Proteases Produced by a Proteolytic Mutant of *Clostridium botulinum* Type E

By A. NAKANE

Department of Bacteriology, Hokkaido University School of Medicine, Sapporo 060, Japan

(Received 20 December 1977; revised 23 February 1978)

A proteolytic mutant from *Clostridium botulinum* type E produced extracellular proteases after the end of exponential growth coinciding with the period of sporulation. Proteases were separated into four fractions by chromatography on a DEAE-cellulose column. One was a sulphhydryl-dependent protease that also apparently required a divalent cation for enzyme activity since it was inhibited by EDTA. This enzyme hydrolysed synthetic amide and ester compounds containing an arginine residue, and showed some activity towards L-lysine methyl ester. It appeared that two of the other proteases were serine proteases and the fourth was a metal protease. These last three proteases did not require a thiol agent and did not hydrolyse any of the synthetic amides or esters examined. Only the sulphhydryl-dependent protease could activate *C. botulinum* type B, E and F toxins. The ability of this enzyme to activate type B and E toxins was markedly lower than that of trypsin. The susceptibility of type B toxin to this protease was lower than that of type E toxin. C₂ toxin was not activated by this enzyme. It is suggested that the sulphhydryl-dependent protease in this proteolytic mutant of *C. botulinum* type E has properties similar to those of proteases from *C. botulinum* types B and F.

INTRODUCTION

Toxins produced by *Clostridium botulinum* may be activated by exposure to some proteolytic enzymes, e.g. trypsin (Duff, Wright & Yarinsky, 1956; Bonventre & Kempe, 1959, 1960). Iida (1964*a, b*, 1970) observed highly active toxins at an early stage of culture of proteolytic strains of types A, B and F. In cultures of non-proteolytic strains of types B, E and F, toxicities remained low even after prolonged incubation, but could be considerably increased by treatment with trypsin. It was suggested that the toxins of proteolytic *C. botulinum* were activated by a proteolytic enzyme(s) produced by the organisms.

Some extracellular proteases have been isolated from *C. botulinum* (DasGupta & Sugiyama, 1972*a*; Elberg & Meyer, 1935; Millonig, 1956). Recently, the relationship between activation of botulinum toxins and the production of proteases by the organisms has been investigated. *Clostridium botulinum* type A produces three distinct proteases, one of which (with a pH optimum at 6) activates type A toxin (Inukai, 1963). DasGupta (1971) and DasGupta & Sugiyama (1972*b*) isolated and purified from a proteolytic type B strain (B-Okra) a trypsin-like enzyme (TLE) with the ability to activate botulinum toxins, and which required a thiol agent and a divalent cation for the enzyme activity. Likewise, sulphhydryl-dependent proteases from *C. perfringens*, *C. histolyticum* and *C. sporogenes*, as well as from proteolytic *C. botulinum* types A, B and F, were found to activate botulinum toxins (Ohishi, Okada & Sakaguchi, 1975; Ohishi & Sakaguchi, 1977).

Recently, proteolytic mutants have been isolated from *C. botulinum* type E (Nakane & Iida, 1977). This report describes some properties of proteases obtained from a proteolytic mutant of type E and some attempts to activate botulinum toxins with these proteases.

METHODS

Preparation of proteases from a proteolytic mutant of C. botulinum type E. Spores of *C. botulinum* type E proteolytic mutant 'strain Abashiri no. 19 (Nakane & Iida, 1977)' were inoculated (to give 10^8 spores ml⁻¹) into TPG medium which consisted of (% w/v): trypticase, 5; Bacto-peptone, 0.5; glucose, 0.2; and L-cysteine.HCl, 0.1; at pH 7.2. The cultures were incubated by standing in air at 30 °C for 96 h and then centrifuged at 5200 g for 20 min. The supernatant fluid obtained was used as the crude fraction. Proteases were partially purified as follows. Solid (NH₄)₂SO₄ was added to 800 ml of the culture supernatant fluids at room temperature to give 60 % saturation. After stirring at room temperature for 3 h, the resulting precipitate was collected by centrifuging at 13000 g for 20 min and resuspended in a small volume of 0.05 M-Tris/HCl buffer, pH 7.8, containing 2 mM-CaCl₂. Similar buffer was used for subsequent dialysis and chromatography of the fraction. The precipitate was dialysed at 4 °C for 18 h and the insoluble material formed was removed by centrifugation. The supernatant fraction (17 ml, containing 247 mg protein) was applied to a DEAE-cellulose column (1.5 × 26 cm) equilibrated with the buffer. Proteases were adsorbed to this column. After washing the column with 450 ml buffer, proteases were eluted in a linear gradient of 0 to 0.6 M-NaCl (150 ml buffer plus 150 ml buffer containing 0.6 M-NaCl).

Preparation of the toxins. Type B toxin from proteolytic strain B-Lamanna (Okra), type E toxin from strains Iwanai and Abashiri (non-proteolytic parent strain of the proteolytic mutant), type F toxin from non-proteolytic strain F-OSU and C₂ toxin from strain D-139 were used in these experiments. Type B, E and F strains were incubated at 30 °C for 18 h in LYG medium comprising (% w/v): lactalbumin, 1; yeast extract, 2; glucose, 0.5; and L-cysteine.HCl, 0.15, at pH 7.2. Strain D-139 was cultivated at 37 °C for 40 h in medium containing (% w/v): trypticase, 2; yeast extract, 3; glycerol, 0.5 (v/v); NH₄Cl, 0.5; and L-cysteine.HCl, 0.1, at pH 7.4. The cultures were used as the toxin preparations.

Toxicity assays and activation of the toxins. The toxicity was assayed by injecting intraperitoneally into each of five mice 0.5 ml samples serially diluted twofold with 0.02 M-phosphate buffer, pH 6.0, containing 0.2 % (w/v) gelatin. The LD₅₀ was calculated by the method of Reed & Muench (1938).

Activation of the toxins was carried out by incubating the following mixture at 37 °C for 1 h: 0.5 ml toxin preparation; 0.4 ml trypsin (5.4 mg protein) or a protease obtained from the proteolytic mutant dissolved in 0.1 M-acetate buffer, pH 6.0, containing 2 mM-CaCl₂; 0.1 ml 2-mercaptoethanol. After cooling to 4 °C, the toxicity was estimated. The same proportions of the constituents were used for mixtures of different volumes.

Determination of proteolytic activities. The enzyme activities were measured in 0.1 M-Tris/HCl buffer, pH 7.8, containing 2 mM-CaCl₂. The proteolytic activity was measured with azocasein as substrate (azocasein-hydrolysing activity) as reported by Prestidge, Gage & Spizizen (1971). The reaction mixture (1 ml) containing 0.5 ml azocasein in buffer, 0.1 ml enzyme solution and 0.4 ml distilled water was incubated at 37 °C for 30 min. The reaction was stopped by adding 2 ml cold 10 % (w/v) trichloroacetic acid and the tubes were centrifuged at 1630 g for 10 min. The supernatant fluid was made alkaline by adding 0.3 ml 10 M-NaOH and the absorbance at 430 nm was determined. Absorbance was linear with respect to both enzyme concentration and incubation time up to values of 0.600. One arbitrary unit of activity was defined as an increase of 1.0 absorbance unit min⁻¹. Amidase activity was assayed with *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPA) as substrate (Nakane & Iida, 1977). Esterase activity was determined by the modified method of Herstrin (1949) with *N*-α-benzoyl-L-arginine ethyl ester (BAEE) as substrate. The reaction mixture (1 ml) contained 0.5 ml 10 mM-BAEE in buffer, 0.1 ml enzyme solution, 0.1 ml 0.1 M-2-mercaptoethanol and 0.3 ml distilled water. After incubation at 37 °C for 30 min, the reaction was terminated by adding 1.5 ml alkaline hydroxylamine solution (a mixture of equal volumes of 3.5 M-NaOH and 2 M-hydroxylamine). The reaction mixtures were allowed to stand at room temperature for 30 min and then 1 ml of each of the following reagents was added successively: trichloroacetic acid (18 % w/v), 3 M-HCl and FeCl₃ (10 % w/v, in 0.04 M-HCl). The absorbance at 530 nm was determined, and a unit of activity was defined as the hydrolysis of 1 μmol substrate min⁻¹.

Other determinations. Protein contents were determined by the method of Lowry *et al.* (1951). Sporulation was estimated by heating the cultures at 60 °C for 20 min and then incubating 0.2 ml of diluted preparations by standing in air at 30 °C for 16 h in a tube (12 × 150 mm) containing 13 ml LYG agar [glucose concentration, 0.2 % (w/v)] to determine the viable count.

Chemicals. Trypsinase was from BBL, and lactalbumin, yeast extract and trypsin (1:250) were from Difco. BAPA and BAEE (both hydrochloride salts), *p*-tosyl-L-arginine methyl ester.HCl, *N*-acetyl-L-tyrosine ethyl ester.H₂O and *N*-benzoyl-L-tyrosine ethyl ester were purchased from Nakarai Chemical Co., Tokyo, Japan. L-Lysine ethyl ester. 2HCl was obtained from Fluka, Buchs, Switzerland. Azocasein, L-lysine methyl ester.HCl and phenylmethylsulphonyl fluoride were purchased from Sigma. DEAE-cellulose (DE 52) was obtained from Whatman.

Fig. 1. Production of extracellular proteases and sporulation in the proteolytic mutant of *C. botulinum* type E grown in TPG medium at 30 °C. (a) Turbidity (○), pH (□) and the number of heat-resistant spores (●) were estimated on samples withdrawn at intervals up to 168 h. (b) Azocasein-hydrolysing activity (○), amidase activity (●) and esterase activity (□) (all expressed in units ml^{-1}) were determined in the supernatant fluids of each sample (see Methods).

Table 1. Change in toxicity of a culture of the proteolytic mutant of *C. botulinum* type E during incubation

Spores of the *C. botulinum* type E proteolytic mutant were inoculated into 200 ml TPG medium, pH 7.2, to give 10^8 spores ml^{-1} . The culture was incubated in air at 30 °C. Samples (5 ml) were withdrawn at intervals and stored at -20 °C. These samples were thawed at room temperature and centrifuged at 5200 g for 20 min. The toxicities of the supernatant fluids, with or without trypsin treatment, were estimated as described in Methods.

Age of culture (h)	$10^{-3} \times \text{Toxicity (LD}_{50} \text{ ml}^{-1})$	
	Without trypsin	With trypsin
14	0	0
27	0	2.0
39	0	2.0
50	0.011	4.7
63	0.036	4.7
72	0.15	4.7
96	0.20	4.7
121	0.30	4.7
144	0.20	0.94

RESULTS

Production of extracellular proteases by the proteolytic mutant of C. botulinum type E in culture

Prolonged culture of the proteolytic mutant of *C. botulinum* type E caused a decrease of turbidity and a rise in pH of the culture fluids (Fig. 1), similar to *C. botulinum* proteolytic types A and B (Iida, 1964a, 1970). Azocasein-hydrolysing, amidase and esterase activities each appeared after 50 h incubation and attained maximum levels at 96 h. Sporulation began almost at the same time as the appearance of these proteolytic activities. Production of the toxin corresponded with cell growth (Fig. 1, Table 1). The maximum level of toxicity after trypsin treatment was obtained at 50 h and remained at the same level until 121 h. A

Fig. 2. Rechromatography of proteases on a DEAE-cellulose column. Fractions covering all proteolytic activities obtained from chromatography on a DEAE-cellulose column (see Methods) were combined and solid $(\text{NH}_4)_2\text{SO}_4$ was added to 60% saturation at room temperature. The resulting precipitate was dissolved in a small volume of 0.05 M-Tris/HCl buffer, pH 7.8, containing 2 mM- CaCl_2 , and dialysed against similar buffer at 4 °C for 16 h. The extract (2.4 ml) was applied to a DEAE-cellulose column (1.5 × 26 cm) equilibrated with similar buffer and eluted with a linear gradient of this buffer containing 0 to 0.3 M-NaCl (—). ---, Protein content (A_{280}); ○, azocasein-hydrolysing activity (units ml^{-1}); ●, amidase activity (units ml^{-1}); □, esterase activity (units ml^{-1}).

marked decrease in toxin titre was observed after prolonged incubation of the culture (Table 1). No toxicity could be demonstrated without trypsin treatment until the bacterial proteolytic activities had appeared. When the enzyme levels were constant, the naturally activated toxin continued to increase with incubation time, though a large part of the toxin in the culture remained as a 'precursor' even in the aged culture.

Separation of proteases

An attempt was made to separate the enzyme activities of an extract of culture fluids precipitated with $(\text{NH}_4)_2\text{SO}_4$ at 60% saturation by chromatography on a DEAE-cellulose column (see Methods). Two peaks of azocasein-hydrolysing activity, which were incompletely resolved, and one peak of amidase activity were detected. The peak of esterase activity coincided with that of amidase activity. No further proteolytic activity was eluted with buffer containing 1.0 M-NaCl. Fractions covering all three proteolytic activities were combined and concentrated by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 60% saturation at room temperature. The dialysed extract was rechromatographed on a DEAE-cellulose column (Fig. 2). The azocasein-hydrolysing activities were separated into three peaks. Elution of the fractions with esterase activity again coincided with elution of the amidase activity, suggesting that these activities relate to a single enzyme, as reported for other types of *C. botulinum* (DasGupta, 1971; DasGupta & Sugiyama, 1972a; Ohishi *et al.*, 1975; Ohishi & Sakaguchi, 1977). These results indicated that at least four proteases were present in the extracted culture of the proteolytic mutant of *C. botulinum* type E.

Some properties of the proteases

Proteases separated by DEAE-cellulose column chromatography were designated I, II, III and IV (Fig. 2). Some properties of these proteases were studied.

Requirement for a reducing agent and effect of inhibitors. Protease II required 2-mercaptoethanol for activity and almost all the activity was lost in the absence of this reducing agent.

Table 2. Activation of type B, E and C₂ toxins by protease II

The reaction mixtures contained: 0.4 ml protease II (1.2 mg protein) or trypsin (5.4 mg protein) in 0.1 M-acetate buffer, pH 6.0, containing 2 mM-CaCl₂; 0.5 ml toxin; 0.1 ml 0.1 M-2-mercaptoethanol. The mixtures were incubated at 37 °C for 1 h, and the reactions were terminated by cooling to 4 °C. The toxicities were then estimated. The amidase and esterase activities of the protease II preparation were 1.3 units ml⁻¹ and 6.1 units ml⁻¹, respectively, and those of trypsin were 1.0 unit ml⁻¹ and 66.5 units ml⁻¹, respectively.

Protease	Toxin ...	10 ⁻³ × Toxicity (LD ₅₀ ml ⁻¹)			
		E-Iwanai	E-Abashiri	B-Lamanna	D-139 (C ₂)
None		0.042	0.58	0.040	0
Protease II		0.64	2.6	0.081	0
Trypsin		1.8	85	2.5	2.9

No absolute requirement for 2-mercaptoethanol for enzyme activities was observed in the other three proteases.

Both amidase and esterase activities of protease II were completely inhibited by 10 mM-EDTA, but not by 5 mM-phenylmethylsulphonyl fluoride, an inhibitor of serine proteases. The activity of protease I was incompletely inhibited by 10 mM-EDTA, but was not affected by 5 mM-phenylmethylsulphonyl fluoride. The activities of proteases III and IV were 60 to 70% inhibited by 5 mM-phenylmethylsulphonyl fluoride, though no effect was observed when 10 mM-EDTA was added.

Substrate specificity. Proteases I, III and IV hydrolysed azocasein, but not synthetic amide or ester compounds. Protease II hydrolysed the amide and esters with an arginine residue and was also slightly active against L-lysine methyl ester, but did not hydrolyse azocasein.

pH optima. The amidase and esterase activities of protease II were maximal at pH 6.0 to 6.3 and at about pH 7.0, respectively. Proteases I, III and IV had optimum activity around neutral or alkaline pH.

Participation of proteases in activation of botulinum toxins

After proteases I, II and IV had been concentrated by ultrafiltration and dialysed against 0.1 M-acetate buffer, pH 6.0, containing 2 mM-CaCl₂, each protease was incubated with type E and F toxins. Only protease II was able to activate these toxins. The abilities of protease II and trypsin to activate type E and other *C. botulinum* toxins were compared (Table 2). E-Iwanai toxin and E-Abashiri toxin were activated 15-fold and fivefold, respectively, by protease II, but there was only twofold activation of type B toxin and none of C₂ toxin. Substantial activation was catalysed by trypsin in each case.

DISCUSSION

Four proteases have been detected chromatographically in the culture of a proteolytic mutant of *C. botulinum* type E. One is a sulphhydryl-dependent protease apparently requiring a divalent cation for its activity (protease II); thus, this is similar to TLE from *C. botulinum* type B (DasGupta & Sugiyama, 1972*a, b*), a sulphhydryl-dependent protease (SHP) from *C. botulinum* type F (Ohishi & Sakaguchi, 1977) and clostripain from *C. histolyticum* (Michell & Harrington, 1968). Protease II differed from trypsin in that it did not hydrolyse L-lysine ethyl ester, though it showed some activity with L-lysine methyl ester. Unlike chymotrypsin, no hydrolysis of *N*-acetyl-L-tyrosine ethyl ester or *N*-benzoyl-L-tyrosine ethyl ester was observed. On the other hand, proteases I, III and IV did not hydrolyse any of the synthetic amides or esters tested. From the results on the effect of inhibitors, it is concluded that protease I requires a metal ion and that proteases III and IV are both serine proteases. The effects of inhibitors on proteases III and IV were similar and the possibility remains that these enzymes are identical.

A relationship between protease production and sporulation has been reported for *Bacillus subtilis* (Prestidge, Gage & Spizizen, 1971) and other micro-organisms (Schaeffer, 1969). The present finding that proteolytic activities appeared at the period corresponding to the onset of sporulation suggests a relationship between the two phenomena.

Of the proteases from the proteolytic mutant of *C. botulinum* type E studied here, only protease II was capable of activating type B, E and F toxins. Sakaguchi & Sakaguchi (1967) proposed that activation of botulinum toxins was caused by the esterase activity rather than the peptidase activity in trypsin. The quantitative difference in activation of the toxins by trypsin and protease II might be due to the 10-fold higher esterase activity of the trypsin preparation, but this may not be the full explanation. It has been reported that TLE from *C. botulinum* type B was incapable of 'full activation' of type E progenitor toxin (DasGupta & Sugiyama, 1972*b*), and that a large quantity of SHP proteins from *C. botulinum* type F were necessary to activate that toxin completely (Ohishi & Sakaguchi, 1977). It is noteworthy that protease II, TLE and SHP all have low specificity to substrates containing a lysine residue. It is possible that hydrolysis of a lysine bond(s) in peptide chains of the toxins plays an important role in 'full activation' of botulinum toxins as reported by Miura (1974). Of the *C. botulinum* toxins tested, C₂ toxin was not activated by protease II. Attempts are now being made to elucidate whether C₂ toxin has extremely low susceptibility to the sulphhydryl-dependent protease of the proteolytic mutant of *C. botulinum* type E, or is activated by a different mechanism from that of other botulinum toxins.

I thank Professor Iida for his interest and counsel.

REFERENCES

- BONVENTRE, P. F. & KEMPE, L. L. (1959). Toxicity enhancement of *Clostridium botulinum* type A and B culture filtrates by proteolytic enzymes. *Journal of Bacteriology* **78**, 892–893.
- BONVENTRE, P. F. & KEMPE, L. L. (1960). Physiology of toxin production by *Clostridium botulinum* types A and B. IV. Activation of the toxin. *Journal of Bacteriology* **79**, 24–32.
- DASGUPTA, B. R. (1971). Activation of *Clostridium botulinum* type B toxin by an endogenous enzyme. *Journal of Bacteriology* **108**, 1051–1057.
- DASGUPTA, B. R. & SUGIYAMA, H. (1972*a*). Isolation and characterization of a protease from *Clostridium botulinum* type B. *Biochimica et biophysica acta* **268**, 719–729.
- DASGUPTA, B. R. & SUGIYAMA, H. (1972*b*). Role of a protease in natural activation of *Clostridium botulinum* neurotoxin. *Infection and Immunity* **6**, 587–590.
- DUFF, J. T., WRIGHT, G. G. & YARINSKY, A. (1956). Activation of *Clostridium botulinum* type E toxin by trypsin. *Journal of Bacteriology* **72**, 455–460.
- ELBERG, S. & MEYER, K. F. (1935). The extracellular proteolytic system of *Clostridium parabotulinum*. *Journal of Bacteriology* **37**, 541–564.
- HERSTRIN, S. (1949). The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine, and its analytical application. *Journal of Biological Chemistry* **180**, 249–261.
- IIDA, H. (1964*a*). Experimental studies on toxin production of *Clostridium botulinum*. I. *Japanese Journal of Bacteriology* **19**, 458–461.
- IIDA, H. (1964*b*). Experimental studies on toxin production of *Clostridium botulinum*. II. *Japanese Journal of Bacteriology* **19**, 463–468.
- IIDA, H. (1970). Activation of *Clostridium botulinum* toxin by trypsin. In *Proceedings of the First U.S.–Japan Conference on Toxin Microorganisms*, pp. 336–340. Edited by M. Herzberg. Washington, D.C.: Government Printing Office.
- INUKAI, Y. (1963). Activation of the toxin in the culture of *Clostridium botulinum* type A. *Japanese Journal of Veterinary Research* **11**, 87–93.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- MICHELL, W. M. & HARRINGTON, W. F. (1968). Purification and properties of clostridiopeptidase B (clostripain). *Journal of Biological Chemistry* **243**, 4683–4692.
- MILLONIG, R. C. (1956). Isolation of an aminopeptidase from type B *Clostridium botulinum*. *Journal of Bacteriology* **72**, 301–307.
- MIURA, T. (1974). Isolation and characterization of an esterase-active enzyme from pronase with special reference to activation of *Clostridium botulinum* type E progenitor toxin. *Japanese Journal of Medical Science and Biology* **27**, 285–296.
- NAKANE, A. & IIDA, H. (1977). Proteolytic mutants obtained from *Clostridium botulinum* type E. *Applied and Environmental Microbiology* **34**, 99–101.
- OHISHI, I. & SAKAGUCHI, G. (1977). Response of type B and E botulinum toxins to purified sulf-

- hydriyl-dependent protease produced by *Clostridium botulinum* type F. *Japanese Journal of Medical Science and Biology* **30**, 179–190.
- OHISHI, I., OKADA, T. & SAKAGUCHI, G. (1975). Responses of *Clostridium botulinum* type B and E progenitor toxins to some clostridial sulfhydryl-dependent proteases. *Japanese Journal of Medical Science and Biology* **28**, 157–164.
- PRESTIDGE, L., GAGE, V. & SPIZIZEN, J. (1971). Protease activities during the course of sporulation in *Bacillus subtilis*. *Journal of Bacteriology* **107**, 815–823.
- REED, L. J. & MUENCH, H. (1938). A simple method of estimating fifty per cent end points. *American Journal of Hygiene* **27**, 493–497.
- SAKAGUCHI, G. & SAKAGUCHI, S. (1967). Some observation on activation of *Clostridium botulinum* type E toxin by trypsin. In *Botulism 1966*, p. 266. Edited by M. Ingram and T. A. Roberts. London: Chapman Hall.
- SCHAEFFER, P. (1969). Sporulation and the production of antibiotics, exoenzymes, and exotoxins. *Bacteriological Reviews* **33**, 48–71.