Purification and Properties of Clostridium botulinum
Type F Toxin

K. H. YANG and H. SUGIYAMA*
Department of Bacteriology and Food Research Institute, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 27 January 1975

Clostridium botulinum type F toxin of proteolytic Langeland strain was purified. Toxin in whole cultures was precipitated with (NH₄)₂SO₄. Extract of the precipitate was successively chromatographed on diethylaminoethyl-cellulose at pH 6.0, O-(carboxymethyl)cellulose at pH 4.9, Sephadex G-200 at pH 8.1, quaternary aminoethyl-Sephadex at pH 4.9, and finally diethylaminoethyl-cellulose at pH 8.1. The procedure recovered 14% of the toxin assayed in the starting culture. The toxin was homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, double gel diffusion serology, and isoelectric focusing. Purified toxin had a molecular weight of 150,000 by gel filtration and 155,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Specific toxicity was 9.6 x 10⁴ mean lethal doses per absorbancy (278 nm) unit. Sub-units of 105,000 and 56,000 molecular weight are found when purified toxin is treated with a disulfide reducing agent and electrophoresed on sodium dodecyl sulfate-polyacrylamide gels. Reciprocal cross neutralizations were demonstrated when purified type F and E toxins were reacted with antitoxins which were obtained with immunizing toxoids prepared with purified toxins.

Clostridium botulinum type F was first isolated in 1958 from a liver paste which was responsible for a botulism outbreak on the island of Langeland, Denmark (8, 14). The organism has caused one botulism episode in the United States (13) and is found in different parts of this country (9, 19). The Langeland strain is proteolytic but some isolates are not. Significant progress in the purification of type F toxin has not yet been reported. The objectives of the present study were the purification of type F toxin and the comparison of some of its properties with those of the well-characterized types A, B, and E toxins.

MATERIALS AND METHODS

Toxin production. C. botulinum type F, strain Langeland, was obtained from M. W. Eklund, National Marine Fisheries Service, Seattle, Wash. Stocks were cultures grown in cooked meat medium (Difco Laboratories, Detroit, Mich.) for 4 days and then stored at 4 °C. All incubations for growth were at 30 °C.

Medium was 1% enzymatic digest of casein (N-Z Amine, type B, Sheffield Chemical, Norwich, N.Y.), 2% proteose peptone (Difco), 1% yeast extract (Difco), 1% glucose, and 0.05% sodium thiglycolate. For toxin production, the medium, minus glucose, was prepared in 15-liter volumes in 5-gallon (18.925 liters) carboys, adjusted to pH 7.4, and autoclaved at 121 °C for 80 min. A 50% aqueous glucose solution was autoclaved separately, and 300 ml was added to the sterilized medium.

A 9-ml amount of toxin production medium was inoculated with a stock culture and incubated for 24 h. Starting with this growth, cultures were developed serially in 90 and 900 ml and finally in the 15 liters of toxin production medium by using as inocula all the growth obtained by 16 h of incubation. Incubation for toxin production was 5 days.

Toxin purification. Whole cultures were made 60% saturated with (NH₄)₂SO₄ by adding and dissolving solid salt. After 2 days at 4 °C, as much as possible of the clear supernatant fluid was removed by siphoning. Toxin in the precipitate portion was recovered by centrifugation at 10,000 x g for 30 min at 4 °C and extracted with 1.5 liters of 0.07 M sodium phosphate buffer, pH 6.0. The extract was clarified by centrifugation, and the solubilized toxin was reprecipitated at 50% saturation of (NH₄)₂SO₄. The toxic precipitate was taken up in 160 ml of the same buffer, and the clarified solution was dialyzed against the buffer. This concentrated crude toxin was the starting material for the chromatographic steps.

Buffers were made by titrations of equimolar solutions of acidic and basic components. Chromatography on diethylaminoethyl (DEAE)-cellulose and O-(carboxymethyl)cellulose (Sigma Chemical Co., St. Louis, Mo.) and Sephadex G-200 and quaternary aminoethyl! (QAE)-Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, N.J.) were at room temperature. The toxic sample obtained from a chromatographic step was concentrated for the next step by precipitation at 80% saturation of (NH₄)₂SO₄.
The precipitate, which developed during overnight holding at 4°C, was collected by centrifugation, dissolved in a small volume of appropriate buffer, and dialyzed against that buffer.

**Tests for purity of toxin.** Sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis (7, 20) was performed with 6- by 100-mm gels. Samples were prepared by adding 20 μl of 10% SDS to 0.2 ml of protein solution and dissolving 50 mg of urea in the mixture. To reduce disulfides of toxin, 5 μl of β-mercaptoethanol was added to the protein-SDS mixture. The mixture was held in a boiling-water bath for 5 min and cooled before addition of urea. Electrophoresis was for 6 h at 8 mA/gel. The gel electrophoresis for determining molecular weights of toxin and its subunits used marker proteins myosin (molecular weight, 220,000), rabbit muscle phosphorylase b (94,000), bovine serum albumin (68,000), and ovalbumin (45,000).

Isoelectric focusing with the water-cooled 8101 Ampholine column (LKB Productor AB, Sweden) was used. A 1% (wt/vol) concentration of ampholytes of 5 to 7 pH range and a sucrose density gradient (Ampholine instruction manual) Electrophoresis was 600 V for 72 h. Toxicity and absorbance at 278 nm (A₁₈₅) were determined on 2-ml fractions.

Ouchterlony double diffusion serology used gels made with 1.5% purified agar (Difco), 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer of pH 7.4, 0.85% NaCl, and 1:10,000 merthiolate (Thimerosol, Nutritional Biochemicals, Cleveland, Ohio). Anti-F serum used in these tests was that of a rabbit immunized with the toxoid obtained by incubating the toxin recovered from DEAE-cellulose chromatography with formalin (18). Immune precipitate lines were developed by holding charged plates at room temperature for 7 days.

**Toxin assays.** Titrations for toxicity were done with HA/IRC strain mice of body weight about 22 g. Estimations of toxicity in the chromatographic fractions were obtained with the intravenous procedure (3) that was based on a standard curve prepared with crude F toxin. When more precise values were needed, the intraperitoneal procedure with mice (16) was used.

**Cross neutralization tests.** Separate rabbits were immunized (18) with toxoids made of purified type E (molecular weight, 150,000) and F toxins. Antitoxins had more than 50 IU per ml when titrated with antitoxin standards obtained from the Center for Disease Control, Atlanta, Ga. When reacted in Ouchterlony tests with crude toxin of the homologous type, the antisera gave only a single immune precipitate line.

Serial, twofold dilutions were made of a working antitoxin dilution of known international unit content. These antitoxin dilutions were mixed with an equal volume of purified, heterologous type toxin of 20 mean lethal doses (LD₅₀) per ml. After 30 min at 30°C, each mixture in the series was tested in a separate group of six mice (0.5 ml intraperitoneally per mouse). Deaths within 96 h were used in the Reed and Muench procedure (15) to calculate the IU that protects one-half of the mice being challenged with 5 LD₅₀ of the heterologous type. Type E toxin was activated with trypsin (16) for the experiment.

**RESULTS**

**Toxin purification.** Toxin recovery in the successive chromatographic procedures are illustrated with representative elution profiles. All the concentrated crude toxin from one 16-liter culture was processed in four runs on DEAE-cellulose (Fig. 1). The toxin recovered from these runs was divided into two and each portion was processed separately with O-(carboxymethyl)cellulose (Fig. 2) and Sephadex G-200 (Fig. 3). The resulting two toxic pools were combined for chromatography on QAE-Sephadex A-50 (Fig. 4) and then the final DEAE-cellulose column (Fig. 5).

Average data obtained during processing of three separate culture lots are summarized in Table 1. The toxin in the final product was 14% of that in the starting culture. The specific toxicity of the product was 9.6 × 10⁴ LD₅₀/A₂₂₅₅ unit.

**Homogeneity of toxin.** Toxic fractions obtained in the final DEAE-cellulose step were pooled. The precipitate obtained with (NH₄)₂SO₄ was dissolved in a small volume of 0.05 M Tris-hydrochloride buffer (pH 8.1), and the concentrated toxin was rechromatographed on DEAE-cellulose at pH 8.1. A single, symmetrical protein peak was eluted; the specific toxicities of the several fractions were the same.

SDS-polyacrylamide gel electrophoresis of the concentrated toxic solution gave a single protein band (Fig. 6a and b). Similarly, isoelectric focusing showed a single protein peak with apex at pH 5.7. Serological homogeneity was indicated by the development of a single, sharp, immune precipitate line when 20 μg of purified toxin was reacted with 6 IU of type F antitoxin (crude) in Ouchterlony immunodiffusion tests.

**Molecular weight.** Purified toxin dissolved in 0.05 M Tris-hydrochloride buffer (pH 8.1) containing 0.1 M NaCl was gel filtered on a Sephadex G-200 column which was calibrated with apoferitin (molecular weight, 480,000), rabbit muscle aldolase (149,000), bovine serum albumin (68,000), ovalbumin (49,000), and chymotrypsin (25,000). The conventional plot of molecular weights of protein markers versus elution volumes was a straight line (1). From the almost identical elution volumes of the toxin and of aldolase, the molecular weight of toxin is indicated to be 150,000.

The single band in SDS-polyacrylamide gel electrophoresis migrated to the distance expected of molecules of 155,000 molecular weight. When the same toxin preparation was
Fig. 1. DEAE-cellulose chromatography of concentrated crude toxin. Column (3.0 by 20 cm) equilibrated with 0.07 M sodium phosphate buffer (pH 6.0); 40-ml sample volume. After a wash with equilibrating buffer starting at (a), batch elutions were started at (b) with same buffer containing 0.15 M NaCl and at (c) with buffer containing 1 M NaCl. Flow rate of 100 ml/h. Symbols: ●, A560; ▲, intravenous toxicity. Toxin in peak eluted by (c) is discarded.

Fig. 2. O-(carboxmethyl)cellulose chromatography of toxin recovered by DEAE-cellulose step. Column (1.5 by 15 cm) equilibrated with 0.05 M citrate-phosphate buffer (pH 4.9); sample volume of 20 ml. After a wash with equilibrating buffer, elution was with a linear NaCl gradient (0 to 0.5 M) in the same buffer. Flow rate of 80 ml/h. Symbols: ●, A560; ▲, intravenous toxicity; ——, molarity of NaCl.
treated with β-mercaptoethanol to reduce disulfides, the band obtained with the unreduced toxin disappeared; in its place were two faster moving bands (Fig. 6c) whose proteins were calculated to be of 105,000 and 56,000 molecular weight.

Cross neutralization. The neutralization of type E and F toxicity by F and E antitoxins, respectively, were studied with a challenge dose of 5 LD₅₀/mouse. The cross neutralization titrations showed that 2 IU of type E antitoxin was needed to protect 50% of mice given the type F toxin challenge. With the type E challenge, 4 IU of type F antitoxin was required to save one-half of the mice.

DISCUSSION

Homogeneity tests done on the final product show its purity. Attempts were made to simplify the purification procedure, but omission or modification of any step or changing the sequence of steps invariably resulted in one or more contaminants. However, the recovery of 14% of toxin in the starting culture volume is
not too different from that obtained in the types A (5) and B toxin (2, 6) purification methods.

The specific toxicity of \(9.6 \times 10^4 \text{LD}_{50}/A_{758}\) unit found for the purified F toxin is significantly lower than the \(6.2 \times 10^7\) of type A toxin that can be calculated from published data (12). It is also less than the values for type B toxin obtained by intravenous assays: \(5.9 \times 10^7\) (6) or average \(7.6 \times 10^7\) (2) minimal lethal doses/\(A_{758}\) unit. The specific toxicity of the F toxin is more like that of activated E toxin: \(2 \times 10^7\) as determined directly (R. C. Heimsch, Ph.D. thesis, University of Wisconsin, Madison) and \(1.2 \times 10^7\) as calculated from available data (11) and assuming 16% N content and \(E_{180}\) of 17 (12).

Botulin toxins in culture fluids are complexes in which the toxic protein is associated with a nontoxic protein. The molecular weights of these complexes range from the 900,000 of type A to the 350,000 of type E. However, the toxic protein in the complexes studied to date are of similar size, with pure type A toxin being 150,000 (5), type B 165,000 (2, 6), and type E 135,000 molecular weight (11). The similar size of pure type F toxin is shown by the 150,000 molecular weight determined by gel filtration and the 155,000 found by SDS-polyacrylamide gel electrophoresis. Attempts were not made to identify the toxic complex of F, but the preparation obtained from the Sephadex G-200 step is likely to be the complexed toxin.

Fully activated type A, B, and E toxins are two-chained proteins of which one subunit is about 100,000 and the other about 50,000 molecular weight, with one or more disulfides acting as an interchain link (2, 7). A comparable two-chain structure is now shown for type F toxin.

A previous report showed that type A, E, and F toxins lose almost all toxicity when treated with a disulfide reducing agent (16). Like A, B, and E toxins, pure type F toxin has significant toxicity when fed to mice, although the killing potency by this route is not as high as the corresponding toxin complex (17). Except for

---

Table 1. Summary of purification of C. botulinum type F toxin

| Purification steps | Vol (ml) | Total LD_{50} | Specific toxicity* | Recovery from preceding step (%) | Recovery from culture fluid (%) |
|--------------------|----------|---------------|-------------------|----------------------------------|---------------------------------|
| Culture fluid      | 16,000   | \(3.2 \times 10^4\) | \(4 \times 10^5\) | (100)                            | (100)                            |
| (NH_{4})_{2}SO_{4}  | 180      | \(1.98 \times 10^4\) | \(3 \times 10^4\) | 62                               | 62                              |
| Concentration      |          |               |                   |                                  |                                 |
| DEAE-cellulose     | 40       | \(1.28 \times 10^4\) | \(4 \times 10^3\) | 65                               | 40                              |
| CM-cellulose       | 10       | \(9.28 \times 10^4\) | \(1.2 \times 10^4\) | 73                               | 29                              |
| Sephadex G-200     | 30       | \(5.91 \times 10^4\) | \(4 \times 10^3\) | 64                               | 19                              |
| QAE-Sephadex       | 30       | \(5.0 \times 10^3\) | \(7.8 \times 10^3\) | 85                               | 16                              |
| DEAE-cellulose     | 28       | \(4.48 \times 10^3\) | \(9.6 \times 10^3\) | 90                               | 14                              |

*Based on intraperitoneal LD_{50} per A_{758} unit.

**CM, O-(carboxymethyl)cellulose.
the characteristic antigenicity, type F toxin is not significantly different from the other botulinum toxin types.

The previous reports of a slight but measurable neutralization of type F toxicity by E antitoxin (8, 9, 14) are confirmed by the present tests, which used pure F toxin and an anti-E serum that was derived with pure E antigen. The reverse neutralization in which type F toxin reduces toxicity of E toxin is demonstrated for the first time. In the reciprocal cross neutralizations, action of F antitoxin on E toxin may be quantitatively more effective than the converse. The suggestion is based on 1 IU of type E antitoxin neutralizing toxicity of about 1,000 LD₅₀ of homologous toxin, whereas 1 IU of F antitoxin neutralizes about 10,000 LD₅₀ of type F toxin (4, 10). Regardless of any quantitative difference, the low-level reciprocal neutralizations could confuse the identification of the type of toxin in a sample that contains only one or two mouse lethal doses in a challenge volume.

ACKNOWLEDGMENTS

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and Public Health Service grants FD-0900 and 00712.

LITERATURE CITED

1. Andrews, P. 1964. The gel-filtration behaviour of proteins related to their molecular weights over a wide range. Biochem. J. 96:596–606.

2. Beers, W. H., and E. Reich. 1969. Isolation and characterization of Clostridium botulinum type B toxin. J. Biol. Chem. 244:4473–4479.

3. Boroff, D. A., and U. S. Fleck. 1966. Statistical analysis of rapid in vivo method for the titration of toxin of Clostridium botulinum. J. Bacteriol. 92:1580–1581.

4. Cooper, M. S. 1964. Antitoxins to C. botulinum, p. 147–164. In K. H. Lewis and K. Cassel (ed.), Botulism, proceedings of a symposium. Public Health Service publ. no. 599-FP-1. U.S. Government Printing Office, Washington, D.C.

5. DasGupta, B. R., L. J. Berry, and D. A. Boroff. 1970. Purification of Clostridium botulinum type A toxin. Biochim. Biophys. Acta 214:343–349.

6. DasGupta, B. R., D. A. Boroff, and K. Cheong. 1968. Isolation of chromatographically pure toxin of Clostridium botulinum type B. Biochem. Biophys. Res. Commun. 32:1057–1063.

7. DasGupta, B. R., and H. Sugiyama. 1972. A common subunit structure in Clostridium botulinum type A, B, and E toxins. Biochem. Biophys. Res. Commun. 48:108–112.

8. Dolman, C. E., and L. Murakami. 1961. Clostridium botulinum type F with recent observations on other types. J. Infect. Dis. 109:107–129.

9. Eklund, M. W., F. T. Poyssky, and D. I. Wieler. 1967. Characteristics of Clostridium botulinum type F isolated from the Pacific coast of the United States. Appl. Microbiol. 18:1316–1323.

10. Harrell, W. K., J. H. Green, and J. F. Winn. 1964. Preparation, evaluation, and use of C. botulinum antitoxins, p. 165–170. In K. H. Lewis and K. Cassel (ed.), Botulism, proceedings of a symposium. Public Health Service publ. no. 599-FP-1. U.S. Government Printing Office, Washington, D.C.

11. Kitamura, M., S. Sakaguchi, and G. Sakaguchi. 1969. Significance of 12S toxin of Clostridium botulinum type E. J. Bacteriol. 98:1173–1178.

12. Knox, J. N., W. P. Brown, and L. Spero. 1970. The role of sulfhydryl groups in the activity of type A botulinal toxin. Biochim. Biophys. Acta 214:350–354.

13. Midura, T. G., G. S. Nygaard, R. M. Wood, and H. L. Bodily. 1972. Clostridium botulinum type F: isolation from venison jerky. Appl. Microbiol. 24:165–167.

14. Moller, V., and I. Scheibel. 1960. Preliminary report on the isolation of an apparent new type of C. botulinum. Acta Pathol. Microbiol. Scand. 48:80.

15. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27:493–497.

16. Sugiyama, H., B. R. DasGupta, and K. H. Yang. 1973. Disulfide-toxicity relationship of botulinal toxin types A, E, and F. Proc. Soc. Exp. Biol. Med. 143:589–591.

17. Sugiyama, H., B. R. DasGupta, and K. H. Yang. 1974. Toxicity of purified botulinal toxin fed to mice. Proc. Soc. Exp. Biol. Med. 147:589–591.

18. Sugiyama, H., I. Ohishi, and B. R. DasGupta. 1974. Evaluation of type A botulinal toxin assays that use antitoxin to crystalline toxin. Appl. Microbiol. 27:333–336.

19. Walls, N. W. 1969. Clostridium botulinum type F: isolation from crabs. Science 162:375–376.

20. Weber, K., and M. Osborn. 1959. The reliability of molecular weight determination by dodecyl sulfate polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406–4412.