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Effect of Zinc and Calcium Ions on the Production of Alpha-Toxin and Proteases by *Clostridium perfringens*

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Clostridium perfringens produced at least three distinct proteases in a synthetic medium containing calcium. Two of them, thiol and ethylenediaminetetraacetic acid disodium salt-sensitive proteases, appeared at an early stage of growth, but the other one, perhaps being identical to the one produced in a calcium-deficient medium, appeared at a late stage. The production of these proteases depended on Ca^{2+} but not on Zn^{2+} in the medium. Alpha-toxin, perhaps being a zinc-containing metalloenzyme, was rather resistant to the proteases, but toxin, produced in a zinc-deficient medium or deprived of zinc with ethylenediaminetetraacetic acid disodium salt, was very sensitive. By adding Zn^{2+}, the toxin lacking zinc may have been converted to the zinc-containing metalloprotein that is resistant to proteases. This may explain why α-toxin activity increased progressively in a zinc-containing medium in spite of simultaneous production of potent proteases and why it disappeared rapidly in a zinc-deficient medium.

We have reported that zinc ion was indispensable for production of *Clostridium perfringens* alpha-toxin or phospholipase C (EC 3.1.4.3) in a synthetic medium (12, 19). Alpha-toxin was produced in parallel with bacterial growth in a Zn^{2+}-containing medium, but in a Zn^{2+}-deficient medium, a comparable amount of less active toxin protein was produced at an early stage of growth, but it disappeared rapidly. When Zn^{2+} was added to the culture or its cell-free culture supernatant fluid in an early stage, the protein became fully active and stable α-toxin (19).

It has been suggested that phospholipase C of *C. perfringens* is a Zn^{2+} metalloenzyme (5, 7, 19), but the structure-activity relationship of the enzyme or the function of Zn^{2+} has not been elucidated. We attempted isolation of the less active toxin protein from the supernatant fluid of culture in a Zn^{2+}-deficient medium, but were hampered by the instability of the protein. It seemed essential, therefore, to identify the factor(s) affecting the stability of the toxin protein.

This report states that *C. perfringens* type A strain PB6K produces several distinct proteases that destroy α-toxin produced in a Zn^{2+}-deficient medium. Among these, both thiol and ethylenediaminetetraacetic acid disodium salt (EDTA)-sensitive proteases, produced in a Ca^{2+}-containing medium from an early stage of bacterial growth, caused rapid destruction of α-toxin produced in a Zn^{2+}-deficient medium. Alpha-toxin without Zn^{2+} is highly sensitive to proteases, but resistant when combined with Zn^{2+}. Unstable α-toxin produced in a Zn^{2+}-deficient medium is converted to stable Zn^{2+}-containing α-toxin upon addition of Zn^{2+} and becomes resistant to proteases.

MATERIALS AND METHODS

Organism. *C. perfringens* type A strain PB6K N5 was maintained in a synthetic medium, SM 67 (11), deprived of Mn^{2+}. This medium contained such divalent cations as Mg^{2+}, Fe^{2+}, Ca^{2+}, and Zn^{2+} at 1.0, 0.025, 0.5, and 0.05 mM, respectively, and 1 mM thioglycolic acid.

Culture methods. To examine the effect of divalent cations on the production of α-toxin and proteases, the medium deprived of Zn^{2+} and Ca^{2+}, both of which were not essential for growth, was used as the basal medium (BM). The seed culture was grown overnight in BM after two to three daily transfers in the same medium. The grown cells were centrifuged and resuspended in BM, which was distributed into test tubes and cultured at 37°C. Three other media containing Ca^{2+}, Zn^{2+}, or both were used; they are named BM + Ca^{2+}, BM + Zn^{2+}, or BM + Ca^{2+} + Zn^{2+}, respectively. The concentrations of Ca^{2+} and Zn^{2+} in these media were the same as those in SM 67.

Bacterial growth was determined by measuring optical density at 660 nm with a 10-mm light path.

Purified α-toxin. Alpha-toxin was purified from the supernatant fluid of culture in a peptone medium by Sephadex G-150 gel filtration and starch block electrophoresis as reported previously (18). The specific activity of the toxin was 500 Lv or 7,500 egg units (EU) per mg of protein.

Zn^{2+}-less α-toxin. Supernatant fluid of a culture grown for 3 h in BM was used as Zn^{2+}-less α-toxin to examine the effect of proteases and Zn^{2+}. Alpha-toxin

activity was 14 EU/ml or 0.68 Lv/ml; protease activity was undetectable. It was kept at −70°C until used.

EDTA-treated α-toxin. To a 0.5-ml portion of purified α-toxin (40 EU/ml), 0.02 ml of 10 mM EDTA was added, and the mixture was incubated for 10 min at 37°C. By this treatment, the toxin activity decreased to 25%, but was restored almost completely by adding Zn^{2+} or Co^{2+} and partially by adding Mn^{2+}.

Assay for α-toxin. Phospholipase C activity was measured by the lecithovitellin method and expressed in EU or Lv as reported previously (13). One EU corresponded to three mouse 50% lethal doses.

Assay for protease activities. Two substrates, casein (Merck & Co., Inc.) and azocasein (Sigma Chemical Co.), were used.

Casein-hydrolyzing activity. To an appropriate volume of enzyme, 0.25 ml of 4% heat-denatured casein in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.0) was added, and the mixture was made to 0.5 ml with 0.1 M Tris-hydrochloride buffer (pH 7.5). After incubation for 2 h at 37°C, 0.5 ml of 8% trichloroacetic acid was added to the reaction mixture. After centrifugation (2,600 × *g* for 30 min), absorbance of the supernatant fluid was measured at 280 nm with a 1-cm light path in a Shimazu D-40 spectrophotometer. One casein unit (1 CU) is the amount of the enzyme producing an absorbance increase of 0.01. A reaction mixture without substrate was incubated and added with 8% trichloroacetic acid and then with substrates, which served as the blank.

Azocasein-hydrolyzing activity. To an appropriate volume of enzyme, 0.1 ml of 2.5% azocasein in 0.1 M Tris-hydrochloride buffer (pH 7.5) was added, and the mixture was made to 0.5 ml with the same buffer. After incubation for 2 h at 37°C, 0.5 ml of 8% trichloroacetic acid was added to the reaction mixture and the precipitate was removed by centrifugation. To 0.5 ml of the supernatant fluid, 0.5 ml of 0.5 N NaOH was added, and absorbance at 440 nm was measured. One azocasein unit (1 ACU) is the amount of enzyme producing an absorbance increase of 0.01 under the specified conditions. The blank was prepared by the same method as that for determining casein-hydrolyzing activity.

Proteases. Besides the proteases produced by *C. perfringens*, four other proteases were used; papain (2× crystal), trypsin (2× crystal), chymotrypsin, and subtilisin BPN′ (type VII) were purchased from Sigma, and 20 ng of each protease corresponded to 2.0, 1.4, 0.4, and 4.0 CU, respectively, under the conditions described above. Papain was used after activation by 5 mM cysteine.

RESULTS

Production of α-toxin in Zn^{2+}-deficient media. Alpha-toxin production paralleled the bacterial growth in BM + Ca^{2+} + Zn^{2+}. In BM + Ca^{2+} and BM, toxin activity appeared at an early stage of growth and disappeared with growth; the toxin activity disappeared more rapidly in BM + Ca^{2+} than in BM (Fig. 1). To clarify the cause of the disappearance of α-toxin, the culture supernatant fluids of BM + Ca^{2+} and

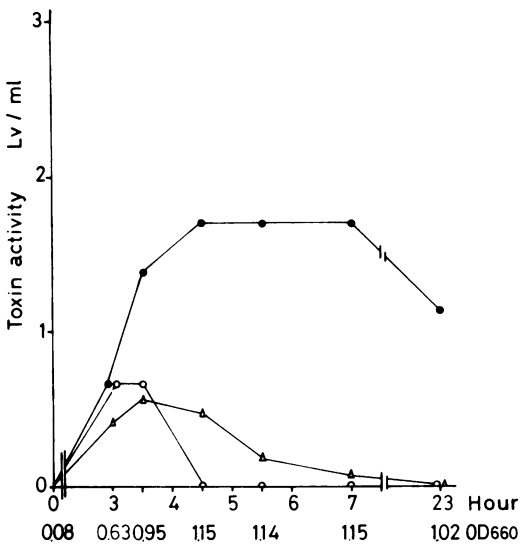

FIG. 1. *Production of α-toxin in Zn^{2+}-deficient media. Bottom numbers denote OD_{660} values (optical density at 660 nm) during growth in BM + Ca^{2+} + Zn^{2+}. Symbols:* △, *BM;* ○, *BM + Ca^{2+};* ●, *BM + Ca^{2+} + Zn^{2+}.*

BM were examined for a substance(s) inactivating α-toxin. The culture supernatant fluid was divided into two 1.52-ml portions. To each group, 0.08 ml of 0.05 M Tris-hydrochloride buffer (pH 7.5) or 1 mM Zn^{2+} was added. The concentration of Zn^{2+} in group II was 0.05 mM, being the same as that in BM + Ca^{2+} + Zn^{2+}; there was no group II for the culture supernatant fluid of BM + Ca^{2+} + Zn^{2+}. Each group was further divided into three subgroups, A (1.2 ml), B (0.15 ml), and C (0.15 ml). To B and C, 0.05 ml of 0.05 M Tris-hydrochloride buffer (pH 7.5) and the supernatant fluid of a 6-h culture of BM + Ca^{2+} were added, respectively. The toxin activity of each group was determined before (A) and after (B and C) incubation for 30 min at 37°C. The toxin activity of A was measured by both EU and Lv and the others only by EU. Lv values of A in group I are plotted in Fig. 1. The supernatant fluid of a 6-h culture of BM + Ca^{2+} did not show any toxin activity even if Zn^{2+} was added.

As shown in Table 1, addition of Zn^{2+} to supernatant fluid of each culture in Zn^{2+}-deficient media resulted in increased toxin activity to the same level as that in BM + Ca^{2+} + Zn^{2+} (A of group II). The toxin activity in BM + Ca^{2+} disappeared after incubation without any supplement, but that in BM did not (B of group I). In BM, toxin was inactivated only when the supernatant fluid of BM + Ca^{2+} was added to group I (C of group I). In the presence of Zn^{2+},

the toxin activity did not decrease (BM + Ca^{2+} + Zn^{2+} and group II).

These results suggest the presence of some factors(s) destroying the toxin protein in culture in BM + Ca^{2+}, but of little or no such factor(s) in BM. The factor(s) was inactivated by treating with *p*-chloromercuribenzoate or by heating (20). It seemed that some proteases produced in BM + Ca^{2+} had inactivated the toxin and that such was not the case in the presence of Zn^{2+}.

Relationship between Zn^{2+} and Ca^{2+} in production of α-toxin and proteases. Table 2 shows the activities of α-toxin and protease in supernatant fluids of 6-h and 24-h cultures in the four media. Protease activity was measured with both casein and azocasein after gel filtration of the supernatant fluid on Sephadex G-25 to remove low molecular substances absorbing light at 280 nm. A 10-ml portion of the supernatant fluid was concentrated to 1 ml with Ficoll and applied on a column (1.5 by 25 cm) of Sephadex G-25 equilibrated with 0.05 M Tris-hydrochloride buffer containing 0.1 M NaCl (pH 7.5). The excluded fractions were pooled; the protease activity was assayed and expressed in units per milliliter (CU/ml and ACU/ml) of the

TABLE 1. *Effects of addition of supernatant of 6-h culture in BM + Ca^{2+} on the activity of α-toxin produced in the three media*[a]

Medium	OD_{660}[c]	Toxin activity (EU/ml)[b]					
		Group I[d]			Group II[d]		
		(A)[e]	(B)[e]	(C)[e]	(A)[e]	(B)[e]	(C)[e]
BM	0.60	8	7	0	17	16	28
	0.85	18	17	0	32	32	40
	1.05	16	13	0	36	32	32
	1.15	9	8	0	18	18	21
	1.20	2	ND[f]	ND	ND	ND	ND
	0.70	0	ND	ND	ND	ND	ND
BM + Ca^{2+}	0.64	11	0	0	12	12	18
	0.90	13	0	0	32	32	40
	1.15	0	ND	ND	0	ND	ND
	1.20	0	ND	ND	0	ND	ND
	1.20	0	ND	ND	ND	ND	ND
	0.67	0	ND	ND	ND	ND	ND
BM + Ca^{2+} + Zn^{2+}	0.63	18	20	28			
	0.95	32	31	40			
	1.15	37	29	40			
	1.14	28	23	26			
	1.15	34	28	42			
	1.02	30	ND	ND			

[a] The activity of A of group I is delineated in Lv/ml in Fig. 1.
[b] EU/ml of original culture supernatant.
[c] OD_{660}, Optical density at 660 nm.
[d] Culture supernatant (1.52 ml) was supplemented with 0.08 ml of 0.05 M Tris-hydrochloride buffer, pH 7.5 (group I), or 1 mM Zn^{2+} (group II).
[e] Toxin activity of each group was determined before (A) and after (B and C) incubation for 30 min at 37°C with (C) or without (B) supernatant of 6-h culture in BM + Ca^{2+}.
[f] ND, Not determined.

TABLE 2. *Effects of Ca^{2+} and Zn^{2+} on the production of α-toxin and proteases*

Medium	Incubation period (h)	OD_{660}[a]	α-Toxin		Protease	
			EU/ml	Lv/ml	ACU/ml	CU/ml
BM	6	1.12	5.0	0.29	3	14
	24	0.39	0.6	<0.02	13	150
BM + Ca^{2+}	6	1.15	<0.5	<0.02	134	107
	24	0.58	<0.5	<0.02	241	303
BM + Zn^{2+}	6	1.10	23.0	0.96	4	16
	24	0.74	22.0	1.38	8	106
BM + Ca^{2+} + Zn^{2+}	6	1.15	32.0	1.12	161	147
	24	0.83	7.6	0.47	207	221

[a] OD_{660}, Optical density at 660 nm.

original culture supernatant fluid.

In Ca^{2+}-containing media, regardless of the presence of Zn^{2+}, high protease activities, both CU and ACU, were observed within 6 h, when little protease activity was detected in Ca^{2+}-deficient media. In overnight cultures, a considerable casein-hydrolyzing activity was detected in both media. Azocasein-hydrolyzing activity was, however, hardly detected in the latter media.

These results show that production of protease, especially azocasein-hydrolyzing protease, depends on the presence of Ca^{2+} but not on that of Zn^{2+}, which is important for production of α-toxin.

Alpha-toxin activity disappeared completely in 6 h in BM + Ca^{2+} but not in BM + Ca^{2+} + Zn^{2+}, in spite of the production of protease to an activity equal to that in the former medium. The result may suggest that the disappearance of α-toxin at an early stage of growth in the former medium was caused mainly by the azocasein-hydrolyzing protease, but it did not destroy α-toxin in the latter medium.

To ascertain this interpretation, effects of Ca^{2+} on the production of α-toxin and protease in Zn^{2+}-deficient media were examined. Figure 2 shows production of α-toxin and of azocasein-hydrolyzing protease. In BM + Ca^{2+}, α-toxin activity reached the peak in 3 h of cultivation and disappeared rapidly, while the protease increased with incubation period. In BM, on the other hand, the protease was not detected at any stage of growth and toxin activity declined gradually after 3 h. When Ca^{2+} was added to the culture in BM during the logarithmic growth phase, however, the protease was produced immediately and toxin activity steeply decreased. Neither α-toxin nor protease activity was altered by addition of Ca^{2+} to the supernatant fluid of the culture in BM.

In Zn^{2+}-containing media, the protease was produced to an extent similar to that produced in the Zn^{2+}-deficient media; namely, Zn^{2+} inhibited neither production nor activity of the protease (data not shown). Alpha-toxin production was nevertheless not repressed by the protease in the Zn^{2+}-containing media. These results evidenced the above suggestion.

Some properties and effects of the proteases produced in BM + Ca^{2+} on α-toxin activity. Proteases produced in BM + Ca^{2+} in 6 and 24 h were fractionated by gel filtration on Sephadex G-150. The supernatant fluid (200 ml) of the 6-h culture was concentrated to 2.5 ml with a Sartorius Membranfilter (SM 13200). The supernatant fluid (180 ml) of an overnight culture was concentrated with ammonium sulfate at 80% saturation, and the precipitate was dissolved in 2.8 ml of 0.05 M Tris-hydrochloride buffer containing 0.1 M NaCl and 1.0 mM thioglycolic acid (pH 7.5). These samples were each applied on a Sephadex G-150 column, equilibrated with 0.05 M Tris-hydrochloride buffer containing 0.1 M NaCl and eluted with the same buffer.

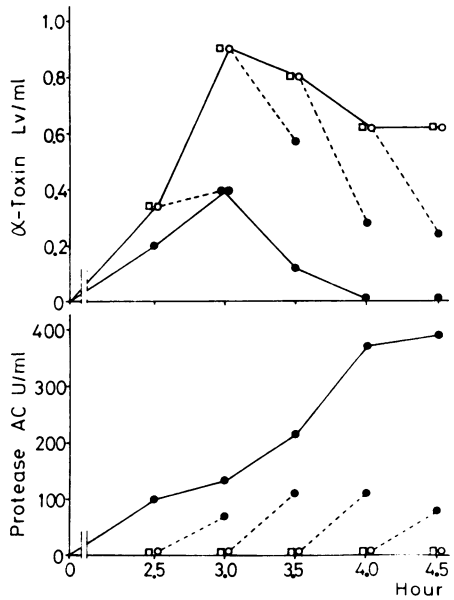

FIG. 2. *Effects of Ca^{2+} on production of protease and α-toxin. The concentration of Ca^{2+} was 0.5 mM. Symbols:* O——O, *BM;* ●——●, *BM + Ca^{2+};* □, *activity in BM determined after addition of Ca^{2+};* O----●, *activity in BM determined after cultivation with Ca^{2+} for 30 min.*

Figure 3 shows the elution profiles of the proteases obtained from 6-h (A) and 24-h (B) cultures. Protease activity was shown by both CU and ACU. With the 6-h culture, little protease was eluted in the void volume and a considerable quantity was eluted in later fractions; with the 24-h culture, a large quantity of protease was eluted in the void volume.

To examine sensitivities of these proteases to some reagents, the effluent was divided into four and three fractions in A and B, respectively, and each was pooled. After preincubation of 0.2 ml of each fraction with 0.05 ml of each reagent for 10 min at 37°C, protease activity was measured with casein (Table 3). From these results, at least three proteases were differentiated: a thiol protease in $A_{62\text{-}80}$ and $B_{62\text{-}73}$, an EDTA-sensitive protease in $A_{39\text{-}49}$, $A_{50\text{-}61}$, and $B_{50\text{-}61}$, and a protease insensitive to iodoacetamide and EDTA in $B_{34\text{-}49}$. Zn^{2+} at 0.1 mM had no effect on activity of any protease, but Ca^{2+} at 1 mM activated the EDTA-sensitive protease. Protease activity in

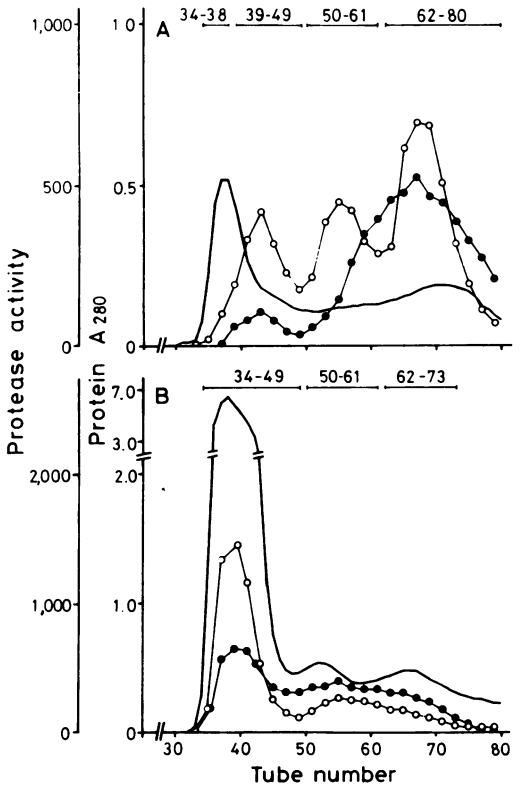

FIG. 3. *Gel filtration on Sephadex G-150 of protease in 6-h (A) and 24-h (B) cultures in BM + Ca^{2+}. Concentrated culture supernatant was applied on a Sephadex G-150 column (1.6 by 80 cm) equilibrated with 0.05 M Tris-hydrochloride buffer containing 0.1 M NaCl, pH 7.5, and eluted with the same buffer. The flow rate was 8 ml/h, and 1.5-ml fractions were collected. Proteases were divided in four and three fractions in A and B, respectively, and each was pooled. Symbols: ——, protein; ●, protease (CU); ○, protease (ACU).*

A_{50-61} was completely inhibited with 1 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid (EGTA), but not with EDTA monocalcium salt (EDTA-Ca) (data not shown). The thiol protease tended to be inactivated on storage, but it was reactivated with cysteine.

Effects of the proteases fractionated as shown in Fig. 3 on Zn^{2+}-less α-toxin with or without added Zn^{2+} were examined (Table 4). The supernatant fluid of a culture grown for 3 h in BM (0.7 ml) was incubated with or without 1 mM Zn^{2+} (0.05 ml) for 5 min at 37°C, and then added with protease and made to 1 ml with 0.05 M Tris-hydrochloride buffer. After incubation for 2 h at 37°C, toxin activity was determined. Every protease derived from 6-h and 24-h cultures in BM + Ca^{2+} inactivated Zn^{2+}-less α-toxin in the absence of Zn^{2+} (Table 4). In the presence of Zn^{2+}, however, the toxin was not inactivated by any protease. The lost activity of Zn^{2+}-less α-toxin was not restored by adding Zn^{2+}. These results suggest that Zn^{2+}-less α-toxin is sensitive to any protease produced in BM + Ca^{2+}, but becomes resistant to the proteases by binding Zn^{2+}.

Comparison of proteases produced in four media. Proteases were fractionated by gel filtration of the supernatant fluid of the 24-h cultures in each medium. The protein in the supernatant fluid (100 ml) of the overnight culture was precipitated with ammonium sulfate at 80% saturation. The precipitate was dissolved in 1.0 ml of 0.05 M Tris-hydrochloride buffer (pH 7.5) containing 1.0 mM thioglycolic acid and applied on a Sephadex G-150 column equilibrated with the same buffer. Figure 4 shows

TABLE 3. *Sensitivities of each protease to some reagents*

Protease fraction	Protease activity (%)[a]				
	None	2 mM IAA	1 mM EDTA	0.1 mM Zn^{2+}	1 mM Ca^{2+}
A_{34-38}	100 (30)	87	38	102	144
A_{39-49}	100 (90)	105	14	113	164
A_{50-61}	100 (81)	73	2	114	210
A_{62-80}	100 (72)	8	128	110	92
B_{34-49}	100 (77)	86	83	109	117
B_{50-61}	100 (45)	85	60	101	163
B_{62-73}	100 (39)	0	79	109	93

[a] Numbers in parentheses show original activities (CU/ml).

TABLE 4. *Effects of proteases produced in BM + Ca^{2+} on Zn^{2+}-less α-toxin*

Protease	CU[a]	Zn^{2+}[b]	Toxin activity (%)[c]
None		−	100
		+	129
A_{39-49}	11	−	10
		+	148
A_{50-61}	10	−	10
		+	148
A_{62-80}	8	−	<10
		+	157
B_{34-49}	9	−	10
		+	152
B_{50-61}	6	−	10
		+	152
B_{62-73}	8	−	21
		+	162

[a] Activity in CU added to Zn^{2+}-less α-toxin (0.7 ml).
[b] Zn^{2+}-less α-toxin was preincubated for 5 min at 37°C with (+) or without (−) Zn^{2+} (0.05 mM) before addition of protease.
[c] Toxin activity (EU/ml) was determined after incubation for 2 h at 37°C and expressed as the percentage of the activity of nontreated Zn^{2+}-less α-toxin.

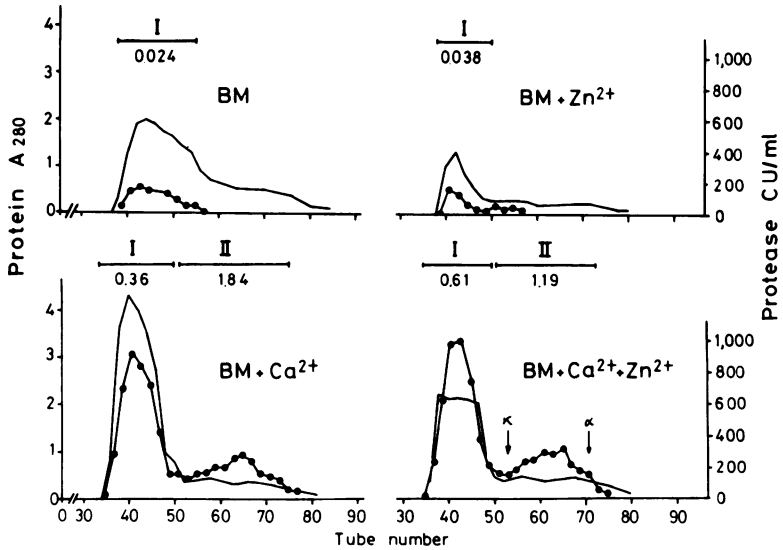

FIG. 4. *Sephadex G-150 gel filtration of proteases produced in four media. Concentrated culture supernatant (1.0 ml) was applied to a Sephadex G-150 column (1.6 by 80 cm) equilibrated with 0.05 M Tris-hydrochloride buffer containing 0.1 M NaCl and 1 mM thioglycolic acid, pH 7.5, and eluted with the same buffer. The flow rate was 8 ml/h, and 1.5-ml fractions were collected. Pooled protease fractions were named proteases I and II according to the order of elution. The numbers given under the bars are the ACU to CU ratios. Elution positions of κ- and α-toxins are shown by arrows. Symbols: ——, protein; ●, protease (CU/ml).*

elution profiles of the proteases from the four media. The activity was assayed with casein as the substrate. These proteases were roughly divided into two fractions, proteases I and II, according to the order of elution. Elution positions of α-toxin and of κ-toxin are shown by arrows. In Ca^{2+}-containing media with or without Zn^{2+}, both proteases I and II were produced. Protease II, containing the EDTA-sensitive and thiol proteases, was not produced in Ca^{2+}-deficient media. These proteases were pooled separately, and the activity was assayed with casein and azocasein in the presence of 1.0 mM cysteine. Although both proteases I and II derived from Ca^{2+}-containing media hydrolyzed azocasein, protease I from Ca^{2+}-deficient media hardly did so. The ratio of ACU to CU of the pooled protease fraction is also shown in this figure.

The effect of the proteases fractionated as shown in Fig. 4 on Zn^{2+}-less α-toxin with or without added Zn^{2+} was examined. The same result as that shown in Table 4 was obtained; every protease inactivated Zn^{2+}-less α-toxin in the absence of Zn^{2+}, but not in the presence of Zn^{2+}. Zn^{2+}-less α-toxin was more sensitive to the protease originating from Ca^{2+}-containing media than to that from Ca^{2+}-deficient media.

Effects of several proteases on α-toxin with or without Zn^{2+}. Some commercial proteases, as well as protease from *C. perfringens*, were examined for their effects on Zn^{2+}-less α-toxin (Table 5). Similar results were obtained with such proteases as papain, trypsin, chymotrypsin, and subtilisin. These results show that Zn^{2+}-less α-toxin is sensitive to the endogenous and exogenous proteases, but resistant to any of these in the presence of Zn^{2+}.

The sensitivity of purified α-toxin, native and EDTA-treated, to various proteases was examined. Purified native α-toxin contained Zn^{2+}, but lost the functional Zn^{2+} by treatment with EDTA (Table 6) (19). EDTA-treated α-toxin was obtained by adding 0.02 ml of 10 mM EDTA to a 0.5-ml portion of purified α-toxin (40 EU/ml) and incubating it for 10 min at 37°C. Each protease was added to purified native α-toxin (0.5 ml) or EDTA-treated α-toxin (0.52 ml), and the total volume was made to 1 ml with 0.05 M Tris-hydrochloride buffer containing 0.1 M NaCl (pH 7.5). After incubation for 30 min at 37°C, toxin activity was measured. In the case of EDTA-treated α-toxin, toxin activity after incubation with 0.03 ml of 10 mM Zn^{2+} for an additional 5 min was also measured. Toxin activity was expressed as the percentage of that of native toxin. Native toxin was little inactivated by any protease.

EDTA-treated toxin, containing an activity of 25% the original that would be reactivated to 93% by addition of Zn^{2+}, was destroyed by any protease to such an extent that its activity was no longer reactivated with Zn^{2+}. When Zn^{2+} was

TABLE 5. *Effects of various proteases on Zn^{2+}-less α-toxin*

Protease	CU[a]	Zn^{2+}[b]	Toxin activity (%)[c]
None		−	100
		+	100
Papain	2.0	−	0
	2.0	+	100
	10.0	+	100
Trypsin	1.4	−	0
	1.4	+	100
	7.0	+	100
Chymotrypsin	0.4	−	0
	0.4	+	100
	2.0	+	100
Subtilisin	4.0	−	0
	4.0	+	100
	20.0	+	100

[a, b, c] See corresponding footnotes to Table 4.

TABLE 6. *Effects of various proteases on native and EDTA-treated purified α-toxin*

Protease	CU	Toxin activity (%)[a]		
		Native toxin	EDTA-treated toxin	
			Without Zn^{2+}	With Zn^{2+}[b]
None		100	25	93
BM + Ca^{2+} I	25.0	100	1	7
BM + Ca^{2+} II	11.0	100	2	3
Papain	2.0	83	0	0
Trypsin	1.4	92	0	12
Chymotrypsin	0.4	100	0	12
Subtilisin	4.0	92	0	0

[a] Toxin activity (EU) was determined after incubation for 30 min at 37°C and expressed as the percentage of that of nontreated native toxin.

[b] Zn^{2+} was added after incubation with each protease.

added to EDTA-treated α-toxin, toxin activity did not decrease when treated with any protease (data not shown).

These results indicate that purified α-toxin possessing Zn^{2+} is rather resistant to various proteases.

DISCUSSION

There have been many reports (1, 3, 6, 8, 10, 17) dealing with various microbial proteases, but little information (5, 15) is available on proteases of *C. perfringens* type A. Therefore, workers have inconsistent views and opinions regarding production, properties, and roles of the proteases produced by this organism.

We found that this organism produces at least three distinct proteases, thiol, EDTA-sensitive, and other proteases, in Ca^{2+}-containing medium. It is conceivable that the EDTA-sensitive protease is Ca^{2+} dependent since its activity is increased with Ca^{2+} and inhibited with EDTA or EGTA, but not with EDTA-Ca. In a preliminary experiment, the EDTA-sensitive protease was inhibited also with 0.1 mM diisopropyl fluorophosphate. This observation may suggest that the fraction contained two or more distinct proteases or such a protease that was inhibited with either EDTA or diisopropyl fluorophosphate. The direct role of Ca^{2+} in production or action of the thiol protease is still not known. Nevertheless, not only the EDTA-sensitive protease but also the thiol protease were detected only when Ca^{2+} was present in the medium. Calcium ion may stabilize or activate the proteases, as has been shown for many other proteases (1, 6, 8–10). The thiol protease and EDTA-sensitive protease of *Clostridium* have been reported in several strains (9, 15). Clostripain (clostridiopeptidase B [EC 3.4.22.8]), a thiol protease produced by *C. histolyticum*, has been studied in detail (9). Calcium is required for activity and stability of this enzyme. In addition to the thiol protease, this organism produced a protease(s) that was not activated with cysteine, but activated with Ca^{2+} (9). Ohishi et al. (15) have reported two distinct thiol proteases from an overnight culture of *C. perfringens*. Since none of the *C. perfringens* proteases has been purified, we can not discuss their chemical relationship.

The extracellular thiol and EDTA-sensitive proteases were the main proteases produced at an early growth stage, but the other one having the largest molecular size was produced at a late stage. The last protease(s) produced in the presence of Ca^{2+} hydrolyzed both casein and azocasein, whereas that produced in Ca^{2+}-deficient media did not hydrolyze azocasein even in the presence of Ca^{2+}. These results suggest that a protease hydrolyzing casein but not azocasein is produced in the presence or absence of Ca^{2+} and another protease hydrolyzing both casein and azocasein is produced in the Ca^{2+}-containing medium. These proteases having a large molecular size are produced at a late growth stage and are sensitive to neither iodoacetamide nor EDTA.

It is clear that the two proteases, thiol and EDTA-sensitive proteases, are the main factors responsible for the rapid disappearance of α-toxin in BM + Ca^{2+}. In BM + Ca^{2+} + Zn^{2+}, however, α-toxin is not destroyed by the same protease. Theta-toxin was also rather sensitive to the proteases produced in Ca^{2+}-containing medium. In the case of θ-toxin, the activity disappeared in BM + Ca^{2+} + Zn^{2+} as well as in BM + Ca^{2+}, as reported previously (12, 21, 22).

Kappa-toxin required both Zn^{2+} and Ca^{2+} (12, 22) and was resistant to the endogenous proteases. Protease produced in BM also inactivated Zn^{2+}-less α-toxin, but its activity was lower than that produced in BM + Ca^{2+}. It is unknown whether the protease is the cause of the slow decrease of the toxin in BM.

It has been suggested but not proved that α-toxin is a zinc-metalloenzyme (5, 7, 19). Our previous studies (19) and this paper suggest strongly that α-toxin is a zinc-metalloenzyme. Alpha-toxin containing no Zn^{2+} is most sensitive to various proteases, but that combined with Zn^{2+} is rather resistant to any protease. Zinc ion not only activates Zn^{2+}-less α-toxin and EDTA-treated toxin, but also arrests inactivation of them by protease. In other words, this cation is required for both activation and stabilization of α-toxin, as is the case in many other metalloenzymes (2, 4, 16, 23–27). It is conceivable that Zn^{2+} makes the toxin molecule rigid and stable without much conformational change.

Alpha-toxin activity lost by EDTA treatment was restored with Zn^{2+} or Co^{2+} almost completely and partially with Mn^{2+} but not with Ca^{2+} or Mg^{2+}. The effects of Co^{2+} and Mn^{2+} on the sensitivity of Zn^{2+}-less α-toxin to the protease produced in BM + Ca^{2+} and to some commercial proteases were compared with those of Zn^{2+}. Cobalt did not show such an effect as did Zn^{2+}; the Zn^{2+}-less α-toxin was destroyed completely even in 0.05 mM Co^{2+}. Manganese (0.05 mM) slightly arrested the disappearance of the toxin activity (data for Co^{2+} and Mn^{2+} not presented).

It was proved that zinc ion plays an important role in both the activity and stability of α-toxin. Therefore, studies on effects of Zn^{2+} and other related cations on various biological activities of α-toxin will certainly give a useful clue to resolution of the structure-activity relationship of the toxin.

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