INFECTION AND IMMUNITY, Jan. 1992, p. 71-78
0019-9567/92/010071-08$02.00/0

Vol. 60, No. 1

Production, Purification, and Characterization of Botulinolysin, a Thiol-Activated Hemolysin of *Clostridium botulinum*

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Received 9 July 1991/Accepted 7 October 1991

A hemolysin, botulinolysin, produced by *Clostridium botulinum* was purified to homogeneity and characterized. First, a strain of *C. botulinum* type C, strain C-203 Tox, which produced a large amount of hemolysin, was selected, and optimal culture medium and conditions for its production of hemolysin were determined. The hemolysin produced in the culture supernatant of this strain under optimal conditions was purified by a combination of ammonium sulfate precipitation, DEAE-Sepharose CL-6B column chromatography, Sephadex G-75 gel permeation chromatography, and SP-Toyopearl 650M cation-exchange column chromatography, with a recovery of 12%. The purified hemolysin gave a single protein band in polyacrylamide gel electrophoresis (PAGE) with and without sodium dodecyl sulfate (SDS). The protein in this band in PAGE with SDS was estimated to have a molecular weight of 58,000 and was immunostained with a neutralizing monoclonal antibody. In PAGE without SDS, the hemolytic activity corresponded in position to the single protein band. The pI of the hemolysin was 8.4. Amino acid analysis of the purified hemolysin indicated the presence of four half-cystine residues per molecule. The purified hemolysin had a specific activity of 2,100 hemolytic units per μg of protein on rabbit erythrocytes. It was activated by SH compounds, inhibited by cholesterol, and heat labile. The optimum pH for hemolysis was 6.0 to 7.0. Rabbit, human, and guinea pig erythrocytes were the most susceptible to the hemolysin, while sheep, mouse, rat, and chicken erythrocytes were much less susceptible. The purified hemolysin had a lethal effect in mice and was cytotoxic for some cultured cells: its 50% lethal dose in mice was 310 ng, and its 50% cytotoxic dose for Vero cells was 120 ng/ml.

Botulinolysin, one of the toxins produced by *Clostridium botulinum*, is an oxygen-labile hemolysin and is a member of a group of thiol-activated cytolysins (1-4, 8, 9, 11, 12, 27, 29). In this group of 17 toxins (4, 8, 29) produced by four genera of bacteria, *Streptococcus*, *Bacillus*, *Listeria*, and *Clostridium*, are found streptolysin O, pneumolysin, listeriolysin O, and perfringolysin O. These cytolysins share common features (4, 8, 27): they all lyse cholesterol-containing membranes, are reversibly inactivated by oxygen and activated by thiol compounds, are inhibited by low levels of cholesterol, and show lethality (cardiotoxicity) in animals. They are closely related serologically. Collectively, these cytolysins are of interest from the standpoint of their origins, molecular evolution, structure-function relationships, and roles in the diseases caused by the bacterial species which produce them. Although related hemolysins have been identified in eight clostridial species and constitute the biggest subgroup of this group of cytolysins (4, 8, 29), the structure of only perfringolysin O (of these clostridial thiol-activated cytolysins) has been clarified (31); the existence of the other members is known only from hemolytic activities around colonies on blood agar plates and/or hemolytic activities of crude culture fluids. Botulinolysin, produced by *C. botulinum*, is one of the cytolysins that has not yet been purified or characterized (3, 8, 14).

In this paper, we report conditions for the production of botulinolysin suitable for its purification, a method for the purification of botulinolysin to homogeneity, and the unique properties of the purified preparation.

MATERIALS AND METHODS

Bacterial strains and cultivation. The 14 strains of *C. botulinum* types C, D, and E used in this study are listed in Table 1. Strain C-003-9 Tox was obtained from G. Sakaguchi, Department of Veterinary Science, College of Agriculture, University of Osaka Prefecture, Osaka, Japan. Other strains were provided by K. Oguma, Department of Microbiology, Sapporo Medical College, Hokkaido, Japan. Bacteria were cultivated in anaerobic jars at 37°C. The strains were subcultured in the fortified cooked meat (FCM) medium described by Kurazono et al. (19). Cultures were stored at 4°C after 23 h of growth. Samples (0.4 ml) of the stock culture were inoculated into 10-ml volumes of FCM medium, incubated for 23 h, and used as a seed culture for examining the abilities of various strains to produce hemolysin in small-scale tests. For these tests, samples (0.4 ml) of the seed culture were inoculated into 10-ml volumes of FCM medium. In some experiments, liver-liver broth (32) and modified Latham medium (21, 22) were used in place of FCM medium for examining the abilities of various strains to produce hemolysin.

Production of hemolysin for purification. Strain C-203 Tox, which produced the most hemolysin (Table 1), was used for hemolysin production in modified FCM medium (fortified low cooked meat [FLCM] medium containing one-fifth the amount of cooked meat [Difco] in FCM medium [2.5 g instead of 12.5 g per 100 ml] and adjusted to pH 7.0).

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TABLE 1. Production of hemolysins by various strains of *C. botulinum*

Type	Strain	Hemolytic activity[a] (HD_{50}s/ml)
C	(C)[b]-A02	390
	(C)[b]-N71	750
	C-003-9 Tox	900
	C-Stockholm Tox	980
	C-6831 Tox	3,100
	C-203 Tox	3,400
D	D-South African Tox	740
	(D)[b]-151	1,900
	D-1873 Tox	2,200
E	E-Mourai Tox	140
	(E)[b]-Mourai	290
	E-Yakumo Tox	180
	E-Iwanai Tox	260
	(E)[b]-Otaru	1,700

[a] Culture supernatants of 15-h cultures at 37°C in FCM medium were used after filtration through a membrane filter (Acrodisc).

[b] Nonneurotoxigenic strains. The types from which the strains were derived are indicated in parentheses.

Samples (18 ml) of the seed culture in FLCM medium were inoculated into 750-ml volumes of FLCM medium in 1-liter reagent bottles and incubated anaerobically at 37°C for 16 h. Routinely, two bottles were incubated at a time. After incubation, the bacteria and cooked meat were precipitated by centrifugation at 10,000 × *g* for 20 min at 4°C, and the supernatant was used as a starting material for the purification of hemolysin.

Hemolysin assay. Hemolytic activity was routinely determined with rabbit erythrocytes as follows. A mixture of 125 μl of hemolysin solution diluted twofold with 5 mM Tris hydrochloride-buffered (pH 7.2) 0.85% NaCl containing 5 mM cysteine (pH 7.2) and an equal volume of a 1% suspension (10^8/ml) of erythrocytes that had been washed two or three times and suspended in the same buffered saline was incubated at 37°C for 60 min and centrifuged at 1,000 × *g* for 3 min. The supernatant was transferred to a flat-bottom microtiter plate, and its A_{540} was measured (Titertek Multiskan MC; Flow Laboratories, Inc., McLean, Va.). The A_{540} measured after 100% lysis of the untreated erythrocyte suspension with 0.1% Triton X-100 served as a control. One hemolytic unit was defined as the dose of hemolysin that caused 50% hemolysis (HD_{50}). In some experiments, erythrocytes from other animal species were used.

Thiol activation. For examination of the activation of hemolysin with thiols, hemolytic activity was determined with hemolysin diluted with 5 mM Tris hydrochloride-buffered (pH 7.2) 0.85% NaCl with or without 5 mM cysteine or 10 mM dithiothreitol, with the corresponding buffers only being used as controls. The ratio of the HD_{50} in the presence of a thiol to that in its absence was calculated.

Inhibition of hemolysin. Cholesterol was dissolved in ethanol at a concentration of 1 mg/ml. Samples of 75 μl of a solution of hemolysin at a constant concentration of 500 HD_{50}s/ml in 5 mM Tris hydrochloride-buffered (pH 7.2) 0.85% NaCl containing 5 mM cysteine were mixed with samples of 75 μl of various dilutions (10 to 100 ng/ml) of cholesterol prepared from the stock solution in the same buffered saline. A mixture of 75 μl of hemolysin and an equal volume of 0.2% (vol/vol) ethanol in the same buffered saline was used as a control. The hemolytic activity of the mixture was measured, and the concentration of cholesterol that inhibited the hemolytic activity completely was determined.

Ammonium sulfate precipitation. To the culture supernatant, solid ammonium sulfate (biochemistry grade; Nacalai Tesque Co., Kyoto, Japan) was added slowly at 4°C to yield 60% saturation (at 25°C), keeping the mixture at ca. pH 6.5 by adding dilute NH_4OH solution. The mixture was kept at 4°C overnight and centrifuged at 15,000 × *g* for 30 min at 4°C. The precipitate was washed once with chilled 50 mM Tris hydrochloride buffer (pH 6.5) 60% saturated with ammonium sulfate. The precipitate from 1.5 liters of culture was dissolved in 16 ml of 50 mM Tris hydrochloride buffer (pH 6.5) containing 5 mM cysteine at 4°C and centrifuged at 15,000 × *g* for 20 min at 4°C. The supernatant was filtered through a membrane filter (Acrodisc; Gelman Sciences, Inc., Ann Arbor, Mich.; pore size, 0.2 μm), dialyzed against the buffer used for dissolving the precipitate, and centrifuged at 15,000 × *g* for 20 min at 4°C. The resulting supernatant was used as the 0 to 60% saturation ammonium sulfate fraction.

DEAE-Sepharose CL-6B column chromatography. The 0 to 60% saturation ammonium sulfate fraction (21.6 mg of protein per ml; 13.5 ml) was applied to a column (2.5 by 25 cm) of DEAE-Sepharose CL-6B (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated with 50 mM Tris hydrochloride buffer (pH 6.5). The column was washed with 150 ml of the same buffer and developed with a linear gradient of NaCl (0 to 0.5 M; 0.00167 M NaCl increase per ml) at a flow rate of 40 ml/h. Fractions of 13 ml were collected. Fractions containing the hemolytic activity (above 290 HD_{50}s/μg of protein) were collected and concentrated with concentrator and a PM30 membrane from Amicon Corp., Danvers, Mass.

Sephadex G-75 gel permeation chromatography. A sample of about 1.5 ml of the concentrate from the DEAE-Sepharose CL-6B ion-exchange column was applied to a column (1.5 by 87.5 cm) of Sephadex G-75 (Pharmacia LKB) equilibrated with 20 mM Na phosphate buffer (pH 6.0). Material was eluted at a flow rate of 10 ml/h at 4°C, and fractions of 2.5 ml were collected. Fractions containing high hemolytic activity (above 670 HD_{50}s/μg of protein) were combined and concentrated with an Amicon PM30 membrane as described above.

SP-Toyopearl 650M cation-exchange column chromatography. As a final step in purification, a sample (ca. 1.0 ml; 0.8 mg of protein) of the material obtained from the Sephadex G-75 gel permeation step was applied to a column (1.7 by 7.5 cm) of SP-Toyopearl 650M (Tosoh Corp., Tokyo, Japan) equilibrated with 20 mM Na phosphate buffer (pH 6.0). Material was eluted with the same buffer (15 ml) and then with a linear gradient of 0 to 0.5 M NaCl (0.07 M NaCl increase per ml; 50 ml) at a flow rate of 10 ml/h. Fractions of 2.5 ml were collected. The peak fraction containing the highest hemolytic activity was concentrated to ca. 1.0 ml with a PM30 membrane as described above and used as the purified hemolysin (botulinolysin) preparation. All the chromatography procedures for purification described above were carried out at 0 to 4°C, and the preparations obtained were stored at 0 to 4°C.

PAGE. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (20) with a 10% (slab) gel. Proteins were stained with Coomassie brilliant blue R-250 and destained with ethanol-acetic acid-water. The molecular weight was estimated with standard protein markers (Pharmacia LKB) as references. Conventional PAGE with a 7.5% (column) gel containing 4 M urea was carried out with the buffer system described by Reisfeld et al. (26) for basic proteins.

Monoclonal antibodies. Purified botulinolysin treated with Formalin (final concentration, 0.1%) as described previously (23) was used for immunizing mice. Spleen cells of mice immunized with Formalin-treated botulinolysin (first, 20 μg of protein with Freund's complete adjuvant in 0.4 ml intraperitoneally; second, 5 weeks later, 10 μg of protein without adjuvant in 0.1 ml intravenously; and third, 4 days later, 10 μg of protein without adjuvant in 0.1 ml intravenously) and X63-Ag8-6.5.3 myeloma cells maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum were mixed at a ratio of 5:1 and fused with the aid of 50% polyethylene glycol (molecular weight, 4,000; Merck, Darmstadt, Germany) with stirring (15). Hybrid cells grown in the same medium containing 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 10^{-5} M thymidine were cloned by the limiting dilution method (15). Monoclonal antibody-positive culture supernatants were screened by an enzyme-linked immunosorbent assay (ELISA) as described by Hashizume et al. (17) with purified botulinolysin (10 μg of protein per ml) for coating. The washed, cloned monoclonal antibody-positive cells (ca. 10^7 cells per BALB/c mouse) were injected intraperitoneally into mice that had been treated 2 weeks previously with pristane (Aldrich Chemical Co., Milwaukee, Wis.). Ascitic fluid containing monoclonal antibodies was obtained 10 to 14 days after inoculation for use in neutralization tests.

Neutralization tests. Neutralization of hemolysin activity by monoclonal antibodies was examined by mixing 62.5 μl of hemolysin (1,000 or 2,500 HD_{50}s/ml) with an equal volume (62.5 μl) of twofold-diluted monoclonal antibodies and incubating the mixture at 37°C for 60 min. Then, 125 μl of a 1% suspension of rabbit erythrocytes was added, and incubation was continued for 60 min at 37°C. Hemolytic activity was determined as described above.

Immunoblotting. After SDS-PAGE of hemolysin, immunoblotting was performed as described by Towbin et al. (30). Monoclonal antibody 2D3 was used. It was found to be of the immunoglobulin G1 type and to have light-chain type κ by ELISA. It neutralized the hemolytic activity of the culture supernatant of *C. botulinum* but not of *C. tetani* and purified tetanolysin. Monoclonal antibody 2D3, which was produced in ascitic fluid and which neutralized 10,000 HD_{50}s of botulinolysin per ml of ascitic fluid, was diluted fivefold with phosphate-buffered saline (PBS) and used for immunostaining. Blots were developed with peroxidase-labeled second antibody (goat anti-mouse immunoglobulin G; Organon Teknika Corp., West Chester, Pa.) and stained with 4-chloro-1-naphthol.

Isoelectric point determination. Isoelectric focusing was carried out in a Phast system (Pharmacia LKB).

Protein determination. Protein was measured by the Lowry method as modified by Hartree (16).

Amino acid analysis. Purified hemolysin (100 μg) was hydrolyzed by being heated at 110°C in 5.7 M HCl in the presence of 0.2% phenol in an evacuated sealed tube for 24, 48, and 72 h. For determination of the tryptophan content, hemolysin was hydrolyzed with 4 M 2-mercaptoethanesulfonic acid at 110°C for 24 h (25). The half-cystine content was determined by hydrolysis with HCl as described above after oxidation of cystine and cysteine to cysteic acid with performic acid. The hydrolyzed materials were analyzed in a model L8500 Hitachi amino acid analyzer by the method of Spackman et al. (28).

Determination of the NH_2-terminal sequence. The NH_2-terminal sequence of the purified hemolysin was analyzed with electroblotted proteins by the microsequence method of Hirano (18).

Lethal toxicity. Female ddY mice (20 to 25 g), 6 to 8 weeks old, were used. Volumes (0.1 ml) of increasing concentrations (twofold intervals) of hemolysin in PBS containing 5 mM cysteine and 0.2% gelatin were injected intravenously into groups of at least five mice, and the animals were observed at 24 h after the injection. The 50% lethal dose was calculated by the probit method (4).

Trypan blue exclusion test of cytotoxicity. Vero cells (a gift from S. Ueda, Department of Preventive Medicine, Research Institute for Microbial Diseases) were used. Cells in confluent cultures in Dulbecco's modified Eagle's medium with 5% fetal calf serum were treated with EDTA-trypsin, washed with PBS, and suspended in PBS at a density of 2×10^6 cells/ml, and aliquots of 50 μl were incubated with 50 μl of hemolysin diluted twofold in PBS at 37°C for 20 min. Then, 100 μl of trypan blue (0.2% in PBS) was added, the preparation was kept in an ice bath for 20 min, and the total number of cells and the number of stained cells were counted in a hemacytometer. The concentration resulting in 50% stained cells represented the 50% cytotoxic dose.

RESULTS

Production of hemolysins by various strains and optimal conditions for hemolysin production. To determine the optimal conditions for preparing a bacterial culture supernatant as starting material for purification of the hemolysin, we examined hemolysin production by various strains of *C. botulinum* types C, D, and E in liver-liver broth, modified Latham medium, and FCM medium. Little hemolysin was produced in liver-liver broth (ca. 1.5% that produced in FCM medium), and hemolysin production in modified Latham medium was about 10% that in FCM medium. A kinetic study showed that 15 h of culturing in FCM medium resulted in the highest hemolysin production by all the strains tested. Of the strains tested, strain C-203 Tox in the growing state secreted the most hemolysin into the culture supernatant (3,400 HD_{50}s/ml) (Table 1). To reduce the content of proteinaceous materials in the medium, we studied the effect of various concentrations of cooked meat (10 to 100% that in the original medium) on hemolysin production with strain C-203 Tox. We found that reduction of the cooked meat content to one-fifth (2.5 g/100 ml) that in the original medium did not affect hemolysin production and that adjusting the starting pH of the medium to 7.0 resulted in a 25% increase in hemolysin production. Therefore, we used modified FCM medium with one-fifth the cooked meat content and a pH of 7.0 (FLCM medium) for the production of hemolysin for purification. A kinetic study of hemolysin production by strain C-203 Tox in FLCM medium showed that hemolysin became detectable in the culture supernatant during an early stage of growth and increased with time to a peak after about 16 h of incubation under these conditions. Therefore, to obtain the starting material for the purification of hemolysin, we used the culture supernatant of strain C-203 Tox grown in FLCM medium for 16 h.

Ammonium sulfate fractionation. The hemolysin in the culture supernatant was completely precipitated at a 60% saturation of ammonium sulfate. The resulting precipitate was dissolved in 50 mM Tris hydrochloride buffer (pH 6.5) containing 5 mM cysteine, centrifuged to remove insoluble materials, filtered, and dialyzed against the same buffer before the subsequent step of anion-exchange chromatogra-

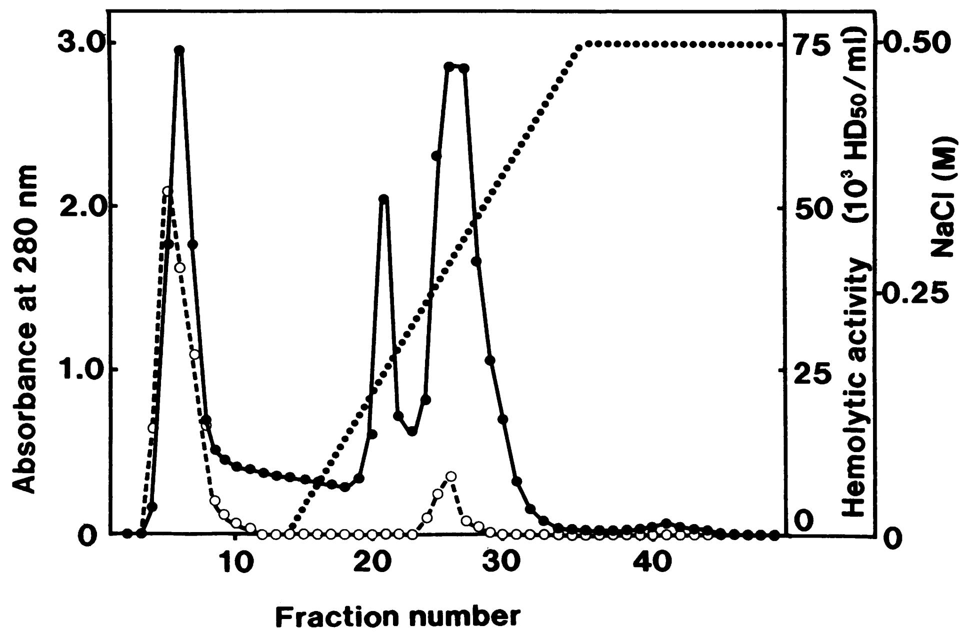

FIG. 1. Elution profile of an ammonium sulfate concentrate of the culture supernatant on a DEAE-Sepharose CL-6B column. The buffer was as described in Materials and Methods. The dotted line indicates the gradient from 0 to 0.5 M NaCl. The flow rate was 40 ml/h. The fraction volume was 13 ml. The sample was 13.5 ml, or 21.6 mg of protein. Symbols: ●, A_{280}; ○, hemolytic activity.

phy. About 70% of the hemolytic activity of the culture supernatant was recovered in this fraction. The hemolysin preparation obtained at this stage had a specific activity of 5.7 HD_{50}s/μg of protein.

Anion-exchange chromatography of the ammonium sulfate fraction on a DEAE-Sepharose CL-6B column at pH 6.5. Figure 1 shows the elution profile obtained on a DEAE-Sepharose CL-6B column of the ammonium sulfate fraction of the culture supernatant after filtration and dialysis. The elution profile showed three major peaks of absorbance (Fig. 1). More than 90% of the hemolytic activity recovered was in the first peak which passed through the column at this pH. Fractions with specific hemolytic activities of greater than 290 HD_{50}s/μg of protein were collected and concentrated by ultrafiltration (Amicon PM30 membrane), and the concentrate was used for the subsequent step of purification. Most of the nonhemolytic materials were removed at this stage. The specific activity was increased about 50-fold by this step. A second, small hemolytic activity peak that was eluted at ca. 0.2 M NaCl was not activated by cysteine. Therefore, it is unlikely that this small peak contained thiol-activated hemolysin.

Gel permeation chromatography through a Sephadex G-75 column of the hemolysin purified by anion-exchange chromatography. The concentrate from the above-described purification step was subjected to gel permeation chromatography through a Sephadex G-75 column. The elution profile (Fig. 2) showed several peaks of absorbance. The peak containing hemolytic activity corresponded to the major peak, with an apparent molecular weight of approximately 55,000 (Fig. 2). Approximately 80% of the hemolytic activity applied to the column was recovered in fractions 21 to 29. Fractions 23 to 25, with specific activities of greater than 670 HD_{50}s/μg of protein, were collected. The hemolysin preparation obtained at this stage had a specific activity of ca. 1,000 HD_{50}s/μg of protein.

Cation-exchange chromatography on an SP-Toyopearl 650M column of the hemolysin preparation from the gel permeation chromatography step. Figure 3 shows the elution profile obtained on an SP-Toyopearl 650M column of the hemolysin preparation from the above-described gel permeation step. A single major peak and several minor peaks of absorbance were obtained (Fig. 3). The hemolytic activity was eluted at 0.26 M NaCl in the position corresponding to the single major peak. The fraction with the highest hemolytic activity was concentrated and used as purified hemolysin.

Purity and properties of the hemolysin preparations. The specific activities and recoveries of hemolysin at each step of purification are summarized in Table 2. The final recovery of hemolysin was 10 to 15% that in the culture supernatant. The specific activity in terms of HD_{50}s per microgram of protein was increased 1,228-fold by the purification steps, the purified hemolysin having a specific activity of 2,100 HD_{50}s/μg of protein. In SDS-PAGE, the purified hemolysin preparation obtained by the above-described method gave a single protein band with a molecular weight of 58,000 ± 2,000 (Fig. 4A), and this single protein band was stained with a monoclonal antibody that neutralized the hemolytic activity of the hemolysin (Fig. 4B). Figure 4A also shows the SDS-PAGE patterns of the hemolysin preparations at various steps of purification. In PAGE without SDS, the purified hemolysin gave a single protein band (Fig. 5A). Tests of the hemolytic activities of eluates from slices of the unstained gel showed that the hemolytic activity was present only in eluates from a few slices in a position corresponding to that of the single protein band in the stained gel (Fig. 5B). Isoelectric focusing showed that the pI of the hemolysin was 8.4.

The hemolytic activity of the purified hemolysin was activated 2.5-fold by 5 mM cysteine or 10 mM dithiothreitol and inactivated completely by cholesterol (100 ng of cholesterol per ml for 500 HD_{50}s of botulinolysin per ml). It was also inactivated by incubation at 45°C for 30 min. The half-life of the hemolytic activity was about 11 days in

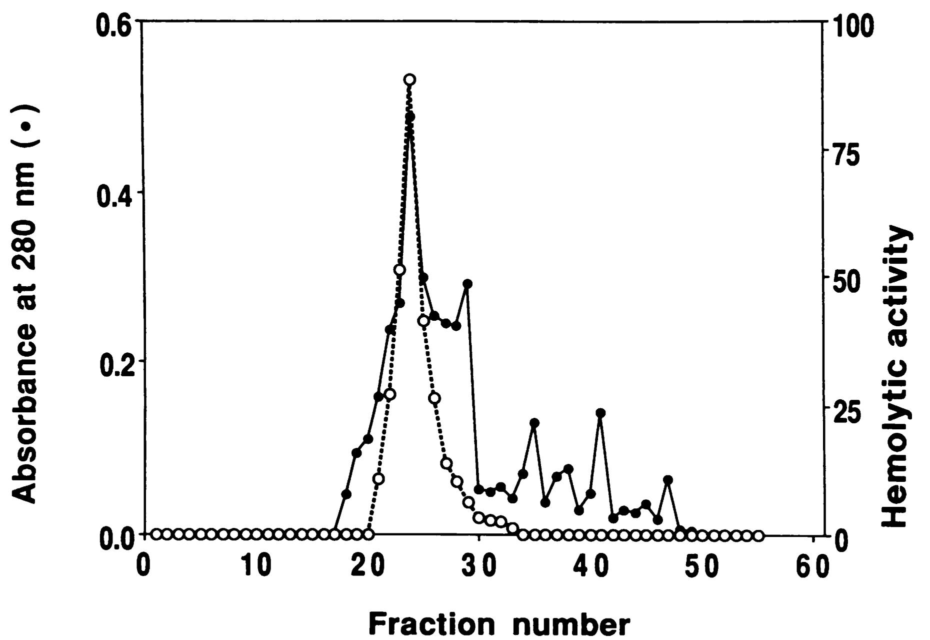

FIG. 2. Elution profile on a Sephadex G-75 column of the partially purified hemolysin from the DEAE–Sepharose CL-6B column. The flow rate was 10 ml/h. The fraction volume was 2.5 ml. The sample was 1.5 ml, or 5 mg of protein.

storage at 4°C. The optimum pH of the hemolytic activity was 6.0 to 7.0, and the activity was most stable at this pH. Below pH 6.0, the hemolytic activity decreased sharply: exposure of the hemolysin to pH 5.0 at 37°C for 30 min resulted in a 50% loss of activity.

Table 3 shows the relative sensitivities of erythrocytes of various species to the hemolysin. The hemolysin showed the highest activity on rabbit erythrocytes; similar, but slightly lower, activities on human and guinea pig erythrocytes; and much lower activities on rat, mouse, and chicken erythrocytes (Table 3).

Table 4 shows the amino acid composition of the purified hemolysin. The hemolysin had high contents of Lys (12.0%), Asp and Asn (12.4%), and Glu and Gln (10%). There were four half-cystine residues per molecule (Table 4). No NH_2-terminal amino acid was detectable by the method of Hirano (18). The purified hemolysin rapidly killed mice: 0.8 μg of the purified hemolysin killed mice in 2 min. The 50% lethal dose

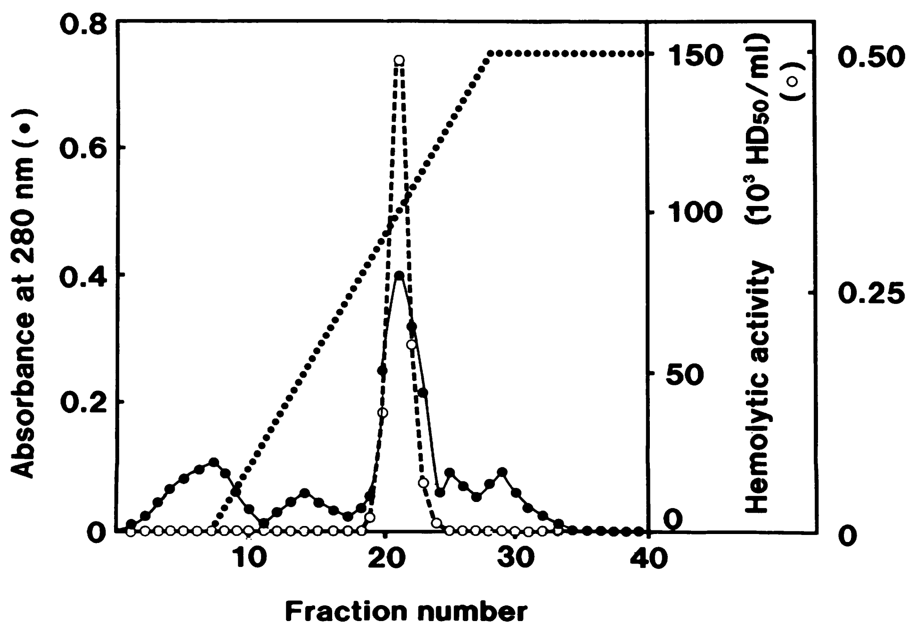

FIG. 3. Elution profile on an SP-Toyopearl 650M column of the partially purified hemolysin from the Sephadex G-75 column. The dotted line indicates the gradient from 0 to 0.5 M NaCl. The flow rate was 10 ml/h. The fraction volume was 2.5 ml. The sample was 1 ml, or 0.5 mg of protein.

TABLE 2. Purification of botulinolysin

Purification step	Total vol (ml)	Total activity, 10^6 $HD_{50}s$ (% recovery)	Total protein, mg (% recovery)	Sp act ($HD_{50}s/\mu g$ of protein)
Culture supernatant	1,230	3.80 (100)	2,210 (100)	1.71
Ammonium sulfate fractionation (0 to 60% saturation)	13.5	2.60 (68)	292 (13)	8.9
DEAE-Sepharose CL-6B chromatography	1.5	2.10 (55)	5.0 (0.22)	420
Sephadex G-75 gel permeation chromatography	1.2	0.87 (23)	0.84 (0.037)	1,035
SP-Toyopearl 650M chromatography	1.1	0.45 (12)	0.22 (0.010)	2,100

of the purified hemolysin for mice was 370 ng. The purified hemolysin also rapidly (in 2 to 5 min) made Vero cells permeable to trypan blue dye. The 50% cytotoxic dose of the purified hemolysin for Vero cells, as determined by the trypan blue exclusion test, was 120 ng/ml.

DISCUSSION

In this study, we established a method for obtaining highly purified botulinolysin in a reasonably high yield and showed that botulinolysin shares features with other thiol-activated cytolysins and also has unique characteristics.

Botulinolysin was purified to homogeneity from a culture supernatant containing a high concentration of hemolysin and a reduced, but sufficient, amount of proteinaceous materials in the medium for high hemolysin production. This culture supernatant was obtained by selecting a *C. botulinum* strain that produced large amounts of hemolysin, modifying the culture medium, and determining the optimal culture conditions for hemolysin production. The main nonhemolytic substances were removed by passing the crude concentrated hemolysin preparation through an anion-exchange column. Consequently, the hemolysin could be obtained as a protein peak in the subsequent step of purification. In fact, this step increased the specific activity 50-fold. The specific activity of our final purified hemolysin preparation, 2,100 $HD_{50}s/\mu g$ of protein, is very high and comparable to those reported for highly purified preparations of other thiol-activated hemolysins (5, 10).

The single protein component obtained by the present method actually had hemolytic activity and was a highly purified hemolysin produced by *C. botulinum*, judging from the following findings: (i) the single band obtained in SDS-PAGE was stained by a monoclonal antibody that neutralized the hemolytic activity of the preparation (Fig. 4B), and (ii) the hemolytic activity was detected only in eluates from gel slices of the single protein band in cathodic PAGE of the purified preparation (Fig. 5).

The purified hemolysin shared the following features with other thiol-activated cytolysins: (i) its hemolytic activity was activated by thiols, (ii) it was heat labile and inhibited by cholesterol, (iii) it had lethal and cytotoxic effects, and (iv) it had a molecular weight (58,000) between 48,000 and 68,000. Therefore, the hemolysin purified in this study was clearly botulinolysin, a member of the group of thiol-activated cytolysins (4, 8) which includes streptolysin O, pneumolysin, listeriolysin O, and perfringolysin O.

Botulinolysin also has the following unique characteristics: (i) it is a basic protein with a pI of 8.4, in contrast to pIs of 4.9 to 7.8 for other thiol-activated cytolysins (3, 4); (ii) it contains four half-cystine residues per molecule, while other members of this group of cytolysins are reported to have one (alveolysin, perfringolysin O, streptolysin O, listeriolysin, and pneumolysin [13, 31]) or two (cereolysin [7]); (iii) its N-terminal amino acid is probably blocked, because it could not be detected; (iv) its pattern of attack on erythrocytes of various animal species differed from those of other thiol-activated cytolysins (3); and (v) it exists as a single form,

TABLE 3. Hemolytic activity of botulinolysin on erythrocytes of various animal species

Animal species	$HD_{50}s/ml$[a]	Relative hemolytic activity
Rabbit	14,540 ± 1,750	1.00
Human	12,649 ± 1,804	0.87
Guinea pig	14,249 ± 182	0.98
Sheep	2,908 ± 97	0.20
Rat	4,801 ± 152	0.33
Mouse	3,344 ± 24	0.23
Chicken	582 ± 34	0.04

[a] $HD_{50}s$ were determined with purified botulinolysin under the assay conditions described for rabbit erythrocytes in Materials and Methods. Data are means ± standard deviations for three determinations.

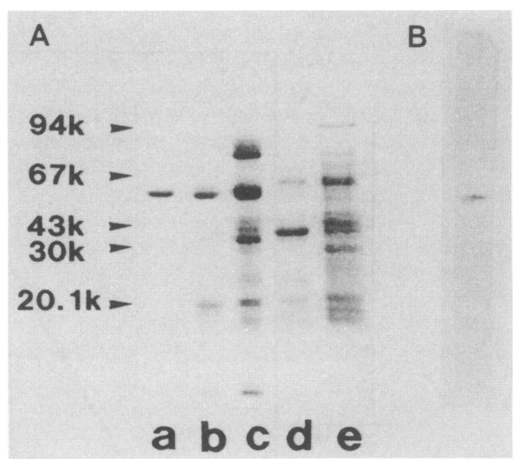

FIG. 4. SDS-PAGE (10% polyacrylamide) of hemolysin preparations at each step of purification (A) and immunostaining of the purified hemolysin with a monoclonal antibody that neutralized botulinolysin (B). Lanes in panel A: a, purified hemolysin (8 μg of protein); b, hemolysin preparation from the Sephadex G-75 column (10 μg of protein); c, hemolysin preparation from the DEAE-Sepharose CL-6B column (20 μg of protein); d, ammonium sulfate concentrate (15 μg of protein); and e, culture supernatant concentrated with an Amicon PM30 membrane (20 μg of protein). Immunostaining of the purified hemolysin was done as described in Materials and Methods. The sample was 3 μg of protein.

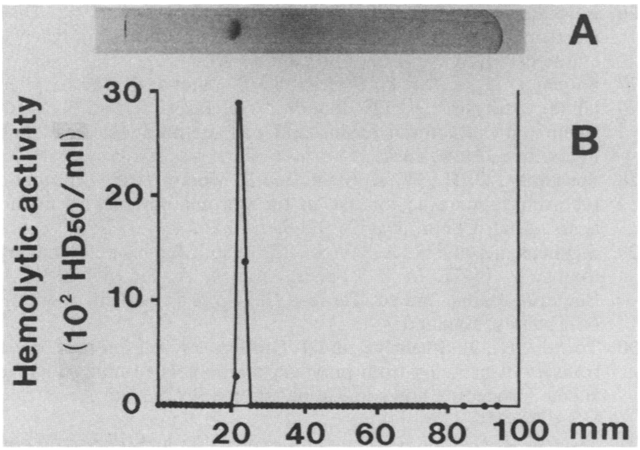

FIG. 5. Electrophoretic pattern (A) and hemolytic activity (B) in cathodic PAGE (column gel, 7.5% acrylamide containing 4 M urea) of the purified hemolysin. Purified hemolysin (25 μg of protein) was analyzed in duplicate samples. Electrophoresis was carried out at a constant voltage of 100 V at 4°C for 5 h. One of the gels was stained (A) and 1.4-mm slices of the other gel were eluted overnight with 2 ml of 5 mM Tris hydrochloride-buffered (pH 7.0) 0.85% NaCl containing 5 mM cysteine at 4°C for analysis of hemolytic activity (B). The horizontal axis of panel B shows distance from the top of the resolving gel.

whereas multiple forms of other thiol-activated cytolysins, such as streptolysin O (1, 5, 27) and tetanolysin (24), have been reported. The variations in the reported characteristics of the thiol-activated cytolysins produced by various bacterial species of diverse genera contrast with their biological similarities, suggesting the existence of conserved concentrated functional domains in thiol-activated cytolysin molecules.

TABLE 4. Amino acid composition of botulinolysin

Amino acid	Number of residues per molecule[a]	Molar ratio
Asp and Asn	65.5	12.4
Thr[b]	31.7	6.0
Ser[b]	33.3	6.3
Glu and Gln	52.8	10.0
Pro	31.7	6.0
Gly	42.8	8.1
Ala	41.7	7.9
Val[c]	40.1	7.6
Met	7.4	1.4
Ile[c]	34.8	6.6
Leu	31.2	5.9
Tyr	16.9	3.2
Phe	15.3	2.9
Lys	63.4	12.0
His	5.3	1.0
Arg	13.2	2.5
Trp[d]	1.6	0.3
Cys[e]	3.7	0.75
Total	532.4	100.8

[a] The number of amino acid residues was calculated on the basis of the molecular weight of 58,000 determined by SDS-PAGE.

[b] Extrapolated to zero time with values obtained after 24, 48, and 72 h of hydrolysis.

[c] Determined by complete hydrolysis (72 h).

[d] Determined by hydrolysis with 4 M 2-mercaptoethanesulfonic acid.

[e] Determined by hydrolysis with HCl after oxidization with performic acid.

The method developed in this study should be useful for further investigations of the structure-function relationship of botulinolysin to elucidate the differences between its characteristics and those of other thiol-activated cytolysins. The purified botulinolysin will also be useful in studies of the biological activities of this hemolysin to determine its role and importance in botulinum infections and bacterial physiology. Studies of the biological activities of botulinolysin and genetic studies of its structure are now in progress in our laboratory.

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