TWO MOLECULAR FORMS OF CLOSTRIDIUM PERFRINGENS
α-TOXIN ASSOCIATED WITH LETHAL, HEMOLYTIC
AND ENZYMATIC ACTIVITIES

The α-toxin of Clostridium perfringens (welchii) contains both the lethal and hemolytic activities. It was identified as phospholipase C (EC 3.1.4.3) catalyzing the hydrolysis of lecithin to a diglyceride and phosphorylcholine (Macfarlane and Knight, 1941).

Pastan et al. (1968) separated the phospholipase C of C. perfringens into two fractions, one preferentially hydrolyzing sphingomyelin to ceramide and phosphorylcholine and the other lecithin. By isoelectric focusing, Bernheimer et al. (1968) also resolved the phospholipase C into two molecular forms having enzymatic (egg-yolk reaction) and hemolytic activities. In these reports, however, no attempts were made to correlate phospholipase C activity with the lethal toxicity. It seemed justified, therefore, to clarify whether the α-toxin is separable into two fractions with respect to the lethal toxicity and, if so, whether both fractions are associated with the hemolytic and enzymatic activities.

In this communication we report a complete separation by isoelectric focusing of the lethal toxicity of the α-toxin into two parts, α1- and α2-toxins, having hemolytic, lecithin-hydrolyzing and sphingomyelin-hydrolyzing activities as well as the antitoxin-binding power.

Crude α-toxin was prepared from a culture filtrate of C. perfringens PB6K (ATCC No. 10543) as described previously (Ohsaka and Sugahara, 1968). Protein content was estimated as albumin equivalent by absorbance at 280 nm. The phospholipase C activity on lecithin was determined by the phosphatase method (Ohsaka and Sugahara, 1968). The phospholipase C activity on sphingomyelin was determined in the presence of Ca2+ by the modified acid-soluble phosphorus method described previously (Ohsaka and Sugahara, 1968) except that the substrate used was sphingomyelin instead of lecithin. One unit of phospholipase C was defined as the amount of the enzyme hydrolyzing 1 μ mole of substrate per min at 37°C (Ohsaka and Sugahara, 1968). The lethal toxicity was assayed by intravenous injections into mice (gpc-Yoken) of 4-5 doses in 0.5 ml amounts of each fraction increasing by 1.4-fold steps. Five animals were injected with each dose. All deaths during 72 hr following injection were ascribed to α-toxin. The LD50 was calculated by the Reed-Muench method. The hemolytic activity was determined according to Akama et al. (1969) in the presence of a specific β-antitoxin (30 BU) and was expressed as the reciprocal of the highest dilution of the sample solution showing complete hemolysis. The antitoxin-binding power (Lv) was determined according to Murata et al. (1965).
Table 1. Purification and yield of α-toxin with respect to

<table>
<thead>
<tr>
<th>Protein</th>
<th>Lethal toxicity</th>
</tr>
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<tbody>
<tr>
<td>Total protein (mg)</td>
<td>Total activity in LD₅₀</td>
</tr>
<tr>
<td>Yield (%)</td>
<td></td>
</tr>
<tr>
<td>Starting material</td>
<td>780</td>
</tr>
<tr>
<td>Ammonium sulfate ppt (20-40%)</td>
<td>213</td>
</tr>
<tr>
<td>Gel filtration on Sephadex G-100 (Tubes No. 58-67)</td>
<td>29.1</td>
</tr>
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</table>

Purification of α-toxin was performed in a cold room (about 4°C). Fractional solution of the crude α-toxin by a decreasing concentration gradient of ammonium sulfate indicated that the material precipitated between 20% and 40% saturation contained most of α-toxin as determined in terms of the antitoxin-binding power (Ohsaka and Sugahara, unpublished work). From this finding, 15 ml of a solution of the crude α-toxin (52 mg protein/ml) was treated with solid ammonium sulfate to 20% saturation (106 g per liter)*, pH of the mixture being adjusted to 7.0. After left standing for 1.5 hr, the precipitate formed was removed by centrifugation and was discarded. The supernatant fluid was then treated with solid ammonium sulfate to 40% saturation (116 g per liter), and the resulting precipitate was collected by centrifugation. The precipitate dissolved in 6 ml of 0.05 M Tris-HCl buffer (pH 7.6) was submitted to gel filtration on Sephadex G-100 as described (Ikezawa et al., 1964). Distributions of lethal toxicity, lecithin-hydrolyzing activity and antitoxin-binding power (Lv) in the fractions obtained during the purification are shown in Table 1. The purification achieved was 15-fold with a 56-58% yield with respect to both the lethal toxicity and lecithin-hydrolyzing activity. As shown in Fig. 1, the lethal toxicity, lecithin-hydrolyzing and sphingomyelin-hydrolyzing activities and antitoxin-binding power were found in the same fraction (tubes No. 58-67). The peak of lethal toxicity, however, did not coincide with those of the others, suggesting the possibility that separate entities are responsible for the lethal toxicity and phospholipaseC activity.

For further fractionation of Sephadex G-100-eluted α-toxin, isoelectric focusing with carrier ampholyte (pH 4-6) was performed in the apparatus supplied by LKB Instruments (Stockholm) according to Vesterberg and Svensson (1966). The results are presented in Fig. 2. Distributions of lethal toxicity, hemolytic activity and antitoxin-binding power (Lv) are shown in Fig. 2b; those of lecithin- and sphingomyelin-hydrolyzing activities in Fig. 2a. We found a complete separation of the lethal toxicity into two parts, α₁-toxin and α₂-toxin (Fig. 2b). The isoelectric points (pI) of α₁- and α₂-toxins were determined to be averagely 5.3 and 5.6, respectively, from duplicate experiments. The hemolytic activity, antitoxin-binding power and lecithin- and sphingomyelin-hydrolyzing activities were closely

lethal toxicity, enzymatic activity and antitoxin-binding power

<table>
<thead>
<tr>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
<th>Total activity in Lv (Lv/mg)</th>
<th>Specific activity (Lv/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5125</td>
<td>6.57</td>
<td>100</td>
<td>3352</td>
<td>4.30</td>
<td>100</td>
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<tr>
<td>2720</td>
<td>12.77</td>
<td>53.0</td>
<td>2535</td>
<td>11.90</td>
<td>75.6</td>
</tr>
<tr>
<td>2856</td>
<td>98.14</td>
<td>55.7</td>
<td>1402</td>
<td>48.19</td>
<td>41.8</td>
</tr>
</tbody>
</table>

Fig. 1. Distribution of lethal toxicity, lecithin- and sphingomyelin-hydrolyzing activities and antitoxin-binding power in eluate from Sephadex G-100.

The ammonium sulfate precipitate was dissolved in 0.05 M Tris-HCl buffer (pH 7.6). The solution containing 213 mg of protein in 6 ml was applied onto a column (3×100 cm) of Sephadex G-100. The column was eluted with the same buffer. Eight-ml fractions were collected. The eluate was assayed for absorbance at 280 mµ (-----), lethal toxicity (----), lecithin (LT)-hydrolyzing activity (-----), sphingomyelin (SM)-hydrolyzing activity (-----), and antitoxin-binding power (-----).

associated with the two peaks of lethal toxicity (Fig. 2a and 2b); yields of all these activities in the purified α₁- and α₂-toxins were about 20%. Separation of α₁- and α₂-toxins was reproducible when the crude α-toxin was directly subjected to isoelectric focusing under the same conditions. Electrophoresis in acrylamide gel at pH 8.3 demonstrated that each preparation of α₁-toxin and α₂-toxin gave a single band, the former migrating toward the anode faster than the latter; the purified preparation of α-toxin from the gel filtration step yielded three bands,
Fig. 2. Isoelectric focusing of α-toxin purified on Sephadex G-100. The electrofocusing column (110 ml capacity) was prepared as described (Vesterberg and Svensson, 1966) using an ampholine solution (pH 4–6). The α-toxin (5 mg) was layered in the center of the column. Electrofocusing was conducted at 4°C for 24 hr with a final potential drop of 1140 volts. 1.5-ml fractions were collected and the pH of each (△△) measured immediately at room temperature. Each fraction was diluted with 3 ml of 0.05 M Tris-HCl buffer (pH 7.5) and then assayed for absorbance at 280 mμ (---), and biological and enzymatic activities.

(a) Distribution of lecithin (LT)-hydrolyzing activity (---○---) and sphingomyelin (SM)-hydrolyzing activity (△△).  
(b) Distribution of lethal toxicity (●●), hemolytic activity (□□) and antitoxin-binding power (△△).  

of which two corresponded to those of α1- and α2-toxins.  

We also noted that sphingomyelin-hydrolyzing activity of every fraction obtained by isoelectric focusing was consistently higher with Ca2+ than with Mg2+. This observation disagreed with the inhibitory effects of Ca2+ reported by Pastan et al. (1968).

It is of much interest to know whether or not the α1- and α2-toxins have an identical antigenicity. Fig. 3 shows the reaction of these toxins against α-antitoxin in the Ouchterlony test. The α2-toxin and α5-toxin formed a single coalescing precipitation band against the National Standard Gas Gangrene Antitoxin (perfringens). A minor difference in antigenicity between the toxins, however, may still be possible. In fact, Iguchi (1940) suggested the possible presence
of multiple lethal toxic components in the culture filtrate of *C. perfringens* type A and the corresponding antibodies in different proportions in antitoxin preparations; he observed varying antilethal potencies for an antitoxin preparation depending on the test toxin used. Should \( \alpha_1 \)- and \( \alpha_2 \)-toxins be different in antigenicity and the potency of such an antitoxin be titrated against a crude \( \alpha \)-toxin preparation, a question may arise as to what the results of such titration indicate; the potency of anti-\( \alpha_1 \)-toxin, that of anti-\( \alpha_2 \)-toxin or both. The antitoxin titer determined relatively to a reference antitoxin can be justified only when the two toxins are used separately as test toxins instead of the crude \( \alpha \)-toxin, as was the case with a snake venom and an antivenine (Ohsaka et al. 1966).

In summary, we separated the lethal toxin ("\( \alpha \)-toxin") of *C. perfringens* into two molecular forms, \( \alpha_1 \)- and \( \alpha_2 \)-toxins. These two toxins were not differentiated in antigenicity but slightly different in electric charge (pI was 5.3 for \( \alpha_1 \)-toxin; 5.6 for \( \alpha_2 \)-toxin). Although the gel filtration on Sephadex G-100 suggested that the lethal toxicity and the lecithin-hydrolyzing activity are effected by distinct entities, the isoelectric focusing failed to prove it. Hemolytic, lecithin- and sphingomyelin-hydrolyzing activities, and antitoxin-binding power (Lv) were found in each of \( \alpha_1 \)- and \( \alpha_2 \)-toxins. Throughout the present work the phosphatase method we had proposed proved to be very useful in determining the lecithin-hydrolyzing activity. Significance of the present finding has been discussed in relation to the standardization of \( \alpha \)-antitoxin.

**REFERENCES**


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