ALPHA TOXOID OF *CLOSTRIDIUM PERFRINGENS*

I. PURIFICATION AND TOXOIDING OF ALPHA TOXIN OF *C. PERFRINGENS*

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SUMMARY: α -Toxin of *Clostridium perfringens* was highly sensitive to formalin; its antigenicity was destroyed rapidly during toxoiding. It was succeeded in toxoiding α -toxin under a strict regulation of protein concentration, formalin concentration, constituents of the medium, temperature and incubation period.

The ratio of egg unit to mouse toxicity changed during toxoiding and a long incubation was required when the egg yolk reaction was used as a measure for the toxicity. The customary usage of the *in vitro* test may have been one of the reasons for the inconsistent antigenicities of α -toxoid in the past.

INTRODUCTION

Many attempts have been made to prepare toxoid from α -toxin of *Clostridium perfringens* (Penfold and Tolhurst, 1937, 1938; Penfold, Tolhurst and Wilson, 1941; Stewart, 1942; Robertson and Keppie, 1943; Adams, 1947; Tytell et al., 1947). Since the immunogenicity of the toxoid preparations was not always satisfactory, there have been suspicion that the antigenicity of the toxin might be easily destroyed by formalinization and that the immunizing potency of "perfringens toxoid" might reside in the undenatured toxin (MacLennan, 1962). A reliable method for preparing *C. perfringens* α -toxoid is now urgently required for prevention of gas gangrene as well as for production of the antitoxin. Since the immunogenicity of toxoid is greatly affected by various conditions during toxoiding, it seemed necessary to reinvestigate each of these conditions.

The present paper describes that α -toxoid with a satisfactorily high antigenicity can be produced consistently from a purified preparation of α -toxin if the conditions for toxoiding were controlled rigidly.

MATERIALS AND METHODS

Culture filtrate: *C. perfringens* PB6KN5 was grown for 4-6 hr in a peptone medium or in a papain-digest medium described by Murata et al. (1965). In some experiments, the peptone medium was added with 5 g per liter of cooked meat prepared by the method of Willis (1964). In the culture supernatant of the latter medium, no θ -toxin was detected. Cells were removed by continuous-flow centrifugation and then by filtration through a Seitz filter-pad EK (Toyo Roshi Co.).

Concentrated toxin: The sterile filtrate of a culture in the peptone medium was added with zinc chloride to 1%. After standing overnight at cold, the resulting precipitate was extracted with disodium phosphate (Hosoya, Deguchi and Kagabe,

1944). Most α -toxin recovered in the extract was precipitated with ammonium sulphate at 80% saturation. The precipitate was dialyzed against cold water and kept at -70°C until use. The concentrated toxin from the culture in the medium containing cooked meat was designated as WLMZN and the one from that containing no cooked meat as WLZN.

Purification of α -toxin: 1. Gel filtration: This was carried out by the method described by Ikezawa, Yamamoto and Murata (1964) with slight modifications. The concentrated toxin was dialyzed against 0.05 M tris buffer (pH 7.6) at cold and 50–100 ml of the toxin containing 300–500 Lv per ml was passed through a column of Sephadex G-100 (5.5 cm $\times$ 100 cm) equilibrated with tris buffer. The column was eluted with the same buffer and the effluent collected in 15-ml fractions. The fraction containing α -toxin were combined and the toxin was concentrated with ammonium sulphate at 80% saturation. The precipitate was dissolved in borate buffer (see below) or in tris buffer and dialyzed against the same buffer at cold. The toxin at this step was called WLMZNS or WLZNS, according to the source. When a highly purified toxin was necessary, the procedure was repeated on the material derived from the effluent containing not less than 100 Lv of α -toxin per ml. The purified toxin at this step will be called WLMZNSS or WLZNSS as before.

Borate buffer (BBS): The buffer consisted of 11.65 g H_3BO_3 , 1.34 g $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ and 2.75 g NaCl per liter (pH 7.1).

2. Acid precipitation: A concentrated preparation (WLZN) containing the toxin of about 300 Lv per ml was added with a saturated ammonium sulphate solution to 25% saturation and the precipitate, if any, was removed by filtration. The supernatant was then cooled to 2°C in a cold bath (0°C). The pH was then adjusted to 3.8 with N H_2SO_4 and the solution kept at cold with continuous stirring for 1 hr. The resulting precipitate was collected by centrifugation at cold, dissolved in a chilled phosphate buffer solution (M/10, pH 7.0) in no larger than a twentieth of the original volume and dialyzed against the same buffer at cold (called WLZNA). To prepare toxoid, the toxin purified by acid precipitation was dialyzed against borate buffer before formalinization.

Assay method: 1. α -Toxin was titrated usually by the lecithovitellin method and, if necessary, by intravenous injection into mice, as described by Murata et al. (1965). The toxin titer by the former method was expressed in terms of "egg unit" (EU) of van Heyningen (1941) and by the combining power, "Lv". One Lv was defined as the amount of α -toxin equivalent to 1 International Unit of the National Standard Gas Gangrene Antitoxin (*C. perfringens*). In the present investigation, Lv/5 determined by the method by Murata et al. (1965) was multiplied by 5. However, in this paper, the direct toxicity determined by the mouse method described below was expressed in "certainly lethal dose (CLD)". Each of serial dilutions of the toxin graded with 3-fold intervals was injected into two mice in 0.5-ml amounts. The smallest amount of the toxin killing two mice in 72 hr was called "CLD". One EU was equivalent to about 2–6 CLD.

2. The amount of α -toxoid was titrated by the lecithovitellin method with the National Standard Antitoxin and expressed in terms of the combining power (Seal and Stewart, 1941). The diluent and the egg yolk suspension were described by Murata et al. (1965). A stabilized toxin in glycerol saline (Murata et al., 1965) was used as an indicator toxin (test toxin). The test was carried out in 10 $\times$ 110-mm tubes. The procedures are shown in Table 1. One Lb was defined as an amount of toxoid com-

Table 1. An example of the determination of the combining power (Lb) of α -toxoid

Test series (ml)										
Toxoid sample	0.86	0.72	0.60	0.50	0.40	0.32	0.25	0.20	0.16	0.12
BBS containing 0.2% gelatin	0.14	0.28	0.40	0.50	0.60	0.68	0.75	0.80	0.84	0.88
Standard Antitoxin 0.8 u/ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Incubation for 2 hr at room temperature										
Indicator toxin	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Incubation for 1 hr at room temperature										
Egg yolk suspension	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Incubation for 2 hr at 37 C										
Results	+++	+++	+++	++	+	—	—	—	—	—
Control series (ml)										
Standard Antitoxin 0.4 u/ml		0.20	0.25	0.32	0.40	0.50	0.60	0.72	0.86	
BBS containing 0.2% gelatin		1.30	1.25	1.18	1.10	1.00	0.90	0.78	0.64	
Indicator toxin		0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
Incubation for 1 hr at room temperature										
Egg yolk suspension		0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
Incubation for 2 hr at 37 C										
Results		+++	+++	++	++	+	—	—	—	

+ indicates the grade of turbidity and — indicates no turbidity.

Calculation of the results. In the control series 0.5 ml of the indicator toxin shows an end-point reaction with 0.2 unit of antitoxin. In the test series a mixture of 0.5 ml of the indicator toxin and 0.4 ml of the toxoid shows the same reaction with 0.4 unit of antitoxin. Therefore, 0.4 ml of the toxoid sample has combined with $0.4 - 0.2 = 0.2$ unit of antitoxin, i. e. the toxoid sample is $0.2 \div 0.4 = 0.5$ Lb per ml.

binning one International Unit of the National Standard Antitoxin under the conditions described in Table 1.

3. θ -Toxin was titrated by the method described by Murata et al. (1968). The titer was expressed by θ -unit as the reciprocal of the highest dilution showing complete hemolysis.

4. κ -Toxin was titrated by the azocoll method of Kameyama, Akama and Murata (to be published) and the potency expressed in terms of κ -unit (κ U) relative to that of a stabilized reference toxin.

5. Hyaluronidase was tested only qualitatively by Di Ferrante's method (1956).

6. Detoxification tests of α -toxin.

a) By mouse injection: A 0.5-ml sample of a toxoid was injected intravenously into each of 5 mice (gpc/s) weighing about 15–19 g and the animals were observed for deaths daily for 3 days.

b) By guinea pig injection: A 2-ml of the sample was injected subcutaneously into each of at least 4 guinea pigs weighing about 400 g and the animals were observed for local reactions and deaths for 7 days.

c) By rabbit injection: A 0.2-ml portion of a serially diluted sample was injected intracutaneously into the back skin of at least 2 male rabbits. Development of necroses was observed for 2 days.

d) By the lectithovitellin method: The same method described above was used.

7) Immunological analysis.

For immunological analyses of the toxin and toxoid, the National Standard Antitoxin was used.

a) The gel-diffusion method by Ouchterlony (1953): Plates were prepared by pouring a 1.0% agar solution in 0.85% saline containing 1:10,000 merthiolate into Petri dishes to a depth of 2–3 mm. Circular wells (8 mm diameter) were made with

Fig. 1. Diagram showing the gel diffusion method combined with egg yolk reaction.

metal cylinders after the agar had solidified and were lined with a thin layer of agar. Plates with 5 to 7 wells were used. The distance between neighboring wells was 18 mm. After toxin or toxoid samples and the antitoxin were placed in the wells, the Petri dishes were kept at room temperature and examined daily for the development of precipitin lines.

b) The gel-diffusion method combined with the egg yolk reaction: The procedure was the same as that described above except that a half part of each Petri dish was poured with the egg yolk agar prepared by mixing a 1% agar solution in 5% saline containing 0.002 M CaCl_2 with a 1.5% egg yolk suspension (Fig. 1).

8. Determination of protein content: The protein contents of chromatographical fractions were estimated by the Cu-Folin method and expressed in bovine serum albumin (BSA) equivalent.

RESULTS

Purification of α -Toxin

Figure 2 shows the pattern of separation of the toxins by gel filtration. As will be seen, hyaluronidase and most θ - and κ -toxins were separated from α -toxin. The effluent was pooled in several fractions according to the toxin content. The immunological behaviors of such fractions are shown in Fig. 3 (a). The peak fraction of α -toxin showed a precipitation line distinct from that with θ - or κ -fraction and common with that with a highly purified toxin (WLZNSS). The line produced with α -toxin was identifiable by the gel diffusion technique combined with the egg yolk reaction, as illustrated in Fig. 3 (b). As will be seen, the egg yolk reaction was clearly inhibited at the precipitation line produced by the α -toxin. The toxin was concentrated for further experiments by precipitation with ammonium sulphate at 80% saturation.

Fig. 2. Separation of toxins of *Clostridium perfringens* by chromatography with G 100.

80 ml of concentrated toxin containing 300 Lv/ml was applied.

* μ -Toxin was eluted in tubes 32-46. Determination of the toxin was carried out only qualitatively.

Separation of α -toxin was more complete when gel filtration was repeated on a concentrated, pooled fraction of α -toxin (e.g. Fr. 55-65 in Fig. 2). Table 2 shows an example of the results of purification. The activities of θ - and κ -toxin in the α -toxin fraction were negligible when the fraction was diluted to the concentration used for examining of the biological activity and the immunogenicity, viz. 50 Lv of α -toxin per ml.

Figure 4 shows the immunological behaviour of a partially purified preparation of α -toxin by acid precipitation. Separation was not so satisfactory as above.

Table 3 summarizes the production of α -toxin by the two methods in a preparative scale. Since gel filtration was more satisfactory than acid precipitation both in yield and in purity of the resulting toxin preparation, most of the further experiments were carried out with toxin purified by gel filtration.

Fig. 3. Immunodiffusion reaction of various toxin fractions separated by chromatography.

- 0.1 ml/well
1. Antitoxin 200 u/ml 2. κ -Fraction 3. θ -Fraction 4. WLZN 200 Lv/ml 5. WLZNSS 200 Lv/ml 6. α -Fraction²⁾
- 0.1 ml/well
1. Antitoxin 250 u/ml 2. WLZN 250 Lv/ml 3. WLZNS 250 Lv/ml
- 1) Specimens were taken from a chromatography similar to Fig. 2.
- 2) 500 Lv/ml.

Table 2. Purification of α -toxin by chromatography on Sephadex G-100
Experiment A

Step	α -toxin		κ -toxin		θ -toxin	
	Specific activity Lv/mg PN ¹⁾	Recovery %	Specific activity κ U/mg PN ¹⁾	Recovery %	Specific activity θ U/mg PN ¹⁾	Recovery %
Culture filtrate WL 66-11	348	100	3,870	100	6,957	100
Concentrated toxin WLZN	593	68.8	3,102	32.4	9,481	55.0
Purified toxin WLZNS	1,786	35.9	13	0.02	3,657	3.6
Experiment B						
Concentrated toxin, WL 67-1~6 ZN ²⁾	893	100 ³⁾	2,571	100 ³⁾	18,285	100 ³⁾
Purified toxin WLZNS	2,676	86.6	17	0.19	1,096	14.8
Purified toxin WLZNSS	1,942	53.3	6	0.07	311	4.2

1) Protein nitrogen (PN) was determined by semi-micro Kjeldahl method on the materials precipitated with 5% trichloroacetic acid.

2) A pool of 6 batches of concentrated toxin.

3) Calculated from the concentrated toxin WLZN.

Fig. 4. Immunodiffusion reaction of α -toxin prepared by various methods.Table 3. Specific activities and recoveries of α -toxin preparations purified by various methods

Methods of purification	Toxin No.	Lv/mg PN ¹⁾	Recovery (%)
Concentration by ammonium sulphate	WL65-2~8ZN	162	100
	WL66-3ZN	257	100
Chromatography on Sephadex G 100	WL64-11~13ZNS	588	50.3
	WL64-14~15ZNS	1,415	85.8
	WL65-2~8ZNS	690	50.1
	WL66-3ZNS	864	60.6
	WL66-11ZNS	1,786	52.2
	WL67-1~6ZNS	2,676	86.6
Acid	WL64-6~8ZNA	218	32.8
precipitation	WL65-2~8ZNA	1) 200	62.2
		2) ND ²⁾	15.0

1) Protein nitrogen (PN) was determined by semi-micro Kjeldahl method on the materials precipitated with 5% trichloroacetic acid.

2) ND: Not determined.

Detoxification of α -Toxin in Culture Filtrate

Several batches of culture filtrate were added with formalin at 0.4% and incubated at 37 C. The rapidity with which the toxicity in terms of EU decreased varied greatly from one culture to another. For some cultures, it took more than 1 month as shown in Table 4. Further addition of formalin was sometimes necessary for complete disappearance of the egg yolk reaction. On such an occasion, the combining power was lost greatly.

Conditions for Toxoiding the Purified Toxin

Since preliminary experiments showed that the antigenicity of a purified α -toxin in terms of the combining power was destroyed rapidly by formalin, the effect of L-

Table 4. Detoxification of α -toxin in culture filtrates

Toxin	Medium	Toxicity		Amount of formalin	Incubation period in day ¹⁾	Combining power of toxoid Lb/ml
		EU/ml	Lv/ml			
WP 1	PD ²⁾	200	10	0.4%+0.6% ³⁾	14+21 ⁴⁾	5.5
WP 3-4	PD	170	10	0.4%+0.6%	5+11	5
WP 5-6	PD	200	—	0.4%+0.6%	4+9	5
WP 7	PD	170	—	1.0%	7	≪5
WL 23	Peptone	100	7	0.4%	5 ⁵⁾	4

- 1) Period required for detoxification.
- 2) Papain digest.
- 3) Formalin was added in two portions.
- 4) The figure after + shows the incubation period after the second addition of formalin.
- 5) Egg yolk reaction was positive though non-toxic for mice.

lysine on toxoid formation was investigated, as suggested by Linggood et al. (1963). Figure 5 (a) shows that when L-lysine was added to a concentration of 0.05 M, the *in vitro* antigenicity remained unchanged for 10 days, during which time the toxicity was lost completely, whereas the antigenicity decreased to less than 20% in a medium without L-lysine. The detoxified material containing 240 Lb/ml after incubation for 10 days did not cause any symptoms in mice or guinea pigs, nor did necrosis develop in rabbits.

Fig. 5. Effect of L-lysine concentration on toxoiding.
Toxin: WL65—2~10ZNS, Purity, 47.6 Lv/mg protein, diluted to contain 100 Lv/ml in BBS containing L-lysine as indicated.
Formalin concentration: 0.3%. Incubation temperature: 37 C.
(a) Change in the direct toxicity in EU/ml. (b) Change in the combining power in Lb/ml.

α -Toxin purified by acid precipitation was amenable to toxoiding in a similar way to that mentioned above.

Then the amount of formalin required to detoxify α -toxin in the presence of L-lysine was investigated. As shown in Fig. 6, the optimum concentration of formalin was about 0.2% for a purified toxin used at a concentration of 100 Lv/ml (about 1.4 mg protein per ml). Figure 7 shows that the speed of detoxification and the rate of decrease in the antigenicity varied with the concentration of formalin as well

Fig. 6. Effect of formalin concentration on toxoiding.

Toxin: WL66-1~3 ZNS, Purity, 70.5 Lv/mg protein, diluted to contain 100 Lv/ml.

L-Lysine; 0.05 M. Formalin was added as indicated.

Arrows ($\downarrow$) show the point of complete detoxification of the direct toxicity by egg yolk method.

*Still showed positive egg yolk reaction after 16 days' incubation.

Fig. 7. Effects of concentration of formalin and toxin on toxoiding.

Toxin: WL66-4ZNS, Purity, 94.3 Lv/mg protein.

Formalin concentration: (a) 0.4% (b) 0.3% (c) 0.2%.

○—○ 100 Lv/ml, ●—● 50 Lv/ml.

* %: Combining power in percentage to the starting material.

Other conditions: Refer to Fig. 6.

Fig. 8 Effect of temperature on toxoiding.
Toxin: WL 66 pZNS, Purity, 92.6 Lv/mg protein, dilute to contain 100 Lv/ml.
Formalin concentration: 0.3%.
Other conditions: Refer to Fig. 6.

as protein.

Figure 8 shows the effect of temperature on toxoiding. It appeared that both the speed of toxoiding and the loss of antigenicity were larger at 37 C than at 30 C.

Change in the Relation between the Toxicity in Mouse and Egg Unit during Toxoiding

During the above experiment, it was recognized that the change in the egg unit did not always run parallel with that of the toxicity in mouse. To confirm this, the toxicities were checked by both the methods frequently during toxoiding both the culture filtrate and the purified toxin. Before detoxification, 1 EU was always equivalent to 2-6 CLD. However, the ratio of CLD/EU changed by formalin as shown in Tables 5 and 6. The mouse toxicity dropped rapidly, while the egg yolk reaction was found positive for a longer period.

Table 5. Detoxication of α -toxin in a culture filtrate of *C. perfringens*

Day	0	1	2	3	4	5	7	8	9	11	14	17	21	25	29
Egg unit	160	54	62	40	35	30	26	22.5	18	9.2	6.8	4.2	4.4	—	1.6
CLD	540	18	18	18	<2										
Lb	8*	10	8	9.6	9.4	7.5	9.4	9.4	9.6	8.0	6.3	6.4	7.5	6.4	—

*Expressed in terms of Lv value. per ml

Table 6. Detoxification of a partially purified α -toxin

Day	0	1	2	3	4	5	7
Egg unit	4,654	1,568	1,004	160	72	30	10
CLD	21,644	>4,860	540	60	<2	<2	<2
Lb	365*	403	284	300	250	200	200

per ml

Toxin: WLM 68-2~3 ZNS, 78.7 Lv/mg protein.

*Expressed in terms of Lv value.

DISCUSSION

There have been pessimistic opinions as to the possibility of converting α -toxin to a toxoid, as reviewed by MacLennan (1962), though several reports supported the effectiveness of the toxoid preparations for human (Stewart, 1943; Adams, 1947; Tytell et al., 1947; Danielson, 1947). The main criticism against the α -toxoid was apparently directed to the inconsistency of the antigenicity of the toxoid preparations hitherto produced.

The experiments presented here show that α -toxin is easily destroyed by formalin and a strict control of the conditions during toxoiding is necessary to prevent the antigenicity from attenuation. Especially, the amount of formalin and incubation period should be cautiously adjusted in relation to the protein concentration (Fig. 7). The fact that such conditions influence the antigenicity of toxoid is known with other bacterial toxins (Glenny, 1931; Holt, 1951; Murata et al., 1952; Linggood et al., 1963). However, the present results show that α -toxin is more sensitive to formalin than other bacterial toxins. When a preparation of diphtheria toxin of 500-1,000 Lf per mgN was incubated for about 3 weeks with 0.3-0.4% of formalin, the destruction of the antigenicity was not so large as was the case with α -toxin of a similar purity. Such a lability of α -toxin to formalin may explain the inconsistent antigenicities of the toxoid preparations reported in previous reports. In many instances, formalin was added to the culture filtrates without careful consideration of the conditions stated above. It should be pointed out that culture media now available for toxin production by *C. perfringens* consist of meat digests or commercial peptone at a high concentration (van Heyningen, 1948; Adams and Hendee, 1945; Logan et al., 1945; Murata et al., 1956). With such media, longer incubation was necessary for toxoiding and during which time the attenuation of the antigenicity might have been unavoidable especially when the conditions were not controlled properly.

In addition, many workers used the egg yolk reaction as a measure of toxoiding, and the animal tests were carried out as the final confirmation. Therefore, on some occasions, the incubation period might have been prolonged than is necessary for toxoiding α -toxin, as illustrated in Tables 5 and 6. With unformalinized toxins, one egg unit was usually equivalent to 2-6 CLD irrespective of the source or purity of the toxin. As the toxoiding proceeded, the ratio of CLD/egg unit became smaller. The toxicity to mouse was lost rapidly while the positive egg yolk reaction persisted longer. On some occasion a specimen containing more than 30 EU/ml did not kill a mouse when 0.5 ml was injected intravenously (Tables 5 and 6). The rapidity with which the egg yolk reaction turned negative seems to be different depending on the

kind of culture filtrates. With crude culture filtrate containing nitrogen at a high concentration, it is apparent that the positive reaction remained longer and an incubation of more than 1 month was required to reach negative egg yolk reaction (Table 4). Further addition of formalin up to 1% was often required in the case of the culture filtrate derived from the papain-digest medium. Such a condition may be unfavorable for the immunogenicity even when the combining power was maintained, as shown with diphtheria toxoid (Murata et al., 1952). Therefore, it is desirable to use the mouse method to check detoxification to shorten the incubation period and to prevent the antigenicity from undue destruction.

The results in this report proved clearly that the combining power of α -toxoid was preserved if the purified toxin was toxoided under a strict control of the protein concentration, the constituents of the medium, formalin concentration, temperature and incubation period. The immunogenicity in animal will be reported in a future communication.

The method of purifying and concentrating the toxin reported here is suitable for large-scale production because of its simplicity in the procedure, high yields and reproducibility. In addition, the purity of the toxin was satisfactorily high for immunological use. Most of other toxic principles present in the culture filtrate was easily removed by a single step of gel filtration. Such a purified toxin as used here is not only useful for toxoid formation but also for the study of the biological activities as well as of the immunological significance of the toxin.

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