INFECTION AND IMMUNITY, May 1996, p. 1589–1594 Vol. 64, No. 5
0019-9567/96/$04.00+0

Molecular Composition of *Clostridium botulinum* Type A Progenitor Toxins

KAORU INOUE,[1] YUKAKO FUJINAGA,[1] TOSHIHIRO WATANABE,[2] TOHRU OHYAMA,[3] KOUICHI TAKESHI,[3] KOHJI MORIISHI,[4] HIROSHI NAKAJIMA,[1] KATSUHIRO INOUE,[2] AND KEIJI OGUMA[1]*

Department of Bacteriology, Okayama University Medical School, Okayama 700,[1] Department of Food Science, Faculty of Bioindustry, Tokyo University of Agriculture, Abashiri 099-24,[2] Hokkaido Institute of Public Health, Sapporo 060,[3] and Department of Veterinary Science, National Institute of Health, Shinjuku-ku, Tokyo 162,[4] Japan

Received 27 June 1995/Returned for modification 23 August 1995/Accepted 2 February 1996

The molecular composition of progenitor toxins produced by a *Clostridium botulinum* type A strain (A-NIH) was analyzed. The strain produced three types of progenitor toxins (19 S, 16 S, and 12 S) as reported previously. Purified 19 S and 16 S toxins demonstrated the same banding profiles on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), indicating that they consist of the same protein components. The nontoxic components of the 19 S and 16 S toxins are a nontoxic non-hemagglutinin (HA) (molecular mass, 120 kDa) and HA. HA could be fractionated into five subcomponents with molecular masses of 52, 35, 20, 19, and 15 kDa in the presence of 2-mercaptoethanol. The molar ratios of neurotoxins, nontoxic non-HAs, and each HA subcomponent of the 19 S and 16 S toxins showed that only HA-35 of the 19 S toxin was approximately twice the size of that of the 16 S toxin, suggesting that the 19 S toxin is a dimer of the 16 S toxin cross-linked by the 35-kDa subcomponent. The nontoxic non-HA of the 12 S toxin, but not those of the 19 S and 16 S toxins, demonstrated two bands with molecular masses of 106 and 13 kDa on SDS-PAGE with or without 2-mercaptoethanol. It was concluded from the N-terminal amino acid sequences that 106- and 13-kDa proteins were generated by a cleavage of whole nontoxic non-HA. This may explain why the 12 S and 16 S (and 19 S) toxins exist in the same culture. We also found that the HA and its 35-kDa subcomponent exist in a free state in the culture fluid along with the three types of progenitor toxins.

Clostridium botulinum strains produce immunologically distinct neurotoxins (types A to G) which inhibit the release of acetylcholine at the neuromuscular junctions and synapses. The molecular masses of all types of neurotoxins are approximately 150 kDa. The neurotoxins associate with nontoxic components in the culture fluids and form large complexes which are designated progenitor toxins. In type A, three different-sized progenitor toxins with molecular masses of 900 kDa (19 S), 500 kDa (16 S), and 300 kDa (12 S) were observed by sucrose density gradient centrifugation. However, only two peaks appeared on gel filtration; the 19 S and 16 S toxins could not be separated by gel filtration (15). The type B, C, and D strains produce both the 16 S and 12 S toxins, whereas types E and F and type G produce only the 12 S or 16 S toxin, respectively (12). In all these types, the nontoxic components of the 19 S and 16 S toxins have hemagglutinin (HA) activity but that of the 12 S toxin does not. Therefore, it was postulated that the 12 S toxin is formed by association of a neurotoxin with a nontoxic component having no HA activity, which is designated here nontoxic non-HA, and that the 19 S and 16 S toxins are formed by conjugation of the 12 S toxin with HA. The nontoxic components are considered to be very important to the development of food poisoning because they protect the neurotoxin from the acidity and proteases in the stomach (12).

The molecular masses of the nontoxic non-HAs of all types of progenitor toxins have been determined to be approximately 140 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular masses of HAs, however, are not clear, because no one has yet succeeded in separating and purifying the HAs from the progenitor toxins. We demonstrated that the HA of the purified type C 16 S toxin consists of subcomponents of 53, 33, 22 to 23, and 17 kDa. We also determined the whole nucleotide sequences of the genes for neurotoxin, nontoxic non-HA, and HA (5, 8, 18, 19). It was concluded from these observations that the 53- and 22- to 23-kDa HA subcomponents were expressed as a 70-kDa single polypeptide and that after translation this polypeptide is split into 53- and 22- to 23-kDa polypeptides. The 22- to 23-kDa subunit consists of several proteins with slightly different molecular masses.

Somers and DasGupta also demonstrated by SDS-PAGE that type A HA derived from the purified progenitor toxins consists of 57-, 35-, 21.5-, and 17.5-kDa subcomponents (13). However, it is not clear whether this HA preparation comes from the 19 S and/or 16 S toxin, and the following questions still remain to be answered. (i) Why do the 19 S, 16 S, and 12 S toxins exist in the same culture? (ii) Are there any differences in the nontoxic non-HAs among the 19 S, 16 S, and 12 S toxins? (iii) Dose the HA of the 19 S toxin have the same subcomponents as those of the 16 S toxin? (iv) What is the molar ratio of neurotoxin, nontoxic non-HA, and each subcomponent of HA of the progenitor toxins? Since no one has yet analyzed the detailed compositions of the 19 S, 16 S, and 12 S toxins after purifying them, we did so to answer some of these questions.

* Corresponding author. Mailing address: Department of Bacteriology, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700, Japan. Phone: 81-86-222-1815. Fax: 81-86-222-1815.

MATERIALS AND METHODS

Purification of toxin. The *C. botulinum* type A strain, A-NIH, was used. The cells were cultured at 35°C overnight in 50 ml of cooked meat medium (pH 7.2; Difco Laboratories, Detroit, Mich.) supplemented with 1% proteose peptone (Difco Laboratories), 1% polypeptone (Nihon Pharmaceutical Co., Ltd., Osaka, Japan), 1% lactalbumin hydrolysate (Difco Laboratories), 1% yeast extract (Difco Laboratories), and 0.1% L-cysteine hydrochloride monohydrate. A high-titer toxin was produced by the cellophane tube method with a cylindrical glass bottle (38 by 8 cm) and a U-shaped Visking tube, as originally reported by Stern and Wentzel (14) and later slightly modified by Syuto and Kubo (16). The outer medium (950 ml) consisted of 0.8% peptone (Kyokuto, Tokyo, Japan), 2% polypeptone, 1.8% lactalbumin hydrolysate, and 0.64% yeast extract. The inner medium (50 ml) consisted of 20% glucose, 4.2% NaCl, and 0.1% L-cysteine hydrochloride monohydrate. A 1-ml sample of the culture from the 50 ml of cooked meat medium as described above was inoculated into the inner medium of the Visking tube, and the bottle was incubated at 35°C for 5 days. For one batch of toxin purification, 10 cultivating bottles were used. After incubation, the cells were removed by centrifugation. The supernatant was fractionated with a 60% saturation of ammonium sulfate and was then left standing overnight. The precipitates, collected by centrifugation at 15,000 × *g* for 10 min, were dialyzed overnight against 50 mM sodium phosphate buffer (pH 6.0). Since the type A culture fluid contains RNA which forms aggregates with the toxin under acidic conditions, this RNA was removed by protamine treatment (15). Thereafter, the preparation was dialyzed against 50 mM sodium acetate buffer (pH 4.2), and precipitates appearing during dialysis were removed by centrifugation at 15,000 × *g* for 20 min. From the resultant supernatant (crude toxin preparation), the progenitor toxins were purified by column chromatography and sucrose density gradient centrifugation.

All the chromatography steps were performed at room temperature, and the dialysis steps were performed at 4°C. The crude toxin preparation was first applied to SP-Toyopearl 650M (Tosoh, Tokyo, Japan) equilibrated with 50 mM sodium acetate buffer (pH 4.2). The column (1.4 by 26 cm) was washed with 100 ml of this solution, the concentration of NaCl was increased linearly to 0.5 M, and 2-ml fractions were collected. Five protein peaks (peaks 1 to 5) were eluted with the NaCl gradient. The fractions of peak 4 containing HA-positive 16 S and 19 S toxins and peak 5 containing HA-negative 12 S toxin were separately pooled, concentrated with a 70% saturation of ammonium sulfate, and then applied to a Sephacryl S-300 column (1.9 by 54 cm) (Pharmacia Biotechnology AB, Uppsala, Sweden) equilibrated with 50 mM sodium acetate buffer (pH 4.2) containing 0.5 M NaCl. The samples were collected in 1.6-ml fractions. The fractions of 16 S and 19 S toxins and the fractions of 12 S toxin were each pooled, concentrated by saturated ammonium sulfate, and rechromatographed on a Sephacryl S-300 column (1.5 by 100 cm) equilibrated with 10 mM phosphate buffer (pH 6.0) containing 0.2 M NaCl. After elution with the same buffer, each peak was again pooled, concentrated with saturated ammonium sulfate, dialyzed against 50 mM phosphate buffer (pH 6.0), and then subjected to sucrose density gradient centrifugation at pH 6.0.

Sucrose density gradient centrifugation. Sucrose density gradient centrifugation was performed by the procedure of Sugii and Sakaguchi (15). In an attempt to isolate the 19 S, 16 S, and 12 S progenitor toxins, a linear density gradient of 5 to 20% sucrose in 50 mM sodium phosphate buffer (pH 6.0) was prepared in a 5-ml polycarbonate tube (Beckman, Inc., Palo Alto, Calif.) with Gradient Master 106-200B (Towa Kagaku Co., Ltd., Tokyo, Japan). For isolation of neurotoxin from the 19 S and 16 S toxins, 5 to 20% sucrose in 50 mM Tris-HCl (pH 8.8) was used. Samples were loaded on top of the sucrose gradient and then centrifuged at 123,000 × *g* at 15°C for 6 h. After centrifugation, the bottom of the tube was penetrated by the needle of the fraction recovery systems (Beckman, Inc.) and 13-drop fractions were collected.

Determination of toxin and HA titers. The preparations were diluted in serial 10-fold steps with 20 mM sodium phosphate buffer (pH 6.0) containing 0.2% (wt/vol) gelatin, and 0.5 ml of each dilution was intraperitoneally injected into three white mice (ddy, about 20 g). The mice were observed for 1 week, and the minimal lethal dose (MLD) per milliliter was measured.

The HA titer was obtained with microtitration in multiwell plates. The toxin solution (50 μl) was diluted in serial twofold steps with 10 mM phosphate-buffered saline (PBS) (pH 7.4) and mixed with an equal volume of 2% suspension of washed human erythrocytes (group O). After incubation at room temperature for 2 h, the reciprocal of the highest dilution at which hemagglutination was positive was denoted as the HA titer (2^n).

Preparation of antisera. Antiserum against a free HA purified from the culture fluid was prepared in rabbits. The HA preparation (100 μg/ml) was mixed with an equal volume of Freund incomplete adjuvant, and two 2-ml aliquots of each emulsion were injected subcutaneously into two rabbits at 3-week intervals. The rabbits were then given injections of two 2-ml doses of the HA preparation (5 μg/ml) through intravenous route at 5-day intervals, and the sera were harvested 5 days after the last injection.

SDS-PAGE. SDS-PAGE was performed by the method of Laemmli (9). In an attempt to analyze the composition of the progenitor toxins, a 5 to 20% acrylamide gradient gel (Tefco Co., Ltd., Nagano, Japan) was used, and the composition of the purified neurotoxin, HA, and HA-35 preparations were analyzed with a 5 to 15% acrylamide gradient gel (Bio-Rad, Hercules, Calif.). On the other hand, a 7.5 or 12.5% acrylamide linear gel was used for analysis of the N-terminal amino acid sequences of each component of the progenitor toxins. Protein bands were stained with Coomassie brilliant blue R-250.

Determination of amino acid sequence. The N-terminal amino acid sequences of each protein were determined. The bands, separated by SDS-PAGE with the 7.5 or 12.5% acrylamide linear gel were electroblotted to polyvinylidene difluoride membranes (ProBlott; Applied Biosystems, Foster City, Calif.) with a semi-dry blotting apparatus (Nippon Eido, Tokyo, Japan) by the method of Hirano and Watanabe (7). The bands were stained, cut out, and then sequenced with a pulsed-liquid phase protein sequencer (model 477-A; Applied Biosystems). All samples were sequenced more than twice.

Immunoblotting. The bands separated by SDS-PAGE with the 12.5% acrylamide linear gel were electroblotted to a polyvinylidene difluoride membrane. The membrane was immersed overnight in PBS (pH 7.4) containing 0.05% Tween 20 and 10% skim milk (blocking buffer) to block nonspecific reactivity. The membrane was incubated for 1 h with 1:1,000-diluted anti-HA serum in blocking buffer and then washed in PBS (pH 7.4) containing 0.05% Tween 20. After incubation with 1:1,000-diluted peroxidase-labelled swine anti-rabbit immunoglobulin G (Dako A/S, Glostrup, Denmark) in blocking buffer for 1 h, immunoreactive bands were detected by the enhanced chemiluminescence Western blotting (immunoblotting) detection system (Amersham International plc, Little Chalfont, United Kingdom).

Densitometric analysis. Densitometry was performed with a scanning imager (300SX; Molecular Dynamics, Sunnyvale, Calif.). The intensity of the protein band was estimated by measuring the area under the peak. The molar ratio of each band was calculated by dividing the intensity of band by each molecular mass, relative to the neurotoxin, which was expressed as 1.0. Measurement was performed three times with different preparations, and the means of these values were obtained.

Determination of protein concentration. The protein concentration was determined by the Bradford method (2) with the protein assay (Bio-Rad) with bovine serum albumin as the standard.

RESULTS

Purification of progenitor toxins and neurotoxin. The progenitor toxins were purified by column chromatography and sucrose density gradient centrifugation. On SP-Toyopearl 650M, five protein peaks (peaks 1 to 5) were eluted by the NaCl gradient (Fig. 1). Peaks 1 and 2 possessed neither toxic (less than 10^3 MLD/ml) nor HA activity. Peak 3 possessed only HA activity (2^8), and peak 5 possessed only toxic activity (more than 10^6 MLD/ml). Peak 4 possessed both toxin (more than 10^6 MLD/ml) and HA activities (2^7). On the basis of the previous report (15), it was postulated that peak 4 contains HA-positive progenitor toxins (19 S and 16 S) and peak 5 contains an HA-negative progenitor toxin (12 S). The fractions of peaks 4 and 5 were separately pooled and concentrated with ammonium sulfate. By applying them to Sephacryl S-300 columns twice, we obtained the purified 19 S and 16 S toxin mixture and the 12 S toxin preparation. Thereafter, the 19 S toxin and 16 S toxin were isolated by sucrose density gradient centrifugation at pH 6.0 as reported previously (15). When the 19 S and 16 S toxin preparation was subjected to sucrose density gradient centrifugation at pH 8.8, the peaks corresponding to the neurotoxin and the nontoxic proteins were obtained; neurotoxin sedimented as 7 S.

Purification of peak 2 and peak 3 proteins. Peak 2 and peak 3 fractions (Fig. 1) were separately pooled, concentrated with a 70% saturation of ammonium sulfate, and further purified by gel filtration on Sephacryl S-300 under the same condition as that of the rechromatography for purifying the progenitor toxins. The peak 2 preparation showed a single peak of approximately 35 kDa with no HA activity. The peak 3 preparation demonstrated two peaks; only the first of these (approximately 300 kDa) showed a high HA titer ($\sim 2^6$). The second peak contained a contaminant with no HA activity.

SDS-PAGE profile and N-terminal amino acid sequence. The SDS-PAGE profiles of the 19 S, 16 S, and 12 S toxins and the neurotoxin obtained by sucrose density gradient centrifugation were analyzed (Fig. 2). The SDS-PAGE profiles of the

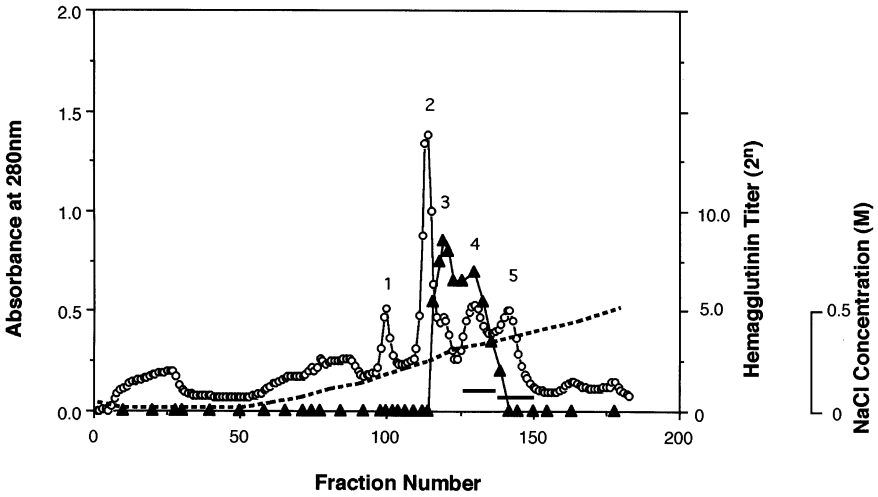

FIG. 1. SP-Toyopearl 650M ion-exchange column chromatography. The ammonium sulfate fraction from the culture fluid was applied to a column equilibrated with 50 mM sodium acetate buffer (pH 4.2). Elution was performed by increasing the concentration of NaCl to 0.5 M, and 2-ml fractions were collected. The fractions indicated by a solid bar were pooled. ○, A_{280}; ▲, HA titer. The dashed line represents the concentration of NaCl.

peak 2 and peak 3 proteins and the HA-positive preparation (mixture of 16 S and 19 S toxins), purified by column chromatography, were also analyzed. The molecular mass of each protein band was estimated by electrophoretic mobility relative to protein standards, and their N-terminal amino acid sequences were determined. On the basis of those SDS-PAGE profiles and their N-terminal amino acid sequences, the following conclusions were made. (i) The SDS-PAGE profiles of the 19 S and 16 S toxins were identical, indicating that these toxins have the same components. (ii) The band of 124 kDa in the absence of 2-mercaptoethanol (2-ME) (lanes 2, 3, and 4 in Fig. 2A; lanes 2, 3, and 4 in Fig. 2B) and the bands of 93 and 55 kDa, which were separated from the 124-kDa band in the presence of 2-ME (lanes 5, 6, and 7 in Fig. 2A; lanes 7, 8, and 9 in Fig. 2B) are neurotoxins and their heavy-chain (Hc) and light-chain (Lc) components, respectively (1, 3, 4, 17). (iii) The band of 120 kDa (lanes 2, 3, 5, and 6 in Fig. 2A; lanes 4 and 9 in Fig. 2B) is nontoxic non-HA (6, 19). (iv) The bands of 52, 35, 31, 20, 19, and 15 kDa are the subcomponents of HA (lanes 2, 3, 5, and 6 in Fig. 2A; lanes 4 and 9 in Fig. 2B) (5, 18).

Only one sequence each was obtained from all the bands except HA-19. From HA-19, major (HA-19a) and minor (HA-19b) sequences were obtained. HA-35 and HA-31 shared identical N-terminal amino acid residues. The 31-kDa band was detected in the absence of 2-ME (lanes 2 and 3 in Fig. 2A; lane 4 in Fig. 2B) but disappeared in the presence of 2-ME (lanes 5 and 6 in Fig. 2A; lane 9 in Fig. 2B) as reported previously (13).

The purified peak 3 protein demonstrated six bands of 52, 35, 31, 20, 19, and 15 kDa in the absence of 2-ME (Fig. 2B, lane 5) and five bands of 52, 35, 20, 19, and 15 kDa in the presence of 2-ME (Fig. 2B, lane 10), and the N-terminal amino acid sequences of each band were identical to those of the corresponding HA subcomponents of the 16 S toxin. Therefore, peak 3 protein was concluded to be HA (free HA), which binds neither neurotoxin nor nontoxic non-HA. The purified peak 2 protein demonstrated the same mobility as that of HA-35 (and HA-31) on SDS-PAGE (lanes 6 and 11 in Fig. 2B), and their N-terminal amino acid sequences were also identical. Therefore, the peak 2 protein was concluded to be HA-35 (free HA-35) which exists free in the culture fluid.

In addition to the bands of neurotoxin and HA subcomponents, the 19 S and 16 S toxins demonstrated only one band (120 kDa), corresponding to the nontoxic non-HA. However, the 12 S toxin demonstrated two bands, of 106 and 13 kDa (lanes 4 and 7 in Fig. 2A; lanes 3 and 8 in Fig. 2B) besides the neurotoxin band. The N-terminal amino acid sequence of the 13-kDa component was identical to those of the nontoxic non-HAs of the 19 S and 16 S toxins. These data indicated that nontoxic non-HA of the 12 S toxin has a cleavage on its N-terminal region, leading to production of 13- and 106-kDa fragments. This was confirmed by the amino acid sequence deduced genetically. Recently, we determined the entire nucleotide sequence of the gene of nontoxic non-HA (6). The N-terminal amino acids of the nontoxic non-HAs of the 19 S and 16 S toxins determined by protein sequencing were located within the open reading frame starting at Met-1, and those of the 13- and 106-kDa components of the 12 S toxin were located within the open reading frame starting at Met-1 and Phe-145, respectively. They showed absolute agreement with the deduced amino acid sequence except X in the protein sequence of the 13-kDa component.

Immunoblot analysis. Immunoblotting was performed by reacting the antisera to type A free HA and type C nontoxic components (including both HA and nontoxic non-HA) with the purified type A 19 S and type C 16 S toxins and with the purified peak 2 (free HA-35) and peak 3 (free HA) proteins. Antiserum to type C nontoxic components was prepared previously by us (10). Anti-type A HA antiserum reacted with 52-, 35-, and 19- to 20-kDa subcomponents of both type A 19 S toxin and peak 3 protein, and the 35-kDa protein of peak 2 (Fig. 3, lanes 3, 4, and 5). Although this serum reacted only slightly with HA-15 (this is the same phenomenon reported previously [13]), these results supported the conclusion that peak 3 and peak 2 proteins are free HA and HA-35, respectively, as described above. The serum also could react with HA-53 and HA-22–23 but not with HA-33 of type C 16 S toxin (lane 2). On the other hand, anti-type C nontoxic component serum, which reacted with all subcomponents of HA and nontoxic non-HA of type C 16 S toxin (lane 6), cross-reacted with the nontoxic non-HA, HA-52, and HA-19–20 of type A 19 S toxin and free HA but not with type A HA-35 (and HA-15) and free HA-35 (lanes 7, 8, and 9), indicating that nontoxic components have antigens common to types A and C but the

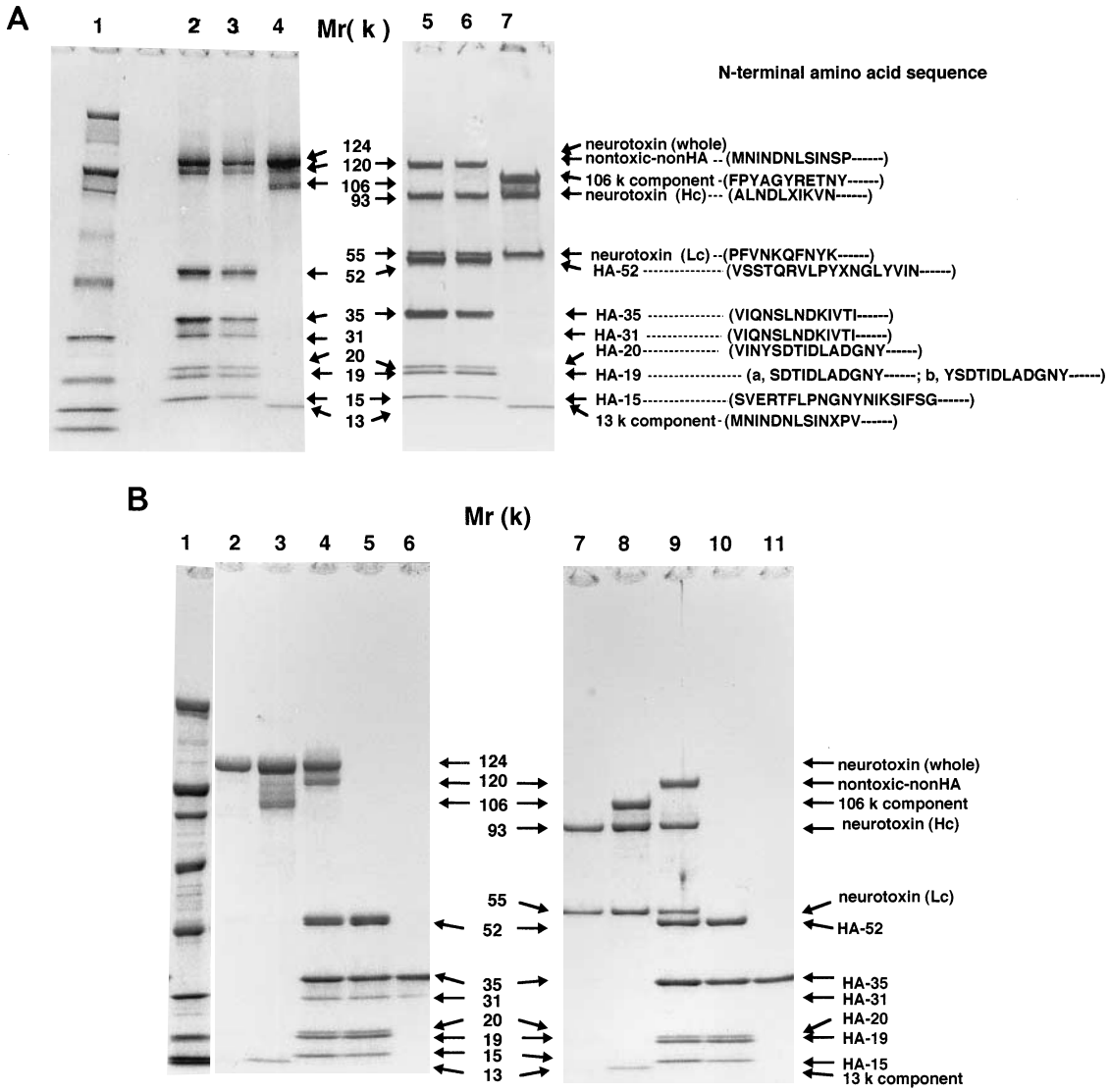

FIG. 2. (A) SDS-PAGE patterns of the purified progenitor toxins and molecular masses (Mr) of each component in kilodaltons. The purified 19 S, 16 S, and 12 S toxins and standard proteins were heated at 100°C for 7 min in the absence (lanes 1 to 4) or presence (lanes 5 to 7) of 2-ME in sample buffer. Electrophoresis was performed on a 5 to 20% polyacrylamide gradient gel. The gel was stained with Coomassie brilliant blue R-250. Lanes: 1, standard proteins; 2 and 5, 19 S progenitor toxin; 3 and 6, 16 S progenitor toxin; 4 and 7, 12 S progenitor toxin. The molecular mass markers used were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). N-terminal amino acid sequences of each band determined are also shown by the single-letter code. X, not determined. (B), SDS-PAGE patterns of neurotoxin, free HA (peak 3 in Fig. 1), and free HA-35 (peak 2 in Fig. 1) preparations. Electrophoresis was performed on a 5 to 15% polyacrylamide gradient gel without (lanes 1 to 6) or with (lanes 7 to 11) 2-ME. Lanes: 1, standard proteins; 2 and 7, neurotoxin; 3 and 8, 12 S progenitor toxin; 4 and 9, HA-positive toxin preparation (19 S and 16 S toxins); 5 and 10, free HA; 6 and 11, free HA-35.

antigenicity of type A HA-35 and type C HA-33 is quite different. The same results were obtained by when the type A 16 S toxin was used instead of the type A 19 S toxin.

Densitometric analysis. Densitometric analysis indicated that molar ratios of the neurotoxins, nontoxic non-HAs and each subcomponent of HAs of the 19 S and 16 S toxins were similar with the exception of the HA-35; the molar ratio of HA-35 of the 19 S toxin was about twice that of the 16 S toxin (Table 1).

DISCUSSION

HA-positive progenitor toxins (19 S and 16 S) and the HA-negative progenitor toxin (12 S) were purified from the culture fluid of the type A strain by cation-exchange column chromatography and gel filtration under acidic conditions. In addition, HA and HA-35, which exist free without binding with other proteins, were obtained. Separation of the 19 S and 16 S toxins was followed not by gel filtration but by sucrose density gradient centrifugation as reported previously (15). Both the 19 S and 16 S toxins demonstrated 124-, 120-, 52-, 35-, 31-, 20-, 19-, and 15-kDa bands on SDS-PAGE without 2-ME. Therefore, we concluded that the 19 S and 16 S toxins have of the same components. The banding profiles of HAs and their amino acid sequences obtained by us were very similar to those reported by Somers and DasGupta (13), although the estimated molecular masses were slightly different, and we found two bands (20 and 19 kDa) at the position of 21.5 kDa. In addition, HA-19

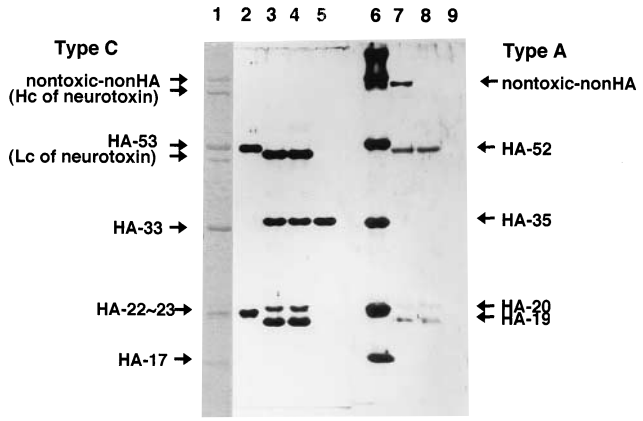

FIG. 3. Immunoblot of type A 19 S toxin, type C 16 S toxin, type A free HA, and type A free HA-35. Samples were separated by SDS-PAGE on a 12.5% acrylamide gel in the presence of 2-ME, blotted onto a polyvinylidene difluoride membrane, and successively reacted with anti-type A HA (lanes 2 to 5) or anti-type C nontoxic-components serum (lanes 6 to 9) and peroxidase-labeled anti-rabbit immunoglobulin serum. The SDS-PAGE pattern of the type C 16 S toxin is shown in lane 1 as a reference. Other lanes: 2 and 6, type C 16 S toxin; 3 and 7, type A 19 S toxin; 4 and 8, type A free HA; 5 and 9, type A free HA-35.

demonstrated two N-terminal amino acid sequences, both lacking several of the N-terminal residues from that of HA-20. These results indicate that several proteins with slightly different molecular masses of 19 to 20 kDa exist, as observed for type C HA-22–23 (5). (The molecular mass of HA-22–23 was calculated to be approximately 20 kDa this time [Fig. 3].)

As described above, we concluded that the 19 S and 16 S toxins contain the same components. Since the molecular masses of the 19 S and 16 S toxins were estimated to be 900 and 500 kDa, respectively (12), it was speculated that the 19 S toxin is a dimer of the 16 S toxin. We tried to obtain the molar ratios of neurotoxins, nontoxic non-HAs, and each subcomponent of HAs of the 19 S and 16 S toxins by densitometric analysis of the SDS-PAGE gels. Although the values obtained varied somewhat for each experiment, we concluded that the molar ratios of the components of 19 S and 16 S toxins were similar, with the exception of the HA-35. The molar ratio of HA-35 of the 19 S toxin was consistently higher (about twofold) than that of the 16 S toxin. Therefore, it was postulated that the 19 S toxin is formed by cross-linking of two 16 S toxins through HA-35. The immunoblot analysis with anti-type A HA and anti-type C nontoxic components sera revealed that the antigenicities of type A HA-35 and type C HA-33 are quite different among the nontoxic components of types A and C. Since the 19 S toxin is produced by the type A strain but not by the type C strain, we speculated that the difference between type A HA-35 and type C HA-33 might explain why only the type A strain produces the 19 S toxin; HA-35 may be a key protein to form the 19 S toxin.

TABLE 1. Densitometry of each component of the 16 S and 19 S progenitor toxins

Protein	Molar ratio[a]	
	19 S	16 S
Neurotoxin	1.0	1.0
HA-52	2.24	2.55
HA-35	7.76	3.99
HA-19~20	3.42	3.22
HA-15	2.71	2.62
Nontoxic non-HA	1.41	1.16

[a] The molar ratios were calculated by dividing the intensity of each protein band by each molecular mass. The molar ratio of neurotoxin was expressed as 1.0. Electrophoresis was performed in the presence of 2-ME. Since HA-19 and HA-20 could not be separated by the densitometer, the ratio of these subcomponents were calculated as one component. The values are the mean of three recordings.

The nontoxic component of the 12 S toxin demonstrated two bands of 106 and 13 kDa on SDS-PAGE. The total value of 106 plus 13 kDa agrees with that (120 kDa) of the whole nontoxic non-HA of the 19 S and 16 S toxins. From the N-terminal amino acid sequences determined by both protein and genetic analyses, we concluded that the nontoxic non-HA of the 12 S toxin is cleaved at its N-terminal region after translation to form these two fragments. Recently, we found the same phenomena in type C (unpublished data) and type D (11) toxins. These facts may explain why the 12 S and 16 S (and 19 S) toxins exist in the same culture. The formation of cleavage in nontoxic non-HA may prevent the binding of HA to the 12 S toxin or may cause the dissociation of HA from the 16 S toxin. The 106- and 13-kDa components of the 12 S toxin moved together on both gel filtration and sucrose density gradient centrifugation, but they were separated on SDS-PAGE with or without 2-ME. Therefore, neurotoxin, the 106-kDa component and the 13-kDa component may bind to each other, not by a disulfide bond(s) but by noncovalent bonds.

In this study, we obtained several answers to the questions described above (see Introduction). However, the three-dimensional conformation of the 19 S and 16 S toxins is still not completely clear. It may be necessary to clone and determine the whole nucleotide sequence of the HA and to clarify the regulation of the gene expression; our findings suggest that whole HA and HA-35 subcomponent are strongly produced compared with other proteins. In addition, it is important to investigate whether some functional differences exist among the nontoxic components of the three different-sized progenitor toxins.

ACKNOWLEDGMENT

This work was supported by grant 06044152 from the Ministry of Education of Japan.

REFERENCES

1. **Binz, T., H. Kurazono, M. R. Popoff, M. W. Eklund, G. Sakaguchi, S. Kozaki, K. Krieglstein, A. Henschen, D. M. Gill, and H. Niemann.** 1990. The complete sequence of botulinum neurotoxin type A and comparison with other clostridial neurotoxins. J. Biol. Chem. **265:**9153–9158.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
3. **DasGupta, B. R., and M. L. Dekleva.** 1990. Botulinum neurotoxin type A: sequence of amino acids at N-terminus and around the nicking site. Biochimie **72:**661–664.
4. **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.
5. **Fujinaga, Y., K. Inoue, S. Shimazaki, K. Tomochika, K. Tsuzuki, N. Fujii, T. Watanabe, T. Ohyama, K. Takeshi, K. Inoue, and K. Oguma.** 1994. Molecular construction of *Clostridium botulinum* type C progenitor toxin and its gene organization. Biochem. Biophys. Res. Commun. **205:**1291–1298.
6. **Fujita, R., Y. Fujinaga, K. Inoue, H. Nakjima, H. Kumon, and K. Oguma.** 1995. Molecular characterization of two forms of nontoxic-nonhemagglutinin components of *Clostridium botulinum* type A progenitor toxins. FEBS Lett. **376:**41–44.
7. **Hirano, H., and T. Watanabe.** 1990. Microsequencing of proteins electrotransferred onto immobilizing matrices from polyacrylamide gel electrophoresis: application to an insoluble protein. Electrophoresis **11:**573–580.
8. **Kimura, K., N. Fujii, K. Tsuzuki, T. Murakami, T. Indoh, N. Yokosawa, K. Takeshi, B. Syuto, and K. Oguma.** 1990. The complete nucleotide sequence of the gene coding for botulinum type C1 toxin in the C-ST phage genome. Biochem. Biophys. Res. Commun. **171:**1304–1311.
9. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of

the head of bacteriophage T4. Nature (London) **227:**680–685.
10. **Oguma, K., B. Syuto, H. Iida, and S. Kubo.** 1980. Antigenic similarity of toxins produced by *Clostridium botulinum* type C and D strains. Infect. Immun. **30:**656–660.
11. **Ohyama, T., T. Watanabe, Y. Fujinaga, K. Inoue, H. Sunagawa, N. Fujii, K. Inoue, and K. Oguma.** 1995. Characterization of nontoxic-nonhemagglutinin component of the two types of progenitor toxin (M and L) produced by *Clostridium botulinum* type D CB-16. Microbiol. Immunol. **39:**457–465.
12. **Sakaguchi, G., and I. Ohnishi.** 1984. Structure and function of botulinum toxins, p. 435–443. *In* J. E. Alouf, F. J. Fehrenbach, J. H. Freer, and J. Jeljaszewicz (ed.), Bacterial protein toxins. Academic Press, London.
13. **Somers, E., and B. R. DasGupta.** 1991. *Clostridium botulinum* types A, B, C1, and E produce proteins with or without hemagglutinating activity: do they share common amino acid sequences and genes? J. Protein Chem. **10:** 415–425.
14. **Stern, M., and L. M. Wentzel.** 1950. A new method for the large scale production of high-titer botulinum formol-toxoid types C and D. J. Immunol. **65:**175–183.
15. **Sugii, S., and G. Sakaguchi.** 1975. Molecular construction of *Clostridium botulinum* type A toxins. Infect. Immun. **12:**1262–1270.
16. **Syuto, B., and S. Kubo.** 1981. Separation and characterization of heavy and light chains from *Clostridium botulinum* type C toxin and their reconstruction. J. Biol. Chem. **256:**3712–3717.
17. **Thompson, D. E., J. K. Brehm, J. D. Oultram, T. J. Swinfield, C. C. Shone, T. Atkinson, J. Melling, and N. P. Minton.** 1990. The complete amino acid sequence of the *Clostridium botulinum* type A neurotoxin, deduced by nucleotide sequence analysis of the encoding gene. Eur. J. Biochem. **189:**73–81.
18. **Tsuzuki, K., K. Kimura, N. Fujii, N. Yokosawa, T. Indoh, T. Murakami, and K. Oguma.** 1990. Cloning and complete nucleotide sequence of the gene for the main component of hemagglutinin produced by *Clostridium botulinum* type C. Infect. Immun. **58:**3173–3177.
19. **Tsuzuki, K., K. Kimura, N. Fujii, N. Yokosawa, and K. Oguma.** 1992. The complete nucleotide sequence of the gene coding for the nontoxic-nonhemagglutinin component of *Clostridium botulinum* type C progenitor toxin. Biochem. Biophys. Res. Commun. **183:**1273–1279.

Editor: R. E. McCallum