ROLE OF PROTEOLYTIC ENZYME IN TOXIN PRODUCTION BY *CLOSTRIDIUM BOTULINUM* TYPE A

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INTRODUCTORY

It is now well known that the toxin of *Clostridium botulinum* type E can be altered by some proteolytic enzyme so that its activity is increased. On the other hand, attempts to activate types A and B toxins in the same manner have been unsuccessful. More recently, however, BONVENTRE and KEMPE observed that the toxicity of young cell extract of type A or B organisms was much enhanced by treatment with trypsin, and they suggested the presence of so called potential toxin or protoxin in these extracts.

In the previous work,[9] the present author also confirmed the presence of type A protoxin and observed that culture filtrates of the organisms contained a certain factor which enhances the toxicity of the protoxin preparation. Besides, it was emphasized that this factor would be an extracellular proteolytic enzyme produced by the organisms. Therefore, the present investigation was undertaken to isolate the proteolytic enzyme (s) from the culure and to obtain experimental evidence which would support its participation in toxin production in the culture.

MATERIALS AND METHODS

The strain used in this investigation was *Clostridium botulinum* type A No. 38. The media employed for cultivation of the organisms, titration of toxin, and measurement of proteolytic activity have already been described[8,9]. Procedure for chromatography on DEAE-cellulose column followed that of SOBER et al. The protoxin preparation available to investigate the activation capacity of test fluids was prepared from young cells of *Cl. botulinum* type A No. 38 in the same manner described in the previous report[9]. Activation of the protoxin preparation was carried out under the condition of pH 6.0, 37°C, 90 min.

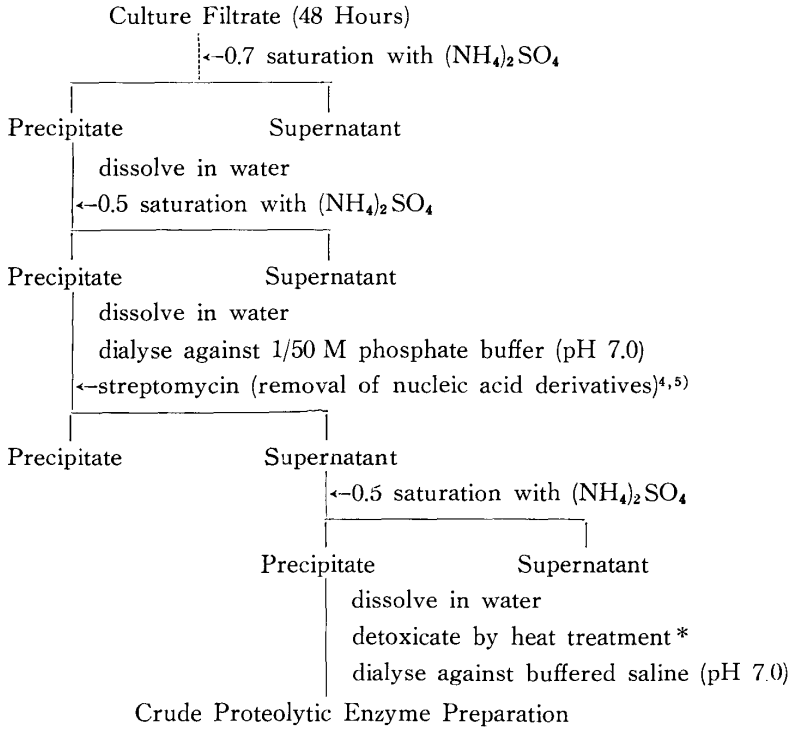
EXPERIMENTS AND RESULTS

Preparation of crude proteolytic enzyme

This work was communicated in part at the 8th Symposium for Bacterial Toxins on July, 1961 in Koyasan.

Fourty-eight hour culture filtrate (without glucose[9]) of the organisms was used as a starting material, since proteolytic activity of the culture had reached maximum at the time of incubation. Detailed procedures for preparation of the crude enzyme are shown schematically in figure 1. The resulting enzyme solution was dark brown, clear, and non-toxic.

FIGURE 1. Procedures for Preparation of Crude Proteolytic Enzyme Produced by *Cl. botulinum* Type A



* pH 8.0, 55°C, 15 min.

TABLE 1. *Activation of Protoxin by Fractions Separated Chromatographically*

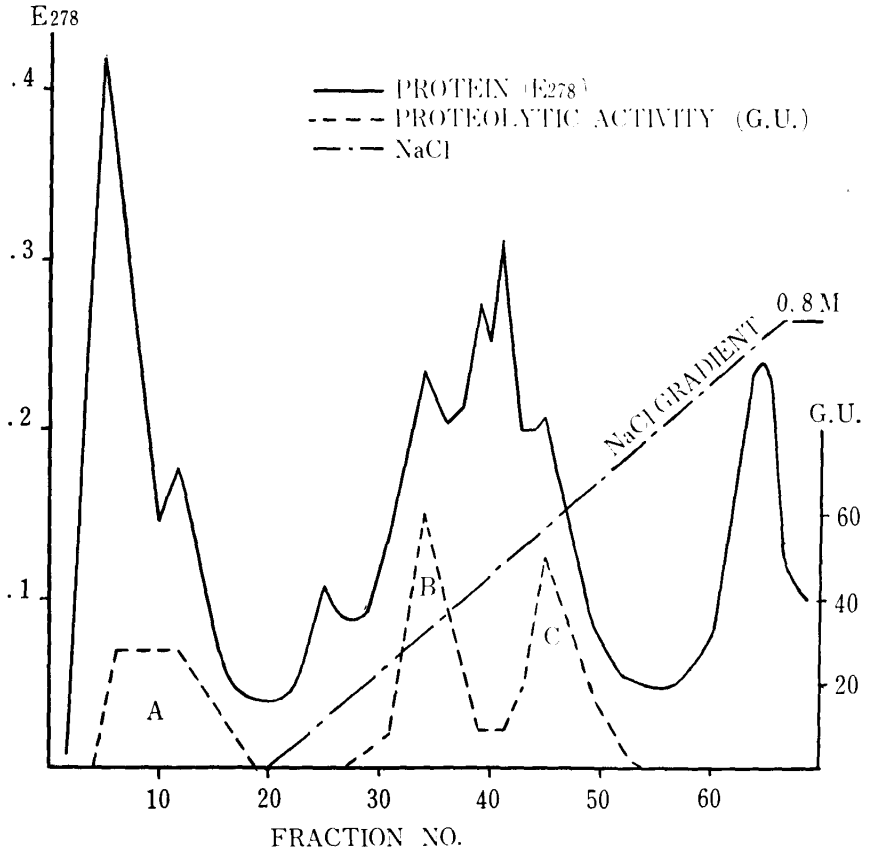
FRACTION NO.	TOXICITY AFTER INCUBATION
1	20,000
5	20,000
10	40,000
20	20,000
25	20,000
34	< 20,000
38	< 20,000
41	80,000
45	160,000
50	160,000
60	20,000
64	20,000
Trypsin (0.1%)	160,000
Control (saline)	20,000

Reaction System: Protoxin solution: Test fluid = 1:1
Condition: pH 6.0, 37°C, 90 min.

Chromatography of crude proteolytic enzyme

The crude enzyme solution was dialysed against 1/50 M phosphate buffer (pH 7.0) and adsorbed on to the top of DEAE-cellulose column equilibrated previously with the same buffer. The chromatogram was developed under a linear gradient of sodium chloride concentration. Each 4 ml of effluent was collected in separate tubes and subjected to determination of protein, proteolytic activity, and activation of the protoxin. The results are summarized in figure 2 and table 1. As seen in the figure, the chromatogram exhibited three characteristic peaks for proteolytic activity. This suggests that the organisms produce at least three different proteolytic

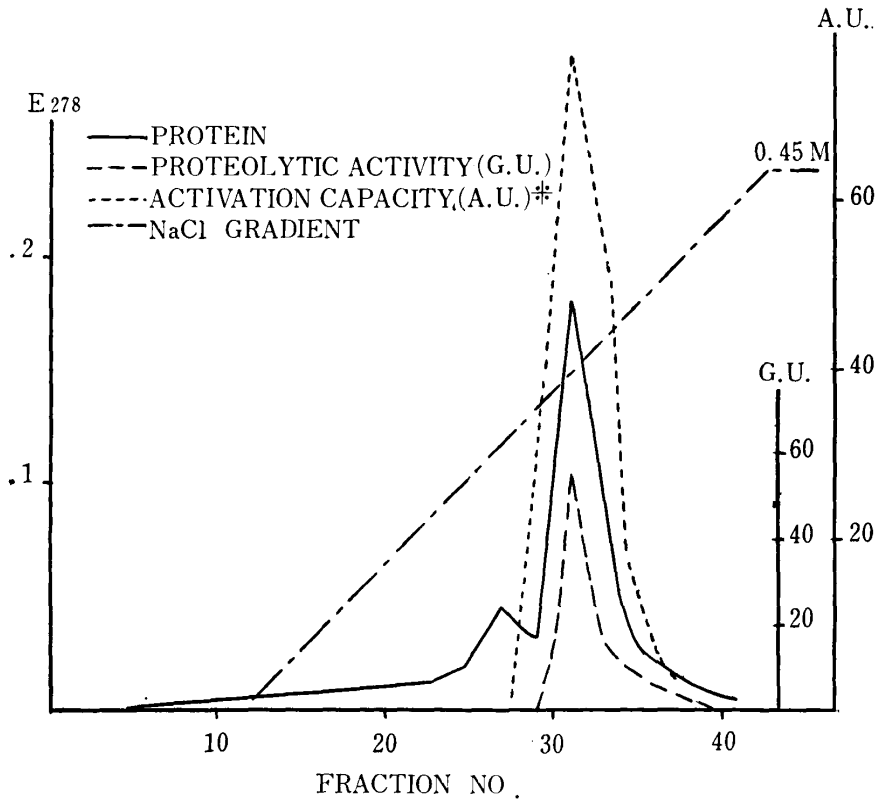
FIGURE 2. *Chromatrgram of Crude Proteolytic Enzyme*



Note: Column size 0.63 $cm^2 \times 23cm$
Buffer 1/50 M phosphate buffer (pH 7.0)
Flow rate 15 ml/hr
Fraction size 4 ml

enzymes (A, B, and C) in the culture. Activation of the protoxin by each fraction separated is shown in table 1. Only fractions containing enzyme C increased the toxicity of the protoxin preparation. These fractions were then collected and subjected to rechromatography. The result is shown in figure 3. In each fraction of No. 29~35, protein concentration, proteolytic activity, and protoxin activating capacity were almost proportional to each other. These results

FIGURE. 3. *Rechromatogram of Enzyme C*



* Reciprocal of Maximum Dilution being capable of Activating the Protoxin

suggested that the protoxin activating factor being in the culture filtrates would be essentially identical with proteolytic enzyme C produced by the organisms.

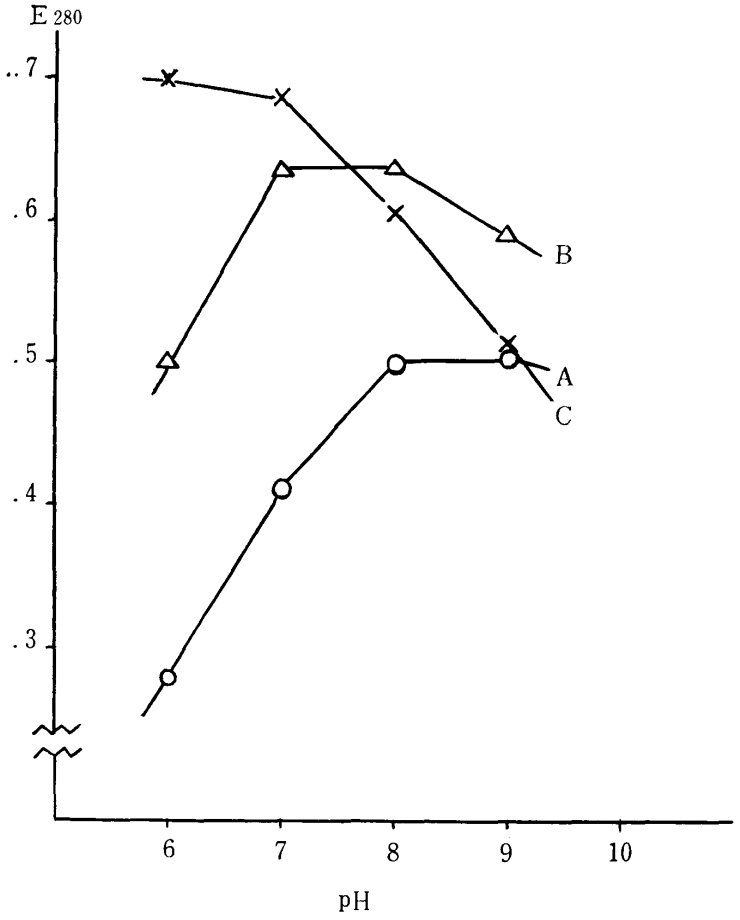
Properties of proteolytic enzymes fractionated by chromatography

Proteolytic enzymes A, B and C named in figure 2 were collected separately and their characteristics were investigated. Proteolytic activities of these three enzymes were completely inhibited by addition of a suitable quantity of EDTA or Cu or serum, which had been inhibitors of proteolytic activity of the culture filtrates. No visible difference was observed among them. Considerable difference among their activities, however, were observed for pH level. Effect of pH on their proteolytic activities was investigated by the method of HAGIWARA using casein as a substrate. The result is shown in figure 4. Optimum pH for activity of each A, B, and C enzyme was respectively 8.5, 7.5 and 6.0 or below.

Effects of proteolytic enzymes A, B, and C on type A toxin

SPERO examined the stability of type A toxin at various pH levels and concluded that the purified toxin was quite stable at pH below 10. On the other hand, it has been observed by many workers that the toxicity of crude preparations of the toxin like culture filtrate

FIGURE. 4. *Effect of* pH *on Proteolytic Activity*



decreased rapidly under alkaline conditions[1,3]. These facts would suggest the contamination of a certain factor, which would destroy the toxin at alkaline pH level, in the preparation used. As indicated in the earlier part of this report, enzymes A and B, produced by the organisms, presented their highest activities at slightly alkaline pH level. The effects of these

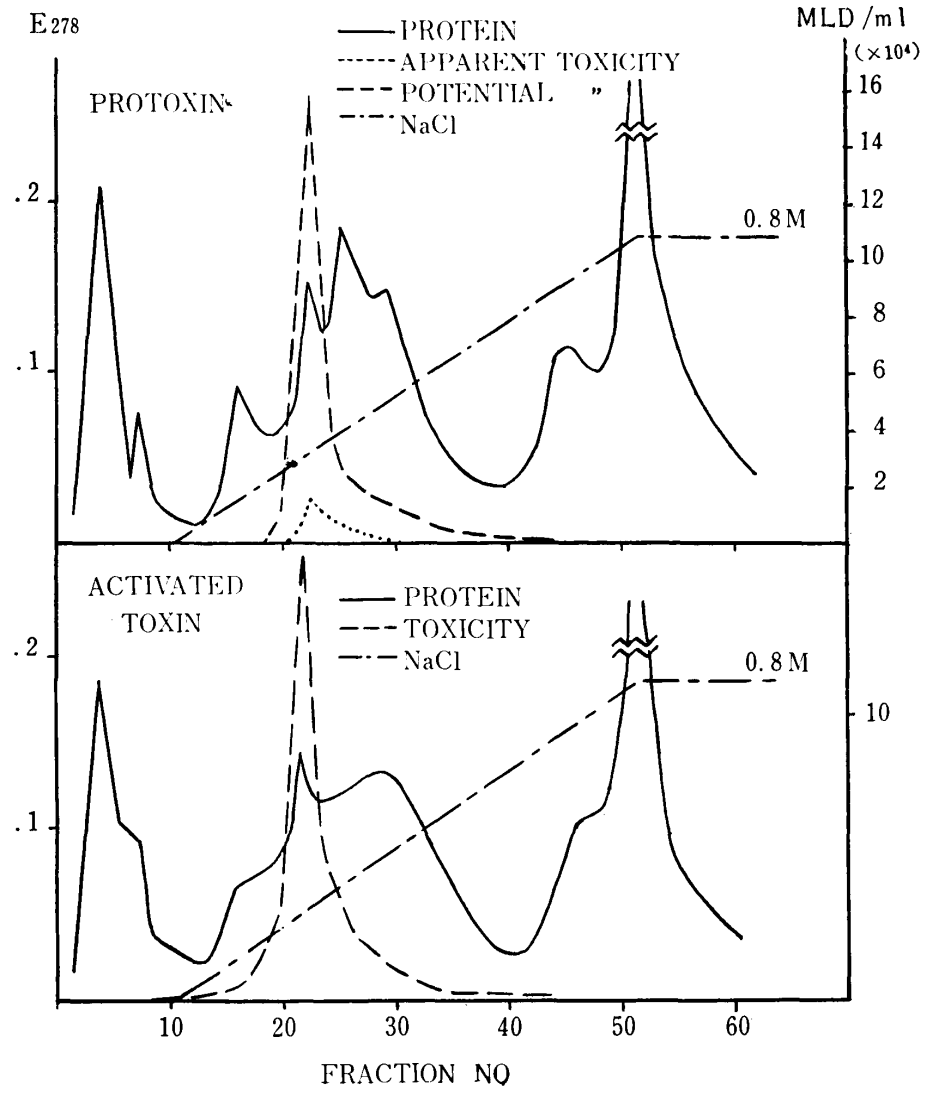
TABLE 2. *Effect of Proteolytic Enzymes on Type A Toxin*

ENZYME	*TOXICITY AFTER INCUBATION*	
	pH 5.8	pH 7.5
Enzyme A	100,000	< 5,000
" B	50,000	< 5,000
" C	100,000	25,000
Trypsin	100,000	< 5,000
Control (saline)	100,000	100,000

Condition : 37°C, 120 min.

enzymes on the toxin were then investigated. The type A toxin used was prepared from the culture filtrates through procedures such as salting out with ammonium sulfate and chromatography on DEAE-cellulose column in an other experiment. (Free from proteolytic activity) The result is shown in table 2. All the enzymes tested depressed more or less the toxicity of the toxin at pH 7.5. Considerable depression, however, was observed in cases of enzymes A and B. In view of these data, it would be concluded that the decrease in toxicity of crude toxin preparation under alkaline condition is, at least partially, due to the action of contaminating proteolytic enzyme (s). This conclusion was also supported by the observation that the addition of a proteolytic inhibitor into the culture filtrates of the organisms suppressed the decrease of toxicity at alkaline pH level.

FIGURE. 5. *Chromatogram of Protoxin and Activated Toxin.*



Chromatographic behaviours of protoxin and activated toxin

The protoxin preparation used in this investigation exerted some toxicity without any treatment. This might be interpreted to mean either that the toxicity is due to an active toxin contaminating in the preparation, or that it is due to a less active protoxin it-self. Then, chromatographic investigations of both protoxin and activated toxin (by enzyme C) preparations were carried out. Procedures for chromatography were virtually identical with those described in the case of the proteolytic enzyme, but the pH of the buffer was changed to 6.0 because of the stability of the toxin. In an experiment for the protoxin preparation, each fraction eluted was submitted to determine toxicities before and after activation by enzyme C (apparent and potential toxicities). As seen in figure 5, both apparent and potential toxicities were observed together in the same fractions and could not be observed separately. Chromatogram of the activated toxin prepared from the protoxin preparation by treatment with proteolytic enzyme C was essentially identical with that of the protoxin preparation. It was found that toxic fractions were eluted at the ionic strength just same to that of toxic fractions in the case of protoxin preparation. These results do not offer any evidence to make a choice between above described alternative interpretations. And, a constituiton of toxic substances of the protoxin preparation is still uncertain.

DISCUSSION

The proteolytic enzyme produced by *Cl. botulinum* type A has been studied by several groups of workers. The great majority of these works, however, have been attempted using crude preparations of the enzyme like the culture filtrate. In the present investigation, the data of chromatographic fractionation of the crude proteolytic enzyme prepared from the culture filtrate of type A organisms indicates clearly its heterogeneity. Three enzymes isolated chromatographically possess different values of pH for their activites. ERBERG and MEYER reported that optimum pH of their enzyme preparation was 7.0. This seems to be an average value of those of three enzymes.

In the previous experiment[9], it was observed that the toxicity of young cell extract of type A organisms could be increased by trypsin while the toxicity of older culture could not be increased. In addition, the trypsin can be replaced by culture filtrates of type A organisms. Optimum pH of the culture for toxin production during cultivation is between 5 and 6. Moreover, optimum pH for activation of the protoxin preparation by culture filtrates of the organisms also exists in the same range of pH value. At this pH level, only C of all proteolytic enzymes isolated chromatographically from the culture filtrate is able to activate the protoxin. Accordingly, it may be concluded that activation phenomenon of the toxin, which would be occurring normally in the culture, is performed by proteolytic enzyme C produced extracellularly by the organisms. A field (place), where the activation of the protoxin would be carried out, is still uncertain. However, the

fact that the proteolytic enzymes produced by the organisms are exoenzyme, so that they can not be found visibly in the cell extracts, seems to suggest the development of activation reaction in an extracellular field or on surface of the cell. Chromatographic differentiation of the protoxin and activated toxin failed to succeed under the experimental condition and could not serve to determine whether the protoxin is really toxic or not. If protoxin is assumed to be quite non-toxic, there might be a possibility that the protoxin preparation used in this investigation does not contain the real protoxin, but a certain intermediate product of the activation reaction. Whether the protoxin is toxic or not will not be solved until the time when a chemical method for investigation of toxicity, not bioassay, can be established. There is no evidence to eliminate a possibility of in-vivo activation of the protoxin, which would be naturally developed by certain proteolytic enzymes of test animals.

Proteolytic enzymes A and B seem to have no relation to the activation phenomenon and rather act destructively on the toxin, especially at alkaline pH level. In the previous report[8] dealing with the effect of carbohydrates on toxin production by *Cl. botulihum* type A, it was concluded that the less toxicity in the absence of available carbohydrate in the culture, at least partially, was caused by the inactivation of synthesized toxin occurring in alkaline environment. It would be reasonable to consider that such inactivation of the toxin is due to the action of the proteolytic enzymes being in the culture.

WAGMAN and BATEMAN observed that when a culture filtrate of *Cl. botulinum* type A was precipitated at different pH levels, two separate dissociation products as toxic as the original supernatant were obtained. SCHANTZ et al. also reported that when crystallized type A toxin was submitted to chromatography, it was separated into several toxic fractions. The present author also observed that the toxin precipitated from a culture filtrate by addition of ammonium sulfate was separated into many toxic fractions on DEAE-cellulose column[10]. These observations seem to suggest a heterogeneity of the toxin in a culture of type A organisms. On the other hand, activated toxin prepared from the protoxin preparation by treatment with enzyme C exhibited a single peak for toxicity in a chromatogram (cf. figure. 5). In view of these data, it seems that a certain fragmentation of the toxin might occur in the culture after activation. This being the case, proteolytic enzymes in the culture concerned with the fragmentation of the toxin would be expected.

On the basis of direct and indirect evidences obtained in this investigation, the most reasonable conclusion describing a process of toxin production by *Cl. botulinum* type A would be that the toxin is at first synthesized intracellularly in an inactive form (protoxin) during anabolic phase of the organisms, liberated into the medium, then, altered to toxic form through the activation by enzyme C, and possibly divided in part in toxic fragments.

SUMMARY

1. Three proteolytic enzymes A, B, and C were isolated from a culture filtrate of *Cl. botulinum* type A No. 38 by chromatography using DEAE-cellulose.

2. Optimum pH for proteolytic activity of these enzymes A, B, and C were respectively 8.5, 7.5 and 6.0 or below.

3. Of these enzymes only C was able to enhance the toxicity of the protoxin preparation.

4. Enzymes A, B, and C acted more or less destructively on type A toxin at alkaline pH level.

5. It was suggested indirectly that a fragmentation of the toxin might occur after activation in the culture.

6. On the basis of the results obtained, a process of toxin production by type A organisms was discussed.

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