PURIFICATION AND SOME PROPERTIES OF KAPPA TOXIN
OF CLOSTRIDIUM PERFRINGENS

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SUMMARY: Kα-Toxin of Clostridium perfringens type A was purified by
gel filtration through Sephadex G-100 followed by column chromatography on
DEAE-cellulose. The purified preparation showed no activities of other toxins
produced by the organism and of such enzymes as neuraminidase, deoxyribonu-
lease, hyaluronidase or proteinase (caseinolytic). The purified toxin behaved
as a single component by immunoelectrophoresis and by centrifugation in suc-
rose density gradient. An approximate molecular weight of 80,000 was esti-
imated. A definite hemorrhage was produced with 0.7 μg of the toxin in the
depilated rabbit skin. Mice were killed with 30 μg; on autopsy severe hemor-
rhage in the lungs was the only visible pathological change. An extensive
destruction of connective tissue resulted from the subcutaneous injection of
the toxin into the guinea pig. Intact muscle was also destroyed severely by
the intramuscular injection of the toxin.

INTRODUCTION

Clostridium perfringens produces multiple toxins (MacLennan, 1962). Kα-Toxin
of C. perfringens is collagenase and considered to be responsible for the develop-
ment of gas gangrene by destroying connective tissues, muscles and blood vessels (Oakley,
1954). However, Evans (1947 a, b) stated that a positive correlation was not always
found between the virulence of C. perfringens and the ability to produce collagenase
and that anti-collagenase was not effective in preventing experimental gas gangrene
in guinea pigs. The substance having the “aggressin” effect may not always be a
protective antigen (MacLeod and Bernheimer, 1965). However, it is not easy to
elucidate the roles in the infection played by various toxins of C. perfringens until
purified toxins are available.

Purification of Kα-toxin was tried by several workers. Bidwell and van Heyningen
(1948) obtained a partially purified preparation by the procedures involving adsorption
on calcium phosphate, charcoal treatment and fractionation with ammonium sulphate.
However, the preparation still contained small amounts of α- and β-toxins, and was
not tested for the hyaluronidase activity. Haberman (1959) tried to purify various
toxins of C. perfringens by methanol fractionation, electrophoresis and column chro-
matography. His Kα-toxin preparation was free from α-toxin and hyaluronidase but
contained some β-toxin.
This paper describes a method of purification of κ-toxin free from other toxins of *C. perfringens* and some properties of the purified toxin.

**MATERIALS AND METHODS**

**Culture filtrate:** A peptone medium (Murata, Yamada and Kameyama, 1956) supplemented with cooked meat was used throughout the experiment. When the meat was added at 5 g per l of the medium, no θ-toxin was produced in the culture filtrate. *C. perfringens* PB6K-N5 (Murata et al., 1965) was grown in the medium for 4–6 hr and cells were removed by continuous-flow centrifugation followed by filtration through a Seitz filter-pad EK (Toyo Roshi Co. Ltd.).

**Concentrated toxin:** Toxins were precipitated with zinc chloride at 1% from the sterile filtrate and extracted with disodium phosphate according to Hosoya, Deguchi and Kagabe (1944). Then κ-toxin was precipitated with ammonium sulphate at 60% saturation. The precipitate was dissolved in a small amount of saline, dialyzed against saline at 4°C and kept at −70°C until use. This solution was called “concentrated toxin”.

**Antitoxins:** The National Standard Gas Gangrene Antitoxin (*C. perfringens*) was used for the titration of α-antitoxin and of κ-toxin for the combining power as well as for the precipitation test in gel. Reference κ-antitoxin was a preparation of horse serum. α-Antitoxin was titrated by the egg yolk method (Ito, 1970) and the titer was expressed by the International Unit (IU). κ-Antitoxin was titrated by the azocoll EP method and the titer was expressed by the British Unit (BU) (Kameyama and Akama, 1970). κ-Antitoxin (G) was a pool of sera of guinea pigs immunized with purified κ-toxin and the content of κ-antitoxin was below 0.04 IU/ml. One ml of the preparation was not able to neutralize 6 θ-doses of a preparation of θ-toxin (see below). α-Antitoxin (R) was obtained by immunizing rabbits with a highly purified α-toxin (Sato, unpublished) and the content of κ-antitoxin was below 0.7 BU/ml.

**Beta toxin (Collagenase) of Clostridium histolyticum:** A purified preparation of collagenase of *C. histolyticum* (Nagai, 1961) was obtained through the generosity of Dr. Nagai.

**Estimation of toxins:** κ-Toxin was usually titrated by the azocoll EP method described by Kameyama and Akama (1970). The titer was expressed in terms of κ-dose (κD) and Lκ for direct and indirect activities, respectively. κD was determined by comparing with the reference κ-toxin as before. One Lκ was defined as the least amount of the toxin releasing the dye from azocoll EP in the presence of 1 BU of the reference κ-antitoxin. The collagenase activity was estimated by the method described before using acid soluble collagen as substrate (Kameyama and Akama, 1970).

α-Toxin was titrated by the egg yolk method described by Murata et al. (1965) and the amount of the toxin was expressed by Egg-Unit (EU) and Lv/5. For the sake of convenience 5 X Lv/5 was called Lv in this report.

θ-Toxin was titrated by the method described by Akama, Yamamoto and Kameyama (1969). One theta dose was defined as the amount of the toxin causing complete lysis of sheep red blood cells in 0.5% suspension in a final volume of 2 ml.

**Determination of activities of other enzymes:** Hyaluronidase was determined by the method of Di Ferrante (1956) and the enzyme content was expressed by U/ml.
Kappa toxin of C. perfringens

raminidase was estimated by the method of Warren (1959) by using N-acetylneuraminic acid as substrate.

Proteinase was determined with casein as substrate by the method of Kunitz (1946).

Deoxyribonuclease was estimated only qualitatively by the method of MacFadyen (1937).

**Determination of hemorrhagic activity:** A 0.2-ml amount each of the toxin dilutions graded with 2-fold intervals was inoculated intracutaneously into the depilated back skin of male rabbits. Reading was made after 24 hr from the inside of the skin as proposed by Kondo et al. (1960). The minimum hemorrhagic dose (MHD) was defined as the amount of the toxin producing a hemorrhagic spot of 10 mm in diameter.

**Determination of lethal activity:** Mice weighing 15-17 g were injected intravenously with 0.2 ml each of the toxin dilutions spaced at 2-fold intervals. At least 3 mice were used for each dilution. The certainly lethal dose (CLD) was defined as the smallest amount of the toxin killing all mice within 1 hr.

**Determination of protein:** Protein content was estimated either by the method of Lowry et al. (1951) with bovine serum albumin as standard or by measuring absorbancy at 280 mµ with a 1-cm cell in a Hitachi photoelectric spectrophotometer.

**Determination of carbohydrate and phosphorus:** The content of carbohydrate was estimated by the method of Dubois et al. (1956). Phosphorus content was determined by the method of Allen (1940).

**Determination of molecular weight:** Molecular weight was estimated by the method described by Seki, Appella and Itano (1968).

**Gel filtration:** Gel filtration of toxins of C. perfringens on Sephadex G-100 was performed essentially by the method described by Ikezawa, Yamamoto and Murata (1964).

**Column chromatography on DEAE-cellulose:** The κ-toxin fractions obtained by gel filtration were combined, concentrated with Ficoll (Pharmacia) and dialyzed against 0.02 M tris-HCl buffer (pH 7.5) at 4°C. The dialysand was chromatographed on DEAE-cellulose. Elution was carried out by stepwise increase in NaCl concentration. κ-Toxin fractions were combined, concentrated with Ficoll and dialyzed against 0.02 M tris-HCl buffer (pH 7.5) at 4°C. The preparation was kept at −20°C until use.

**Sucrose density gradient centrifugation:** Two-tenths ml of a sample solution (3.8 mg/ml) was layered on the top of a linear sucrose gradient (5-20%) in 0.02 M tris-HCl buffer (pH 7.5) of 2.4 ml in a centrifugal tube. The tube was centrifuged at 49,000 rpm in an SW 50 rotor for 8 hr in a Beckman Ultracentrifuge Model L-4. The content was then siphoned with a needle and 13 drop fractions were collected.

**Immunoelectrophoresis:** A plate of 1% Agarose (Nakarai Chem. Ltd.) in veronal buffer (pH 8.6, µ=0.05) was used. The samples were allowed to migrate for 2.5 hr with a potential drop of 100 V and a current of 22 mA/cm². After the run, the Standard Antitoxin (200 IU/ml) was added in the troughs, and the plate was kept at room temperature for 48 hr in a moist chamber.

**Agar-gel double diffusion test:** One per cent agar gel (Difco) was prepared on a glass plate of 10×10 cm to a thickness of 2–3 mm. Circular wells of 8 mm in diameter were cut at a distance of 10 mm between every two wells. The Standard Antitoxin (20 IU/0.1 ml) was placed in the central well. A half part of the plate was...
prepared with agar mixed with azocoll EP (Kameyama and Akama, 1970) as shown in Fig. 5. When κ-toxin diffused through the gel, the part containing azocoll EP was stained red homogeneously.

RESULTS

Purification of κ-toxin

Figure 1 shows the diagram of gel filtration of toxins produced by C. perfringens. α-Toxin was usually separated effectively from κ-toxin; no α-toxin activity was detected in κ-toxin fractions after rechromatography. However, with a few samples

![Diagram](image_url)

Fig. 1. Gel filtration of toxins of C. perfringens on Sephadex G-100.

Thirty ml of a concentrated toxin (1,429 kD/ml) was placed on a column (5.5 x 100 cm) of Sephadex G-100 equilibrated with 0.02 M tris-HCl buffer (pH 7.5). Elution was carried out with the buffer and 15-ml fractions were collected.

- : Protein
- : Hyaluronidase
- : κ-Toxin
- : α-Toxin

| Table 1. Summary of purification of C. perfringens κ-toxin |
|-----------------|-----------------|-----------------|
| **Step** | **Specific activity (kD/mg protein)** | **Yield (%)** |
| 1. Culture filtrate | 0.99 | 100 |
| 2. Extract from ZnCl₂ precipitates | 4.4 | 80.1 |
| 3. Fractionation with (NH₄)₂SO₄ (0.6 sat.) | 68.95 | 61.3 |
| 4. Sephadex G-100 1st | 492.4 | 46.2 |
| 5. Sephadex G-100 2nd | 750.1 | 32.5 |
| 6. DEAE-cellulose* | 845.6 | 22.5 |

* Rechromatography on DEAE-cellulose was not tried in this case.
Fig. 2a. Column chromatography of \( \kappa \)-toxin on DEAE-cellulose.

Two ml (containing 8.1 mg of protein) of the dialyzed solution derived from Sephadex G-100 was applied to a column (1.0 \( \times \) 20 cm) of DEAE-cellulose equilibrated with 0.02 M tris-HCl buffer (pH 7.5). Elution was carried out by increasing in NaCl concentration in the buffer. Four ml-fractions were collected at a flow rate of 40 ml per hour. All the procedures were performed at 4°C.

\[ \text{Protein} \quad \bullet \quad \text{Hyaluronidase} \quad \bullet \quad \text{\( \kappa \)-Toxin} \]

Fig. 2b. Rechromatography of \( \kappa \)-toxin on DEAE-cellulose.

Two ml (containing 5.1 mg of protein) derived from the 1st chromatography on DEAE-cellulose was applied to a column of DEAE-cellulose. All experimental conditions were the same as Fig. 2a.

\[ \text{Protein} \quad \bullet \quad \text{Hyaluronidase} \quad \bullet \quad \text{\( \kappa \)-Toxin} \]
of the concentrated toxin the separation was not so clear as in the figure. Hyaluronidase activity was not separated from κ-toxin by gel filtration, but separated effectively by chromatography on DEAE-cellulose (Fig. 2 a, b).

The extent of purification of κ-toxin and the yield at each step are summarized in Table 1. Eight hundred and fifty-fold purification was attained from the culture filtrate with a yield of 23%. The final preparation contained 846 κD and 537 Lκ per mg protein.

Examinations for Homogeneity

Centrifugation of the purified toxin in sucrose density gradient gave a single peak; a constant specific activity was observed through the peak (Fig. 3). Upon ultracentrifugal analysis, although not strictly symmetrical, a single boundary with an S20w of 5.19 was observed.

The immunoelectrophoresis yielded a single precipitation line as shown in Plate 1. The double-diffusion test gave a single precipitation line at a concentration of 150 κD/0.1 ml (Fig. 4). It is evident that the precipitation line was derived from κ-toxin and antitoxin, since κ-toxin activity on azocoll EP was inhibited completely at the line.
Fig. 4. Double diffusion test of the purified ε-toxin.

Diffusion was allowed in 1% agar gel under the conditions described in the text. The hatched area represents the part stained homogeneously by the release of dye from azocoll EP by the action of free ε-toxin; the double hatched area the presence of intact azocoll EP.

κ: Purified ε-toxin (150 kD/0.1 ml/well, 0.21 mg Prot./0.1 ml).
C: Concentrated toxin (150 kD/0.1 ml).
AT: Standard Antitoxin (α, 20 U/0.1 ml; ε, 63 BU/0.1 ml).

Some Properties of the Purified toxin

The absorption spectrum of the purified toxin showed the maximum at 276-278 mμ and the minimum at 252-254 mμ, respectively. Carbohydrate and phosphorus were not detected at a concentration of 1 mg/ml.

Molecular weight was calculated to be approximately 80,000 according to Seki et al. (1968) using Sephadex G-150 and G-200 (Fig. 5). The molecular weight of the toxin was also determined on the culture filtrate concentrated with Ficoll, since we noted that the procedure of the purification similar to above resulted in polymerization of tetanus toxin (unpublished data). The value estimated with the culture filtrate was 80,300.

pH optimum: The activity of the toxin on azocoll EP was assayed with borate and phosphate buffers of different pH values. As shown in Fig. 6, the optimum pH was around 7.5 in borate buffer and around 6.5 in phosphate buffer. Since the activity was higher in borate buffer, the toxin was assayed at pH 7.5 in borate buffer in further experiments, as described before (Kameyama and Akama, 1970).

pH stability: The toxin was dissolved in phosphate, borate and acetate buffers of varying pH values at a concentration of 0.13 mg/ml. Solutions were kept at 20°C and checked for the activities at the times indicated after dilution with borate buffer.
Fig. 5. Determination of molecular weight by gel filtration on Sephadex G-150 and G-200.
The purified $\kappa$-toxin solution was applied to columns of Sephadex G-150 (2.5×172 cm) and G-200 (2.5×105 cm). Molecular weight of $\kappa$-toxin was calculated graphically. Human immunoglobulin, bovine serum albumin and pepsin were used as the standards.

- - - - : Sephadex G-150, --- : Sephadex G-200
$\blacktriangle$ : Human immunoglobulin $\bigcirc$ : $\kappa$-Toxin
$\blacklozenge$ : Bovine serum albumin $\bigodot$ : Pepsin

Fig. 6. pH-activity curves of $\kappa$-toxin on azocoll EP.
Purified $\kappa$-toxin and azocoll EP were incubated for 3 hr in phosphate buffer (pH 4.5 to pH 9.0) or borax-boric acid buffer (pH 7.2 to pH 9.0). Activity was expressed as per cent that at pH 7.6 in borax-boric acid buffer.
$\bigcirc$---$\bigcirc$ : Phosphate buffer
$\blacklozenge$---$\bullet$ : Borax-boric acid buffer
Fig. 7. pH-stability of κ-toxin

κ-Toxin solution (0.65 mg/ml) was preincubated at various pH values at 20°C for 1 hr or 5 days and adjusted to pH 7.5 with borax-boric acid buffer, and then the enzymatic activity on azocoll EP was determined. The buffers used were acetate buffer (pH 4.1 to 5.9), phosphate buffer (pH 4.6 to 8.1), borax-boric acid buffer (pH 7.1 to 9.0) and boric acid-NaOH buffer (pH 8.0 to 10.0). Activity was expressed as per cent that of a control mixture kept at pH 7.5 (borax-boric acid buffer) at 5°C for 1 hr.

---: 1 hr ----: 5 days
○: Acetate buffer ○: Phosphate buffer
●: Borax-boric acid buffer △: Boric acid-NaOH buffer

(pH 7.5). Figure 7 indicates clearly that the toxin is most stable in borate buffer and least in phosphate buffer.

Heat stability: The toxin was dissolved in borate buffer (pH 7.5) at a concentration of 1 mg per ml. The solution was heated at different temperatures for 10 min and assayed for the activity. The toxin was stable up to 40°C and then lost its activity abruptly, as shown in Fig. 8.

Biological Activities of the Purified toxin

The toxin of approximately 30 μg killed mice within 1 hr. On autopsy, an intense hemorrhage of the lungs was invariably observed. No visible changes were observed in other organs. The lethal activity of the toxin was comparable to that of β-toxin of C. histolyticum.

Guinea pigs of about 400 g were injected subcutaneously with 140 μg of the toxin. The skin become soft and oedematous after 24 hr and serous fluid oozed out from the skin. The animals were killed with ether 3 days after the injection. The skin at the site of lesion was removed easily. An extensive destruction of connective tissue was shown, but no visible changes were observed in the muscle layer. An extensive hemorrhage was observed in the lungs while other organs showed very little, if any, change.
Fig. 8. Heat stability of $\kappa$-toxin.

$\kappa$-Toxin solution at 1 mg per ml in borax-boric acid buffer (pH 7.5) was heated at various temperatures for 10 min and then quickly cooled in running water. Activity was expressed as per cent that of a control solution kept at 5°C.

Fig. 9. Dosage response curve of $\kappa$-toxin given by the intracutaneous injection.

The ordinate represents average diameter of hemorrhagic spots produced in rabbit skin after 24 hr; the abscissa amount of purified $\kappa$-toxin.
Intramuscular injection of more than 140 μg of the toxin caused an extensive destruction of the muscle, as shown in Plate 2. No changes were observed in various organs except for the lungs.

Upon intracutaneous injection of the toxin into rabbits, distinct hemorrhage appeared within 5 min. Necrosis ensued in a few days. Plate 3 shows the pattern of the lesion 24 hr after injection. A linear correlation was obtained between the dose of the toxin and the diameter of the hemorrhagic spot within a certain range (Fig. 9). The minimum hemorrhagic dose of the κ-toxin was 1.4 μg. The hemorrhagic activity of the toxin was comparable to that of β-toxin of C. histolyticum, which in an amount of 1.6 μg produced a lesion of about 10 mm.

An amount of 1.4 μg of the purified toxin was equivalent to 1 κD when determined by the azocoll EP method, whereas 1.6 μg of β-toxin of C. histolyticum, releasing the dye from azocoll EP, was equivalent to 1 κD of the reference κ-toxin under the same conditions. The minimum amounts disintegrating collagen fiber were 0.7 μg and 0.8 μg, respectively, for κ-toxin and β-toxin of C. histolyticum when native collagen fiber was used as substrate (Kameyama and Akama, 1970). These facts showed that the purified κ-toxin was comparable to the enzyme of C. histolyticum in the collagenolytic activity.

Plate 1. Immunoelectrophoresis of the purified κ-toxin.

Immunoelectrophoresis was carried out in a 1% agarose plate in veronal buffer (pH 8.6, μ=0.05). The experimental conditions were described in the text.

A: Purified κ-toxin (2,623 κD/ml; 3.8 mg Prot./ml).
B: Partially purified κ-toxin from Sephadex G-100 1st (250 κD/ml).
C: Concentrated toxin (2,770 κD/ml).
Plate 2. Muscle destruction produced by \( \kappa \)-toxin.

Four hundred \( \mu \)g of purified \( \kappa \)-toxin was injected intramuscularly into the left thigh of the guinea pig and the observation was made after 24 hr.

Plate 3. Hemorrhage produced in the rabbit skin.

Two-tenths ml each of a serially diluted \( \kappa \)-toxin solution was injected intracutaneously and the reactions were observed after 24 hr (A). The \( \beta \)-toxin of \textit{C. histolyticum} was also injected for comparison (B).
The method presented here is suitable for the preparation of \( \kappa \)-toxin of high purity with a good yield. The toxin was free from such other toxins and enzymes probably contributing to the development of gas gangrene by \textit{C. perfringens} (Oakley, 1954; MacLennan, 1962). The toxin was heat-labile protein, being destroyed completely at temperatures above 60°C. The optimal pH was about 7.5 when azocoll EP was used as a substrate. The toxin was unstable in phosphate buffer as already pointed out by Bidwell and van Heyningen (1948). The molecular weight was approximately 80,000. A similar value was obtained with a crude culture filtrate. The fact may eliminate the possibility for polymerization during the purification. Hase \textit{et al.} (1967)* proposed a value of 12,000, but no adequate explanation is now available for the discrepancy.

Though the toxin was not completely homogeneous by ultracentrifugal analysis, the purity may be high enough to use for the studies on the biological activity and on the role of the toxin in experimental gas gangrene.

Various clostridial enzymes have been called "toxin" (MacLennan, 1953); some of them are not suitable for the name because of the absence of the evidence showing their ability to attack the living tissue. It may be reasonable to call collagenase of \textit{C. perfringens} a toxin, since it attacks healthy tissue with such a small amount as 1 µg or less (Fig. 9). Purified \( \kappa \)-toxin in 0.7 µg produced a definite hemorrhage on the rabbit skin. Bleeding from the capillary in the cutis was the only histological change recognizable within a short period after injection (unpublished data). The toxin in 30 µg killed mice with association of severe hemorrhage in the lungs. The biological activities of the toxin were comparable to those of \( \beta \)-toxin of \textit{C. histolyticum} on weight basis.

The pathological changes in the subcutaneous tissue of guinea pigs suggest that the toxin attacks the healthy connective tissue in the skin where collagen fibers constitute the main component. The primary site of attack in the muscle tissue may be the connective tissue supporting the muscle fibers, as described by Robb-Smith (1945). Destruction of the muscle with \( \kappa \)-toxin free from \( \alpha \)-toxin may be caused as a secondary event to the primary lesion at the reticulin supporting muscle fibers, as stated by Oakley, Warrack and van Heyningen (1946). However, this may not be the only cause of muscle damage in gas gangrene. Aikat and Dible (1956) stated that \( \alpha \)-toxin without collagenase activity damaged the muscle tissue. Strunk, Smith and Blumberg (1967) described the muscle damage caused by a purified \( \alpha \)-toxin, though the purity was not checked. We confirmed their observations with a highly purified \( \alpha \)-toxin (unpublished data). However, the appearance of the muscle damaged with \( \alpha \)-toxin was different from that destroyed with \( \kappa \)-toxin in the following points: (1) The grade of hemorrhage was less prominent, and (2) some of the network of connective tissue was still visible even with the naked eye. Further histological examinations with purified toxins are necessary to understand the whole process of the tissue damage in gas gangrene caused by \textit{C. perfringens}.

Although \( \kappa \)-toxin alone kills animals probably as a result of direct effect on the lungs, this may not be the main cause of death in the actual infection. In gas gangrene,

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various other factors should be taken into consideration to explain the causes of death of the infected animals. α-Toxin alone kills animals and the possibility for the production of secondary toxic substances were also suggested (Macfarlane, 1955; Oakley, 1954).

The results of the studies on the role of kappa toxin in the experimental gas gangrene will appear in a separate communication.

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