RESPONSE OF TYPE B AND E BOTULINUM TOXINS TO PURIFIED SULFHYDRYL-DEPENDENT PROTEASE PRODUCED BY CLOSTRIDIUM BOTULINUM TYPE F

IWAO OHISHI and GENJI SAKAGUCHI

College of Agriculture, University of Osaka Prefecture, Sakai-shi, Osaka 591, Japan

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SUMMARY: A sulfhydryl-dependent protease (SHP) was purified from a culture of Clostridium botulinum type F. The enzyme can activate type E progenitor toxin completely but type B progenitor toxin only partially. This may suggest that SHP by itself could completely activate the toxin of proteolytic C. botulinum types A and F in culture. The toxicity of type E progenitor toxin potentiated by the treatment with SHP persisted, whereas that of derivative toxin decreased rapidly by further incubation with SHP. This may indicate that only the progenitor toxin, the complex of the toxic and nontoxic components, activated by SHP withstands the subsequent exposure to the enzyme in cultures of proteolytic C. botulinum.

INTRODUCTION

We demonstrated that sulfhydryl-dependent protease (SHP) produced by some clostridial strains activated preferentially type E over type B progenitor toxin (Ohishi, Okada and Sakaguchi, 1975). The lower sensitivity of type B progenitor toxin to SHP, even to that elaborated by the same parental strain, explains the observation that the toxin of strongly proteolytic C. botulinum type B strain Okra is activable even at a late stage of incubation (Iida, 1964; DasGupta, 1971). The activation ratio of type E progenitor toxin attained with a partially purified clostridial protease was significantly lower than that attained with trypsin (Ohishi et al., 1975). DasGupta and Sugiyama (1972b) also observed a similar divergence with type B and E derivative toxins and a trypsin-like enzyme produced by C. botulinum type B strain Okra and interpreted it as implication of more than a single enzyme in activation and of cleavage of at least two peptide bonds by distinct enzymes in endogenous activation of botulimum toxin.

Our recent investigations have demonstrated that any of type A, B, E, and F botulinum toxins, no matter whether in crude or purified state, is dissociable into two components, toxic and nontoxic (Kitamura, Sakaguchi and Sakaguchi, 1968; Kozaki, Sakaguchi and Sakaguchi, 1974; Sugii and Sakaguchi, 1975; Ohishi...
and Sakaguchi, 1975). The progenitor toxin, the complex of the toxic and nontoxic components, is much more resistant to acid and proteolytic enzymes than the dissociated toxic component or the derivative toxin (Kitamura, Sakaguchi and Sakaguchi, 1969; Ohishi and Sakaguchi, 1975). The nontoxic component thus protects the toxic component from destruction by the gastric juice and digestive enzymes, and only the progenitor toxin acts as an oral toxin (Lamanna and Sakaguchi, 1971).

We purified SHP from a proteolytic strain of *C. botulinum* type F and the response of type B and E toxins to this enzyme was compared with that to trypsin. Purified SHP brought about incomplete activation of type B progenitor toxin as did a partially purified preparation, whereas it brought about complete activation of type E progenitor toxin as does trypsin. Attempts were also made to assess the role played by the nontoxic component in the progenitor toxin molecule in endogenous activation in proteolytic *C. botulinum*.

**Materials and Methods**

Purification of SHP of *C. botulinum* type F: Proteolytic *C. botulinum* type F strain Langeland was grown for 2 days at 30°C in the medium used for toxin production (Ohishi and Sakaguchi, 1974). At steps of purification, SHP was monitored by assaying for the amidase activity with N-benzoyl-ᴅ-arginine ᴵ-𝑛ɪᴛʀᴏαᴍɪlɪᴅe (BAPNA) as a substrate in 0.1 M acetate buffer, pH 6.0, containing 5 mM 2-mercaptoethanol (MCE) and 5 mM CaCl$_2$, unless otherwise is stated.

A whole culture (one liter) was added with solid ammonium sulfate to 65% saturation and allowed to stand overnight at 4°C. The precipitate formed was collected by centrifugation and dissolved in 0.1 M acetate buffer, pH 6.0, containing 5 mM each of MCE and CaCl$_2$, to which a quarter volume of a saturated ammonium sulfate solution was added. The precipitate formed was removed by centrifugation and ammonium sulfate was added to the supernatant up to 60% saturation. The precipitate formed was collected by centrifugation and dissolved in 14 ml of the same buffer, to which 28 mg of dithiothreitol (DTT) (Seikagaku Kogyo Co., Tokyo) was added. This was applied to a Sephadex G-200 column (2.5×95 cm) and eluted with the same buffer. Fractions of every 5 ml were collected. The amidase activity was recovered in a single peak in an elution volume of 250 to 350 ml. These fractions were concentrated by ultrafiltration through Amicon UM-10 membrane (Amicon Co., Lexington, MA) and dialyzed against 0.025 M acetate buffer, pH 6.0, containing 5 mM each of MCE and CaCl$_2$. It was applied to a DEAE-Sephadex, A-50, column (1.5×7.5 cm) equilibrated with the same buffer and eluted by a linear gradient increase in NaCl concentration at a flow rate of 50 ml/hr. Fractions of 10 ml were collected. The fractions possessing high specific activities eluted at an NaCl concentration of about 0.2 M were concentrated by ultrafiltration and dialyzed against 0.01 M acetate buffer, pH 6.0, containing 5 mM each of MCE and CaCl$_2$. The dialyzed
fraction was applied to a CM-Sephadex, C-50, column (1.5×10 cm) equilibrated with the same buffer. Elution was performed by a linear gradient of NaCl from 0 to 0.5 M in 500 ml of the buffer at a flow rate of 10 ml/hr. The fractions containing the amidase activity were concentrated by ultrafiltration to 14 ml, which was re-activated with 28 mg of DTT for 30 min at room temperature. This was applied again to a Sephadex G-200 column (2.5×95 cm) and eluted with the buffer at a flow rate of 20 ml/hr. The elution profiles are shown in Fig. 1. The fractions pointed by the two facing arrows were concentrated by ultrafiltration.

The purified enzyme was added with DTT to 5 mM and stored at 4 C. Prior to use, it was dialyzed against 0.1 M acetate buffer, pH 6.0, containing 5 mM each of MCE and CaCl₂ for 18 hr at 4 C with continuous stirring.

Purification of type B and E progenitor toxins and separation of the derivative toxin: C. botulinum type B strain Okra (some authors call this strain "Lamanna") was grown in the medium for toxin production for 40 hr at 30 C. The progenitor toxin was purified by the same methods as those used for purification of type F progenitor toxin (Ohishi and Sakaguchi, 1974), with the following two improved steps:

In step 3, an equal volume of 0.1 M citrate buffer, pH 4.5, containing 0.5 M NaCl was added to the toxic extract, to which a 2% protamine solution was added (1.0 ml/1,000 of total A at 260 nm). In step 4, the supernatant taken after the protamine treatment was diluted five times with distilled water and applied directly to an SP-Sephadex column. In gel filtration on Sephadex G-200 in the final step of purification, type B toxin was eluted in two peaks of L and M
toxins (Kozaki et al., 1974).

Type E progenitor toxin was purified from C. botulinum type E strain German sprats according to Kitamura et al. (1968).

The derivative toxin was separated from the progenitor toxin (M toxin for type B) by DEAE-Sephadex chromatography at pH 8.0 (Kitamura et al., 1969).

Determinations of toxicity and enzyme activity: Treatment of the progenitor toxin with SHP or trypsin was performed in 0.1 M acetate buffer, pH 6.0, containing 5 mM each of MCE and CaCl₂. To a 0.2-ml portion of a progenitor toxin solution (0.25 mg/ml), 0.2 ml of an enzyme solution, 0.5 ml of the buffer, and 0.1 ml of distilled water were added. The reaction mixture was incubated for 30 min at 35°C unless otherwise stated. The toxicity was determined by the time-to-death method (Boroff and Fleck, 1966; Sakaguchi, Sakaguchi and Kondo 1968). The ratio in percentage of the toxicity obtained by SHP treatment to that obtained by trypsinization was used to express the activating potency.

Protease and amidase activities were assayed by the method described (Ohishi et al., 1975). The esterase activity was determined spectrophotometrically by the method of Hummel (1959) with p-toluenesulfonyl-L-lysine methyl ester (TLME) and p-toluenesulfonyl-L-arginine methyl ester (TAME) as substrates. A reaction mixture contained 0.1 ml of an enzyme solution and 2.9 ml of 1 mM substrate in 0.05 M acetate buffer, pH 6.0, containing 5 mM each of MCE and CaCl₂, previously equilibrated at 30°C. The increase in absorbance at 247 nm was measured in a 1-cm cuvette at 30°C. A molar absorption coefficient (ε) of 409 (Hummel, 1959) was used to calculate μmoles substrate hydrolyzed. One arbitrary unit of activity was defined as the amount of enzyme hydrolyzing one μmole of substrate per min under the specified conditions.

Other determinations: Protein contents were determined by the method of Lowry et al. (1951). Concentration of trypsin was estimated from A²⁸₀nm = 15.6 (Braines, Baird and Elmore, 1964).

Chemicals: Casein (Hammerstein) was the product of E. Merck, Darmstadt. BAPNA, TAME and TLME were purchased from Protein Research Institute, Osaka. Trypsin (Type III, 2x crystallized) was the product of Sigma Chemicals Co., St. Louis, MO.

RESULTS

Purification and Some Properties of SHP

Purification of SHP is summarized in Table I. Approximately 53-fold purification was achieved with a 24% recovery in the amidase activity starting from the first ammonium sulfate precipitate. The purified enzyme gave a single band in disc electrophoresis performed according to Ornstein (1964) and Davis (1964). The relative mobility of the band to bromophenol blue used as a marker dye was 0.54 (Fig. 2). The enzymatic activities of purified SHP toward several
TABLE I
Purification of SHP from a culture of C. botulinum type F

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>$A_{278}$ nm</th>
<th>Amidase (units)</th>
<th>Specific activity*</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole culture</td>
<td>1,000</td>
<td>—</td>
<td>546</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>$(\text{NH}_4)_2\text{SO}_4$ ppt.</td>
<td>15</td>
<td>1,550</td>
<td>456</td>
<td>0.29</td>
<td>83.5</td>
</tr>
<tr>
<td>$(\text{NH}_4)_2\text{SO}_4$ ppt. + DTT</td>
<td>14</td>
<td>—</td>
<td>686</td>
<td>—</td>
<td>125</td>
</tr>
<tr>
<td>G-200 effluent</td>
<td>100</td>
<td>95</td>
<td>568</td>
<td>5.97</td>
<td>104</td>
</tr>
<tr>
<td>DEAE-effluent</td>
<td>32</td>
<td>60.7</td>
<td>305</td>
<td>5.02</td>
<td>55.8</td>
</tr>
<tr>
<td>CM-effluent</td>
<td>14</td>
<td>22.5</td>
<td>85.3</td>
<td>3.81</td>
<td>15.7</td>
</tr>
<tr>
<td>CM-effluent + DTT</td>
<td>14</td>
<td>—</td>
<td>111.3</td>
<td>—</td>
<td>20.3</td>
</tr>
<tr>
<td>G-200 effluent</td>
<td>5</td>
<td>7.2</td>
<td>110.3</td>
<td>15.42</td>
<td>20.2</td>
</tr>
</tbody>
</table>

* Units/$A_{278}$ nm

Fig. 2. Disc electrophoresis of purified SHP. A sample of $42 \mu g$ in protein was applied to a column of 7.5% gel and electrophoresed at pH 9.5 for 100 min. The current applied was 3 mA per column. The gel was stained by treatment with Amidoblack 10B for about 18 hr and then destained with 7% acetic acid.

TABLE II
Enzymatic activities of purified SHP toward different substrates

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Casein</th>
<th>BAPNA</th>
<th>TAME</th>
<th>TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHP</td>
<td>25</td>
<td>25.9</td>
<td>0.96</td>
<td>0.04</td>
</tr>
<tr>
<td>Trypsin</td>
<td>100</td>
<td>0.4</td>
<td>5.0</td>
<td>5.9</td>
</tr>
</tbody>
</table>
TABLE III
Effects of some inhibitors on enzymatic activities of SHP

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>None</th>
<th>NEM</th>
<th>MIA</th>
<th>EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease</td>
<td>4.43</td>
<td>0.23</td>
<td>0.35</td>
<td>0.30</td>
</tr>
<tr>
<td>Amidase</td>
<td>4.60</td>
<td>0.06</td>
<td>0.03</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Figures indicate units/117 μg SHP per ml. Each of NEM (N-ethylmaleimide), MIA (monoiodoacetate), and EDTA (ethylenediaminetetraacetic acid) was added in a final concentration of 10 mM.

Fig. 3. Amidase activities of purified SHP at different pH values. The buffers used were 0.1 M acetate buffer, ●; 0.1 M Tris-acetate buffer, ○; and 0.1 M Tris-HCl buffer, ▲. Each buffer contained 5 mM each of MCE and CaCl₂.

Substrates are shown in Table II. SHP hydrolyzed preferentially TAME over TLME.

As shown in Table III, hydrolysis of amide, casin, TAME and TLME by SHP was inhibited by agents reacting with thiol groups or divalent metal ions. The amidase activity of SHP was the highest at pH 6.0 (Fig. 3).

 Activation of Type E and B Progenitor Toxins by SHP

Effects of increasing concentrations of SHP on type E progenitor toxin were compared with those of trypsin. The highest toxicity was obtained by treating type E progenitor toxin with trypsin at 10 μg/ml and the same toxicity was attained by the treatment with SHP at 2.5 mg/ml (Table IV). The same concentration of SHP activated only partially both type B-L and B-M toxin (Table V). Such incompletely activated type B progenitor toxins were further
TABLE IV

Activation of type E progenitor toxin by SHP

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme concentration $\mu$g/ml</th>
<th>Toxicity after enzyme treatment $\text{LD}_{50}$/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHP</td>
<td>7,500</td>
<td>440,000</td>
</tr>
<tr>
<td></td>
<td>2,500</td>
<td>400,000</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>34,000</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>4,400</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>380,000</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>400,000</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>68,000</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>20,000</td>
</tr>
<tr>
<td>None</td>
<td>—</td>
<td>2,000</td>
</tr>
</tbody>
</table>

TABLE V

Response of type B and E progenitor toxins to SHP and trypsin

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Type E</th>
<th>Type B-L</th>
<th>Type B-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHP (2.5 mg/ml)</td>
<td>100</td>
<td>18.6</td>
<td>28.1</td>
</tr>
<tr>
<td>SHP + trypsin*</td>
<td>—</td>
<td>109</td>
<td>103</td>
</tr>
<tr>
<td>Trypsin (10 $\mu$g/ml)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>None</td>
<td>0.5</td>
<td>0.6</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Figures show the activating potency (see Materials and Methods) in relation to that of trypsin.
* Treated initially with SHP and subsequently with trypsin.

TABLE VI

Effects of NEM, MIA and EDTA on activation of type E progenitor toxin with SHP or trypsin

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>None</th>
<th>NEM</th>
<th>MIA</th>
<th>EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHP (5 mg/ml)</td>
<td>119</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Trypsin (10 $\mu$g/ml)</td>
<td>100</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>None</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figures show the activating potency in relation to that of trypsin. For the abbreviations of the inhibitors and the concentrations, see the legend to TABLE III.
* Not determined.

activated with trypsin to the same toxicities as those attained with trypsin alone, showing no impairment of the progenitor toxins by the treatment with SHP. Such incomplete activation of type B progenitor toxins resulted from the treatment with SHP derived from not only a strain of a different type but also the same parental strain (Ohishi et al., 1975).
Activation of type E progenitor toxin by SHP was inhibited also with thiol-binding and chelating agents as were other enzyme activities (Table VI).

Fig. 4-a. Increase in toxicity of type E progenitor toxin upon treatment with SHP or trypsin. Type E progenitor toxin (50 μg) was treated with SHP (5 mg) or trypsin (10 μg) at 35°C in 1.0 ml of 0.1 M acetate buffer, pH 6.0, containing 5 mM each of MCE and CaCl₂. At intervals, aliquots were removed and assayed for toxicities. Symbols: With SHP, ○——○, and with trypsin, ●——●.

Fig. 4-b. Increase in toxicity of type E derivative toxin upon treatment with SHP or trypsin. Type E derivative toxin (50 μg) was treated with SHP (2.5 mg) or trypsin (5 μg). See Fig. 4-a for symbols.
Comparison between Progenitor and Derivative Toxins with Respect to Activation with SHP

To find the role of the nontoxic component of the progenitor toxin in activation, type E progenitor and derivative toxins were treated with SHP and the resulting toxicities were compared. The rate of increase in the toxicity of progenitor toxin resulting from SHP treatment paralleled to that resulting from trypsinization and the highest toxicity persisted (Fig. 4-a). In contrast to this, the toxicity of derivative toxin was increased by SHP treatment in parallel with Fig. 5-a. Increase in toxicity of type B progenitor toxin upon treatment with SHP or trypsin. Type B progenitor toxin (50 μg) was treated with SHP (5.0 mg) or trypsin (10 μg). See Fig. 4-a for symbols.

Fig. 5-b. Increase in toxicity of type B derivative toxin upon treatment with SHP or trypsin. Type B derivative toxin (50 μg) was treated with SHP (2.5 mg) or trypsin (5 μg). See Fig. 4-a for symbols.
to that caused by trypsinization only up to 10 min and then the former decreased rapidly, while the latter persisted for at least 40 min (Fig. 4-b).

Similar experiments were carried out with type B toxins. As shown in Figs. 5-a and 5-b, neither progenitor nor derivative toxin was fully activated with SHP in 90 or 40 min of incubation.

**DISCUSSION**

Young cultures of proteolytic *C. botulinum* are potentiated by trypsinization, whereas aged cultures are usually not (Bonventre and Kempe, 1959, 1960; Inukai, 1963; Iida, 1964; Holdeman and Smith, 1965; Walls, 1967). A culture of proteolytic *C. botulinum* type B strain Okra, however, is activable with trypsin even after prolonged incubation (Iida, 1964; DasGupta, 1971). Purified progenitor toxin of proteolytic *C. botulinum* type B strain Okra was activable (Kozaki et al., 1974); those of types A and F were not (Sugii and Sakaguchi, 1975; Ohishi and Sakaguchi, 1974). These proteolytic strains of *C. botulinum* types A, B, and F commonly produce sulfhydryl-dependent proteases capable of activating type E and, to a markedly less extent, type B progenitor toxin (Ohishi et al., 1975). It seems justified to consider that proteolytic type A and F strains produce initially inactive toxin molecules, which are subsequently activated completely by an endogenous protease.

Purified SHP of *C. botulinum* type F, if used at a high concentration, activated type E progenitor toxin as completely as trypsin does, but type B progenitor toxin only partially. All these findings obtained in this and other laboratories indicate that the susceptibilities of the progenitor toxins of proteolytic *C. botulinum* types A and F to endogenous SHP are high enough to be activated completely in culture, whereas that of type B progenitor toxin is not high enough to be activated completely. It seems probable from the present results that endogenous activation of the toxin of proteolytic *C. botulinum* could be accomplished with endogenous SHP by itself (cf. DasGupta and Sugiyama, 1972b).

SHP purified from *C. botulinum* type F had an optimum pH of 6 and required a thiol agent and a divalent cation for the enzyme activities and for activation of botulinum toxins. Several proteases have been isolated from cultures of *C. botulinum* (Elberg and Meyer, 1939; Millonig, 1956; Kodama, 1961; Inukai, 1963; DasGupta and Sugiyama, 1972; Tjaberg, 1973a,b). Tjaberg (1973a) isolated two distinct proteases from each of *C. botulinum* types A, B and F. They differed from SHP in the optimum pH and the requirement for a thiol agent. Inukai (1963) found three distinct proteases in *C. botulinum* type A; none of them required a thiol agent, but one of them with an optimum pH at 6 enhanced the toxicity of type A toxin. Only the trypsin-like enzyme purified from *C. botulinum* type B strain Okra (DasGupta and Sugiyama, 1972a), requiring a thiol agent and a divalent cation for its action and having an
optimum pH at 6, appears to be the same enzyme as SHP purified from *C. botulinum* type F.

Type E progenitor toxin was activated completely with SHP and the resulting toxicity persisted, whereas its derivative toxin was also activated but activated derivative toxin was decomposed much more rapidly than activated progenitor toxin. The fact indicates a significant role of the nontoxic component in protecting the toxic component during activation with SHP and the subsequent exposure to the enzyme in cultures of proteolytic *C. botulinum*. It was also demonstrated that the nontoxic component in type B progenitor toxin molecules are not responsible to the incomplete endogenous activation. The progenitor toxin has been defined as the toxin first appearing in food and in culture (Lamanna and Sakaguchi, 1971). It is not known when the toxic and the nontoxic components are bound together after they are synthesized to form the progenitor toxin molecule. They are, however, no doubt bound together before endogenous activation takes place; otherwise the activated toxic component is rapidly destroyed by endogenous SHP in proteolytic strains.

**References**


