified enterotoxin in 0.02 M phosphate buffer, pH 6.8. After 20 min with continuous stirring, the suspension was centrifuged at 10,000 × *g* for 10 min at 25°C. The soluble enterotoxin concentration was determined by measuring the absorbance of the supernatant at 276 nm with a Beckman spectrophotometer, model 25.

The same techniques were used to study solubility at different temperatures and at different pH values. The pH was adjusted by adding small volumes of 3 N H_3PO_4.

Determination of extinction coefficient. The total organic solid content of a purified enterotoxin solution was determined by the chromic acid oxidation method (5) with analytical grade, moisture-free sucrose as the standard. The spectrum of the enterotoxin was determined in 20 mM sodium phosphate buffer, pH 6.8, with the same buffer as the reference. By combination of the organic solid content and the spectrum the $E^{0.1\%}_{1\ cm}$ at 276 nm was found to be 1.33 mg^{-1} cm^2 for the enterotoxin. The extinction coefficient was used for protein determination on the purified enterotoxin.

Other techniques. Protein was determined by the biuret method (6). Polyacrylamide disc gel electrophoresis and rocket immunoelectrophoresis were done as described previously (2).

RESULTS AND DISCUSSION

Figure 1 shows the solubility of the enterotoxin as a function of ammonium sulfate concentration at 25°C in 0.02 M phosphate buffer, pH

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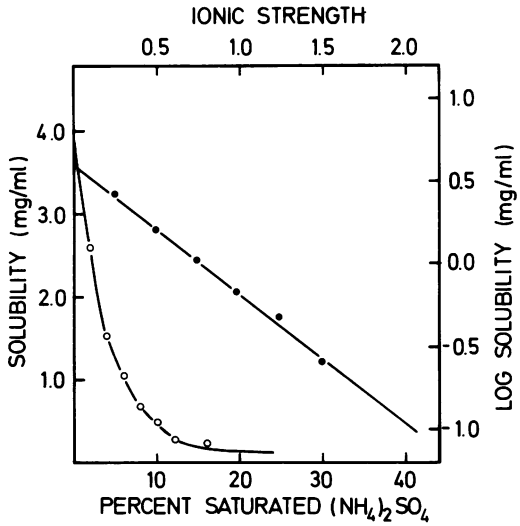

FIG. 1. *Solubility of the enterotoxin in different concentrations of ammonium sulfate in 0.02 M phosphate buffer, pH 6.8, at 25°C. Symbols: ○, Solubility versus percent saturated $(NH_4)_2SO_4$; ●, ionic strength versus log solubility.*

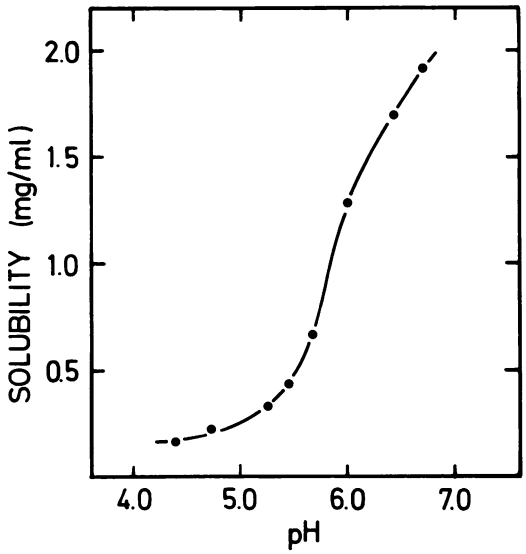

FIG. 2. *Solubility of the enterotoxin in 0.118 M $(NH_4)_2SO_4$ at different pH values at 24°C.*

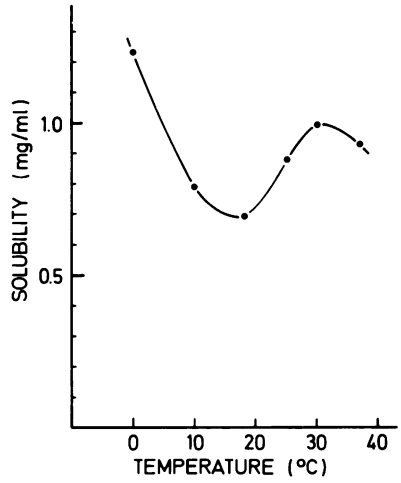

FIG. 3. *Solubility of the enterotoxin at different temperatures in 0.185 M $(NH_4)_2SO_4$–0.02 M phosphate buffer, pH 6.8.*

6.8. Essentially no enterotoxin remained in solution at 40% saturated (1.64 M) ammonium sulfate solution. By dissolving the precipitated protein in as small a volume of 0.02 M phosphate buffer, pH 6.8, as possible, 15% saturated (0.611 M) ammonium sulfate was sufficient to reduce the enterotoxin to 0.2 mg/ml in the supernatant, whereas contaminating proteins did not precipitate at this concentration of ammonium sulfate (Fig. 5). The recovery of the enterotoxin was more than 80% at enterotoxin concentrations higher than 1 mg/ml.

The relation between log solubility of the enterotoxin and ammonium sulfate concentration was found to be $\log S = 0.595 - 0.781\,\mu$ by linear regression, where S is the solubility and μ is ionic strength (3). β, the log solubility at zero μ, is 0.595 ± 0.024, and K_s, the solubility constant, is 0.781 ± 0.025. Therefore, the maximum solubility of enterotoxin in 0.02 M phosphate buffer, pH 6.8, at 25°C is 3.94 ± 0.22 mg/ml.

Solubility of the enterotoxin decreased with decreasing pH (Fig. 2). Solubility of the enterotoxin was <0.2 mg/ml in 0.118 M ammonium sulfate close to the isoelectric point (pI = 4.3). Temperature changes did not affect the solubility of the enterotoxin as much as changes in pH did (Fig. 3). The enterotoxin was about twice as soluble in 0.185 M ammonium sulfate at 0°C as at 18°C (minimum solubility point).

A typical purification of enterotoxin is given in Table 1. A three-step purification procedure gave a homogeneous protein in a yield of 56% with a 12.3-fold purification. The results from the gel filtration chromatography are given in Fig. 4. The first peak after the void volume consisted mainly of nucleic acids since it had a much higher absorbance at 260 nm than at 280 nm. Therefore, chromatography on Sephadex G-100 does not significantly increase the fold purification based on protein.

Figure 5 shows the extent of homogeneity at each step as determined on 7% polyacrylamide gels. The enterotoxin appears to be essentially pure after the second ammonium sulfate precip-

TABLE 1. *Purification of C. perfringens enterotoxin*

Fraction	Vol (ml)	Protein (mg/ml)	Total protein (mg)	Total enterotoxin[a] (mg)	Yield (%)	Purification (fold)
Cell sonicate	16.5	22.7	375	29.3	100	1.0
0–40% ammonium sulfate precipitate	12.6	5.2	66	25.5	86	4.9
0–15% ammonium sulfate precipitate	5.4	4.3	23	21.6	73	12.0
Active fraction after chromatography on a Sephadex G-100 column	51.0	0.34	17.3	16.6	56	12.3

[a] Determined with rocket immunoelectrophoresis.

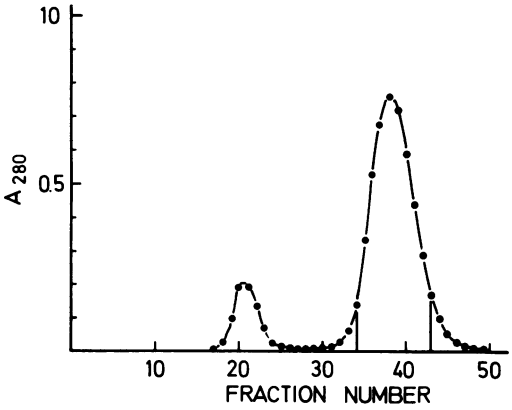

FIG. 4. *Gel filtration of the enterotoxin, after ammonium sulfate fractionation, on a Sephadex G-100 column (2.5 by 70 cm). The flow rate was 20 ml/h, and fractions of 5 ml were collected.*

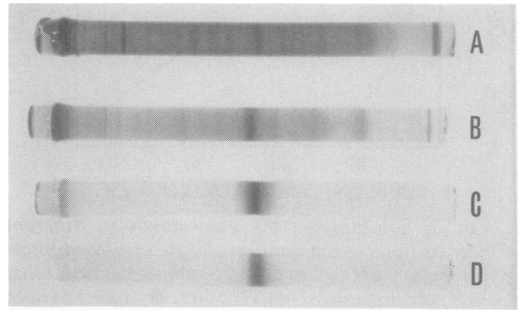

FIG. 5. *Seven percent polyacrylamide gels of the protein solutions after different stages of purification. (A) Cell sonicate (100 μg); (B) 0 to 40% ammonium sulfate precipitate (100 μg); (C) 0 to 15% ammonium sulfate precipitate (50 μg); (D) active fraction (peak II) after gel chromatography on Sephadex G-100 column (50 μg).*

itation since the nucleic acids do not stain with Coomassie brilliant blue.

The purification procedure of Sakaguchi et al. (7) works when the enterotoxin concentration in the cell sonic extract is high (>2 mg/ml) because the maximum solubility of the enterotoxin is about 4 mg/ml and because traces (~2%) of ammonium sulfate are left in the sample under the differential solubilization technique used.

The procedure described here works well at all concentrations of enterotoxin because the enterotoxin is concentrated before the second ammonium sulfate precipitation. With an initial enterotoxin concentration above 1 mg/ml in the cell sonic extract, the first precipitation step may be omitted with good results. The ease of purification of *C. perfringens* enterotoxin will greatly facilitate studies on its chemical, physical, and biological properties.

ACKNOWLEDGMENTS

We thank Gunilla Aasen for technical assistance. J.R.W. is grateful for support by the University of California, Davis and the Norwegian Food Research Institute while on sabbatical leave.

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