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PROTEASES OF CLOSTRIDIUM BOTULINUM

VI. THE ROLE OF TRYPSIN, CLOSTRIDIUM BOTULINUM PROTEASES AND PROTEASE INHIBITORS IN THE FORMATION AND ACTIVATION OF TOXIN IN GROWING CULTURES OF CLOSTRIDIUM BOTULINUM

By

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TJABERG, TORE BJARNE: *Proteases of Clostridium botulinum. VI. The role of trypsin, Clostridium botulinum proteases and protease inhibitors in the formation and activation of toxin in growing cultures of Clostridium botulinum.* Acta vet. scand. 1974, *15*, 487—506. — In this study the influence of bovine serum protease inhibitors, trypsin and proteases produced by different types of Clostridium botulinum has been investigated. Trypsin and botulinum proteases had the capability of increasing the toxicity in growing cultures in Clostridium botulinum types A, B and E. Trypsin increased the toxin level to a greater extent than proteases from Clostridium botulinum types A, B, C and F. Protease inhibitors did not influence the toxin formation to any extent compared with the controls. The combined effects of proteases and protease inhibitors on the development of toxin in Clostridium botulinum type B were also investigated by adding proteases and protease inhibitors to the same culture at different time intervals. Protease inhibitors did not reduce the toxicity of the cultures as compared to the controls. Altogether a complex relationship seems to exist between protoxin, toxin, proteases and inhibitors in the culture, and the order and time sequence of addition seem to be of importance. The results obtained in this investigation indicate that proteases of Clostridium botulinum play a part in the formation and/or activation of toxin in growing cultures of proteolytic strains such as Clostridium botulinum types A and B. As to the activation of protoxin and progenitor toxin produced by non-proteolytic Clostridium botulinum types B and E, botulinum proteases showed a marked capability of increasing the toxicity in these cultures. Trypsinization may be valuable for the detection of Clostridium botulinum types A and B in foods, as well as for type E, where it is commonly used.

Clostridium botulinum; proteases; protease inhibitors; toxin.

Investigations on the role of proteolytic enzymes in the production and activation of toxin from Clostridium botulinum have been inspired by the oral toxicity and protein nature of the toxins. *Schubel* (1923) found that the toxicity of culture filtrates of the organisms was more sensitive to alkali than acid, and unaffected by trypsin and pepsin. Similar results have been obtained by other investigators for toxin from Clostridium botulinum type A (*Bonfenbrenner & Schlesinger* 1924, *Nelson* 1927, *Snipe & Sommer* 1928, *Chistyakov & Rodopulo* 1943, *Littauer* 1951). These observations were done with crude toxin preparations, but even with crystalline toxin *Abrams et al.* (1946), *Lamanna et al.* (1946) and *Putnam et al.* (1946) found that the toxin was destroyed above pH 7 and that the toxin was unaffected by trypsin and pepsin. However, *Halliwell* (1954), using Clostridium botulinum type A toxin, found that papain did not have an inactivating effect, pepsin produced a slight decrease in toxicity, while trypsin greatly reduced the toxicity. *Meyer & Lamanna* (1959) found that toxicity was lost upon exposure of type A toxin to trypsin, chymotrypsin, type B botulinal aminopeptidase and glutamyl transferase from Bacillus subtilis.

The effect of various enzymes on the formation of toxin in cultures of Clostridium botulinum has been under investigation because the maximum concentrations of toxin in a liquid culture of Clostridium botulinum do not parallel the growth of the organisms. This seems to indicate that the release of toxin into the culture medium is due to autolysis of the bacteria rather than excretion of toxin from the growing and multiplying organisms. Different investigators (*Kindler et al.* 1955, *Boroff* 1955, *Bonventre & Kempe* 1960) showed that the toxicity in the culture media developed in the absence of cell multiplication and that maximum toxicity was reached 24 to 72 hrs. after the start of autolysis. Culture filtrates and filtrates of disintegrated cells of Clostridium botulinum types A and B were activated by treatment with trypsin and pepsin (*Bonventre & Kempe*). No increase could be seen after the addition of enzymes to 96 hr. cultures. Based on these results *Bonventre & Kempe* indicated that botulinum toxin in types A and B is produced by activation of a progenitor toxin (*Lamanna & Sakaguchi* 1971) by endogenous proteolytic enzymes (proteases) produced by the organisms themselves.

Sakaguchi & Tokyama (1955 a, b) found that toxin formation in Clostridium botulinum type E developed faster when the cul-

ture was contaminated with a non-toxigenic, proteolytic organism belonging to the genus Clostridium. *Duff et al.* (1956) increased the toxicity of culture filtrates from Clostridium botulinum type E with trypsin. *Dolman* (1957) also reported that proteases from proteolytic, non-toxigenic type E-strains had an activating effect. Similar results were obtained on adding filtrates from cultures of Bacillus licheniformis to cultures of Clostridium botulinum type D (*Prévot & Sillioc* 1962). However, *Legroux et al.* (1947) reported that a proteolytic enzyme from Bacillus antracoides destroyed toxins produced by Clostridium botulinum and Clostridium tetani.

Skulberg (1964) found that, if suspensions of washed cells of types A, B and E were treated with trypsin, a considerable enhancement in toxicity occurred. He found no activation in supernatants of young type A cultures after treatment with trypsin, while a considerable increase in toxicity was seen after trypsin treatment of types B and E supernatants. The influence of trypsin on cell suspensions and culture supernatants of non-proteolytic strains of Clostridium botulinum types B and E was investigated by *Tjaberg* (1973 a). Tryptic digestion of cell suspensions of Clostridium botulinum type B increased the toxin level to a greater extent than trypsinization of the supernatants. For type E, the toxicity in the supernatants increased to the same level as in the cell suspensions. The length of the tryptic activation period influenced the toxicity of cell suspensions and supernatants. *DasGupta* (1971) presented results indicating activation of Clostridium botulinum type B toxin by an endogenous enzyme. Activation of highly purified Clostridium botulinum type E progenitor toxin by a protease produced by a Clostridium botulinum type B culture has been reported by *DasGupta & Sugiyama* (1972). The activation of type E progenitor toxin was always less with type B protease than with trypsin. Recently *Eklund & Poysky* (1972) have reported the activation of a toxic component from Clostridium botulinum types C and D by trypsin.

The aim of this work was to investigate the role of proteases on toxin formation, and on toxin activation in g r o w i n g cultures of Clostridium botulinum types A, B, C and E using proteases produced by Clostridium botulinum and trypsin. In addition, it was of interest to inhibit protease activity in strains of Clostridium botulinum in order to investigate further the mechanism of toxin formation.

MATERIALS AND METHODS

Organisms

The following strains of Clostridium botulinum were investigated: Type A (strain Hall), type B (strain Beans), type C (strain Cid proteolytic) and type E (strain 1537) obtained from culture collection at the Norwegian Food Research Institute, Ås, Norway and type B (strain 2B, non-proteolytic) obtained from Dr. M. W. Eklund, National Marine Fisheries Technology Laboratory, Seattle, Washington, USA.

Enzymes and inhibitors

Trypsin from bovine pancreas (type III, 2x crystallized, lot 220—8121) was obtained from Sigma Chemical Company, St. Louis, Mo., USA. Proteases from Clostridium botulinum types A, B, C and F were prepared according to *Tjaberg* (1973 b). The crude material after ammonium sulphate fractionation was used. Detoxification of Clostridium botulinum types A and B proteases was performed by adding formaldehyde to a final concentration of 0.4 %, and allowing the mixture to react at 37°C until complete detoxification had taken place. The level of the proteases in the various culture fluids was usually of the order of 500—1200 CP-units (*Tjaberg* 1973 b) per 0.025 ml. Bovine serum had been shown to inhibit proteases from Clostridium botulinum types A, B, C and F (*Tjaberg & Fossum* 1973). The whole blood samples from cattle were allowed to coagulate, the serum was collected after centrifugation and stored at —18°C until used.

Cultivation methods

Clostridium botulinum type A (strain Hall) was grown on trypticase yeast extract medium (*Duff et al.* 1957), while the other types were grown on Robertson's meat broth medium (*Robertson* 1915—1916).

Toxin assay

Toxin titrations were made by diluting samples in logarithmic intervals in cold 0.1 M phosphate buffer, with 0.2 % gelatin at pH 6.7. Each dilution (0.5 or 1 ml) was injected intraperitoneally into albino mice (NMRI/BOM spf) weighing about 20 g. The mice were observed for 96 hrs., and the toxin titres calculated as minimum lethal doses per ml (MLD/ml).

Protease assay

The casein precipitation (CP) method of *Sandvik* (1962) was used, and the casein precipitating activity (CP-activity) was expressed as diffusion units (CP-units) (*Sandvik*). According to *Fossum* (1970) 0.025 ml of a 0.1 mg/ml trypsin solution results in 1440 CP-units corresponding to 3.75 Kunitz units (*Tjaberg* 1973 b). A volume of 0.025 ml of a partly purified Clostridium botulinum type A (strain 62) had a proteolytic activity of 512 CP-units with a corresponding value of 21 Kunitz units per 0.025 ml. For type B the activity was 640 CP-units and 17 Kunitz units, for type C 512 CP-units gave 16 Kunitz units and for type F 632 CP-units corresponded to 16 Kunitz units per 0.025 ml (*Tjaberg* 1973 b). These results indicate that various proteolytic enzymes react differently depending on the assay system used.

Experimental

In the first experiment trypsin, Clostridium botulinum proteases or protease inhibitors were added to 9 ml culture media at the start of the incubation period. The following parallels were used: medium with sterile water added, medium with bovine serum added, medium containing trypsin added to give 0.1 mg/ml in the final medium, and four media with proteases from Clostridium botulinum types A, B, C and F added to give a final concentration varying from 500 to 1200 CP-units per 0.025 ml. The additions of the different components were dimensional to give the same medium concentration (90 %) in all tubes. One ml samples were withdrawn from these parallels after 12, 24, 48 and 96 hrs. incubation and tested for growth, proteolytic activity and toxicity.

In the second part of the investigation 150 ml growth medium was used without protease or protease inhibitors added at zero time. After 12, 24 and 48 hrs. of incubation seven samples each of 5 ml were withdrawn and proteases and protease inhibitors added as described above. These samples were incubated at 37°C and tested for growth, proteolytic activity and toxicity after another 1, 12 and 24 hrs. of incubation.

Thirdly, the combined effects of proteases and protease inhibitors on the development of toxicity in Clostridium botulinum type B (strain Beans) were investigated. Tubes with 9 ml Robertson's cooked meat broth were added 1 ml sterile water (A), 1 ml

of type B-protease to give a final concentration of 1200 CP-units (B), 1 ml trypsin to give a final concentration of 0.1 mg/ml (C) and two tubes were added 1 ml protease inhibitor each (D and E).

After inoculation with Clostridium botulinum type B the tubes were incubated at 37°C, and after 24 and 48 hrs. 3 ml samples were withdrawn. Type B-protease or trypsin was added at zero time to tube B and C and later bovine serum to halt all proteolytic activity. Bovine serum was added initially to tubes D and E and trypsin was added to give a final concentration of 0.1 mg/ml and type B-protease to give 1200 CP-units. The addition of the different components was calculated to give the same medium concentration in all tubes. The samples were then incubated at 37°C and tested for proteolytic activity and toxicity after 1, 6 and 24 hrs. of incubation.

Mc Intosh-Fildes anaerobic jars were used and incubation took place at 37°C for types, A, B and C, while type E was cultivated at 30°C.

Bacterial counts were made by using a Petroff-Hausser counting chamber (Depth 0.02 mm).

RESULTS

The bacterial growth in the cultures containing botulinum proteases or trypsin was generally the same as for the control, and the number of cells varied between 2 and 4 million cells/ml after 12 hrs. of incubation. The cultures containing bovine serum showed delayed growth compared to the others after 12 hrs. of incubation, and the cell count never exceeded 600,000 cells/ml. However, at 24 hrs. and later, the bacterial counts for all cultures varied between 60 and 80 million cells/ml.

Fig. 1 shows the toxicity and protease activity at various intervals when proteolytic enzymes, or protease inhibitors were added to Clostridium botulinum types A, B non-proteolytic and E at the start of the incubation period (zero hours). For Clostridium botulinum type B proteolytic the results are not presented as they were similar to the results obtained for type A. The results obtained on addition of proteases from Clostridium botulinum types A, B, C and F were of the same order, and therefore only results from the cultures with type A protease added are shown.

For Clostridium botulinum type A the toxicities of the cultures with trypsin and Clostridium botulinum proteases were

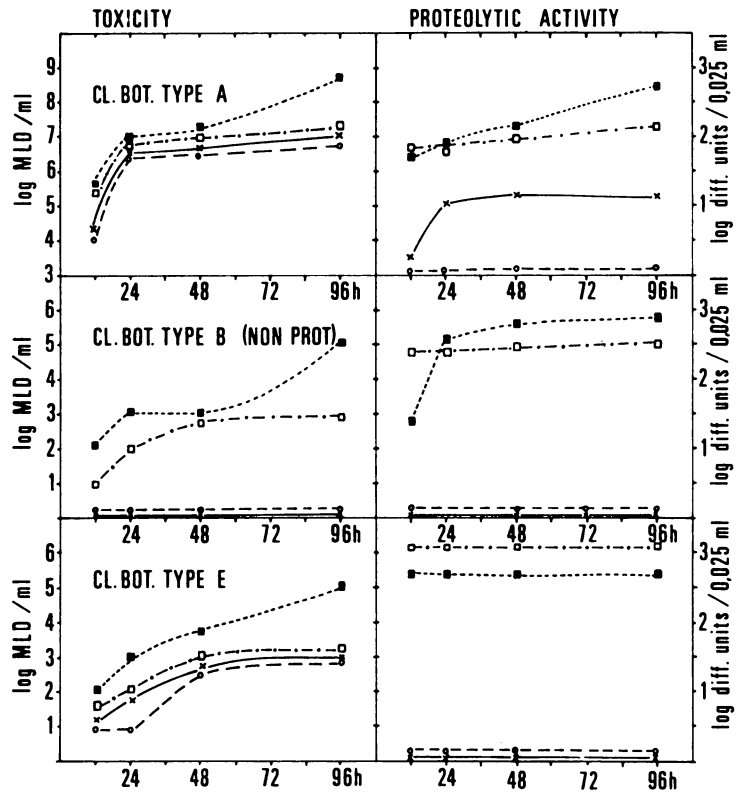

Figure 1. Toxicity and proteolytic activity in cultures of Clostridium botulinum types A (strain Hall), B (non-proteolytic, strain 2B) and E (strain 1537) with various proteolytic enzymes or protease inhibitors added at the beginning of the incubation period. Incubation temperature for types A and B was 37°C and for type E 30°C.
×———× Culture acting as control; o- - - - - o Culture with protease inhibitor added (bovine serum); ■ - - - - - ■ Culture with trypsin added; □-·-·-·-□ Culture with protease produced by Clostridium botulinum type A added, representative for types B, C and F.

higher after 12 hrs. than for the control. No proteolytic activity could be demonstrated in the culture with inhibitors added. The growth of the cultures containing inhibitors was slightly less than for the control. After 48 hrs. there was a tendency for the cultures containing trypsin and proteases from Clostridium botulinum to reach the highest toxin titre, and the same could be seen after 96 hrs. of incubation. The toxin titre of the culture with trypsin was higher than those for the cultures containing botu-

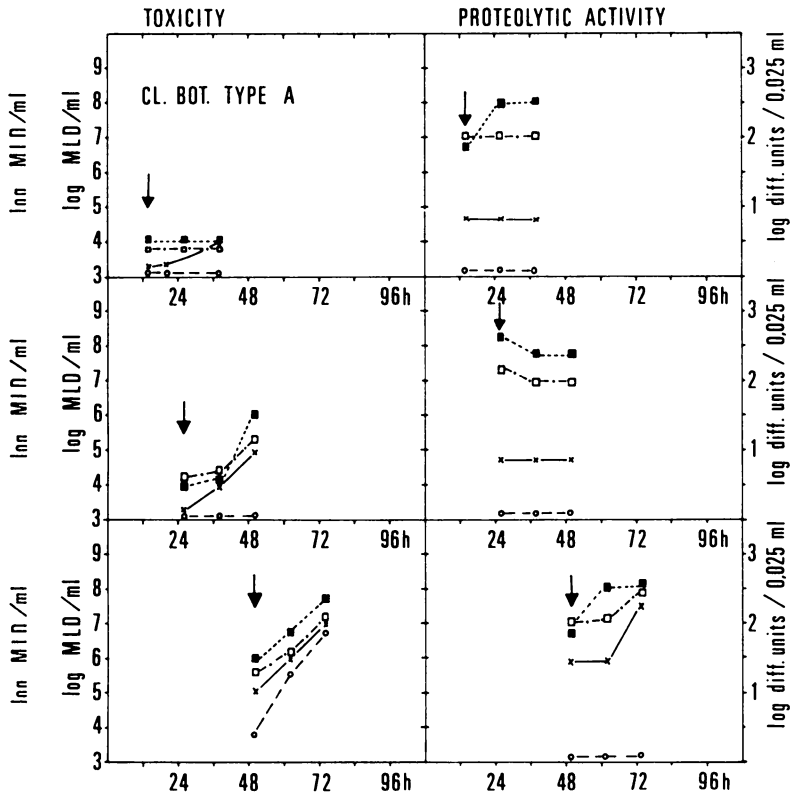

Figure 2. Toxicity and proteolytic activity in cultures of Clostridium botulinum type A (strain Hall) with various proteolytic enzymes and protease inhibitors added. After incubation of the culture for 12, 24 and 48 hrs. the proteases and the inhibitor were introduced and allowed to react for another 1, 12 and 24 hrs.

×———× Culture acting as control; o- - - - - o Culture with protease inhibitor added (bovine serum); ■ - - - - - ■ Culture with trypsin added; □-·-·-·-□ Culture with protease produced by Clostridium botulinum type A added, representative for types B, C and F.

linum proteases. For the non-proteolytic strain of Clostridium botulinum type B no toxic activity could be found in the control or the culture containing inhibitors, while the culture with trypsin reached 100,000 MLD/ml and the cultures with botulinum proteases reached 1,000 MLD/ml following 96 hrs. of incubation at 37°C. The CP-activities in these two cultures were of the same order. For type E, it can be seen that the culture with trypsin added has a higher toxicity than cultures containing proteases

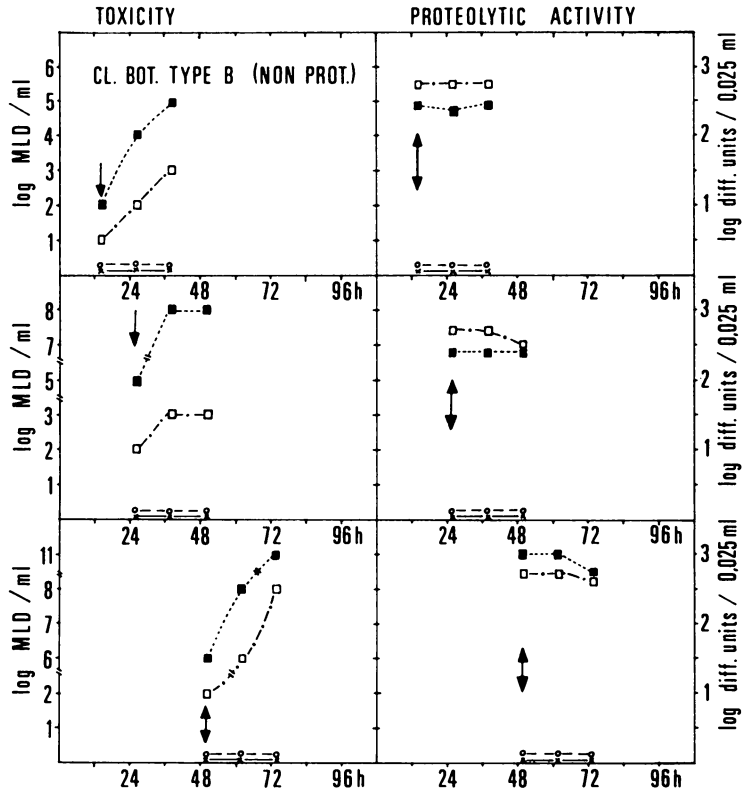

Figure 3. Toxicity and proteolytic activity in cultures of Clostridium botulinum type B (strain 2B, non-proteolytic) with various proteolytic enzymes and protease inhibitors added. After incubation of the culture for 12, 24 and 48 hrs. the proteases and the inhibitor were introduced and allowed to react for another 1, 12 and 24 hrs.
×———× Culture acting as control; o-----o Culture with protease inhibitor added (bovine serum); ■ ----- ■ Culture with trypsin added; □-·-·-·-□ Culture with protease produced by Clostridium botulinum type A added, representative for types B, C and F.

from Clostridium botulinum types A. There was only a small difference in toxin formation in the culture acting as control and the culture containing bovine serum as inhibitor.

Fig. 2 shows the results for a growing culture of Clostridium botulinum type A (strain Hall) with proteases of protease inhibitor added after an initial incubation of 12, 24 and 48 hrs. The added components were then allowed to react with the cultures for 1, 12 and 24 hrs. before being tested for toxicity and

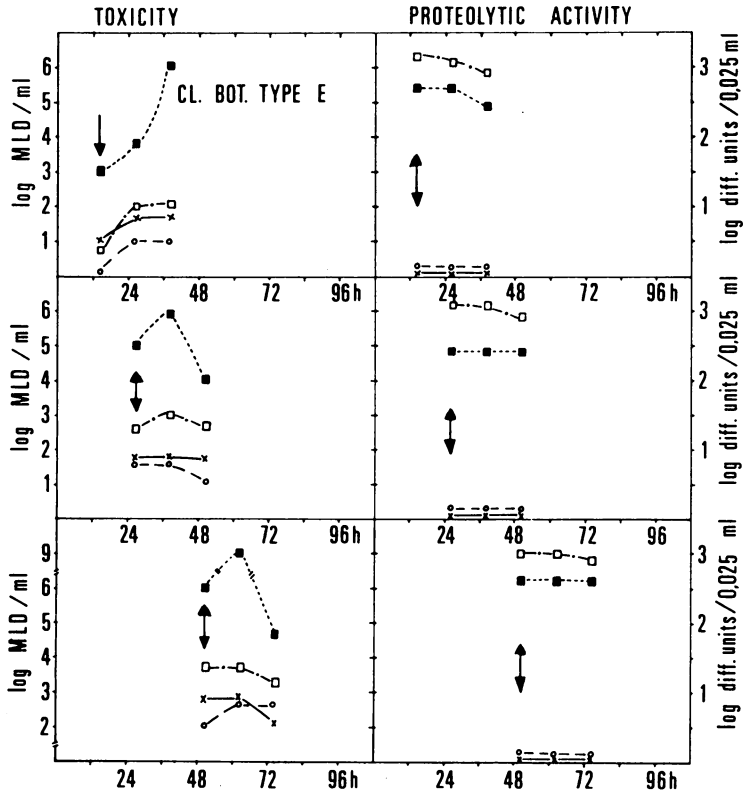

Figure 4. Toxicity and proteolytic activity in cultures of Clostridium botulinum type E (strain 1537) with various proteolytic enzymes and protease inhibitors added. After incubation of the culture for 12, 24 and 48 hrs. the proteases and inhibitor were introduced and allowed to react for another 1, 12 and 24 hrs.

×———× Culture acting as control; o-----o Culture with protease inhibitor added (bovine serum); ■ ----- ■ Culture with trypsin added; □-·-·-·-□ Culture with protease produced by Clostridium botulinum type A added, representative for types B, C and F.

proteolytic activity. Addition of botulinum proteases or trypsin gave increases in the toxicity compared with the control, while the culture with protease inhibitors present had the lowest toxin titre.

The test results of the non-proteolytic type B strains are shown in Fig. 3. The control and the culture containing protease inhibitors did not possess neither toxicity nor proteolytic activity. Trypsin and various botulinum proteases (A, B, C and F) en-

hanced the toxicity of the cultures to a great extent compared with the control. Treatment with trypsin always gave a higher toxin titre than treatment with botulinum proteases, although the CP-activities of the enzymes were of the same order.

Fig. 4 illustrates the results found when adding proteases and inhibitors to cultures of Clostridium botulinum type E at different intervals. The toxicities of the control and the culture with bovine serum were within the same range. The addition of Clostridium botulinum proteases gave no substantial increase in toxicity on adding the proteases after 12 hrs. of incubation while after 24 and 48 hrs. the toxicity was increased when treated with botulinum proteases for 1 hr. Incubation with proteases for 12 and 24 hrs. did not give any rise in toxicity. When adding trypsin after 12 hrs. the toxicity increased 3 to 4 log. units during the 24 hr. incubation period. Adding trypsin to cultures incubated for 24 and 48 hrs. gave an increase in toxicity during incubation for 1 and 12 hrs. respectively, while incubation with trypsin for 24 hrs. caused a decrease compared with the toxicity after 12 hrs. of incubation.

Two strains of Clostridium botulinum type C were tested according to the scheme outlined above, but no toxin formation at all could be found for the proteolytic type C strain. Trypsin and Clostridium botulinum proteases did not give rise to any toxin formation. For the non-proteolytic type C strain neither trypsin, Clostridium botulinum types A, B, C or F proteases caused any increase in toxicity compared to the control.

In Fig. 5 the combined effect of proteases and protease inhibitors on the development of toxin in growing cultures of Clostridium botulinum type B proteolytic can be seen. The cultures with type B-protease or trypsin added at zero time had an increased toxin titre compared to the control, both after 24 and 48 hrs. of incubation. Introducing protease inhibitors into the medium after 24 and 48 hrs. of incubation reduced the proteolytic activity to zero, but did not cause any reduction in toxicity. Trypsin caused a higher toxin titre than did type B-protease. In series D and E the organisms were grown with bovine serum for 24 and 48 hrs. respectively. The toxin level was low, but the same as for the control. Addition of type B-protease caused a 2 log. units increase in toxicity after incubation for 1 hr. Incubation for longer periods (6 and 24 hrs.) gave a distinct de-

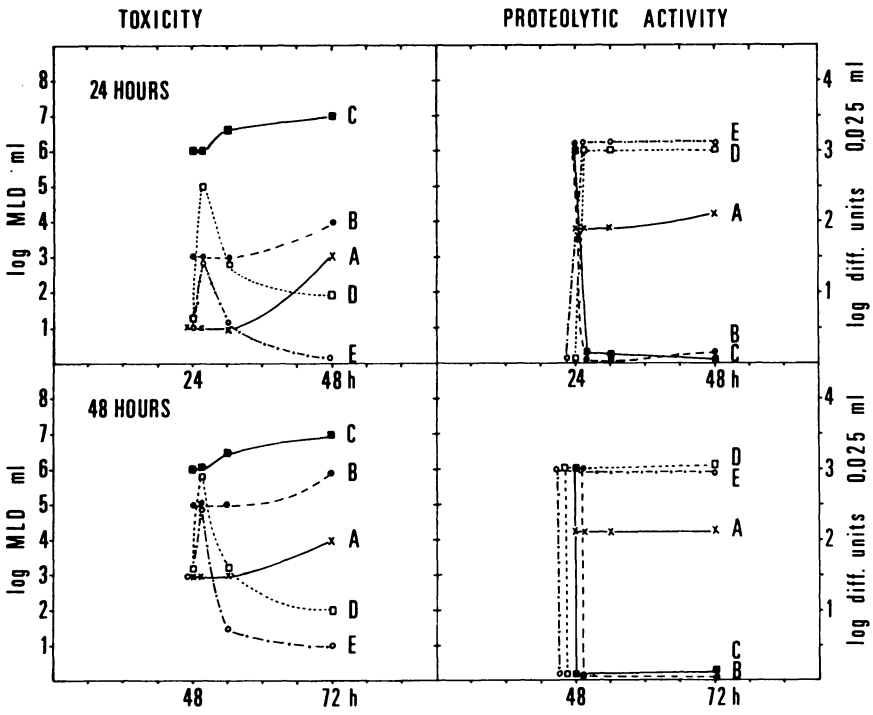

Figure 5. Combined effect of proteases and protease inhibitor on the development of toxicity in Clostridium botulinum type B (strain Beans).

A ×——× Control; B ●- - - - - ● Clostridium botulinum type B-protease added at zero time and protease inhibitor (bovine serum) after 24 and 48 hrs. of growth; C ■——■ Trypsin added at zero time and protease inhibitor (bovine serum) after 24 or 48 hrs. of growth; D □- - - - - □ Protease inhibitor added (bovine serum) at zero time and trypsin after 24 or 48 hrs. of growth; E o·····o Protease inhibitor added (bovine serum) at zero time and Clostridium botulinum type B protease after 24 and 48 hrs. of growth.

crease compared to the maximum level obtained after 1 hr. Trypsin gave a 3—4 log. increase in toxicity after 1 hr. of incubation, followed by a distinct decrease. Protease and protease inhibitors were added to a proteolytic type B culture simultaneously at zero time and after 24 hrs. of incubation. It was found that the toxicity of this culture followed that of the control throughout the time.

DISCUSSION

Under the conditions used in the present investigation it was found that both trypsin and proteases produced by different types of Clostridium botulinum influenced the toxin level in growing cultures of Clostridium botulinum. Trypsin always tended to give a larger rise in toxicity than the proteases produced by various types of Clostridium botulinum. Protease inhibitors were introduced into the culture media to see if such inhibitors could prevent, or retard toxin formation in Clostridium botulinum. Such investigations do not seem to have been reported in the literature.

Introducing trypsin or Clostridium botulinum proteases to Clostridium botulinum type A and proteolytic type B the increase in toxicity was most pronounced for trypsin and slightly higher than the control when using Clostridium botulinum proteases. Inhibition of proteolytic activity in these cultures did not cause a decrease in toxin formation, and the control and the culture with inhibitors added generally gave the same result. This indicates that proteolysis extracellularly does not have to occur in order to form toxin.

The enhancement of toxicity by proteases in the proteolytic types of Clostridium botulinum indicates that progenitor toxins are present, although Clostridium botulinum proteases only increased the toxicity to a small extent. The results obtained are in agreement with *Bonventre & Kempe* (1960) who found that activation of progenitor toxin by trypsin occurs in proteolytic types A and B.

Trypsin activation caused a considerable increase in toxicity in the non-proteolytic types B and E strains tested. The type B strain had to be activated by trypsin or botulinum proteases to show toxicity, while the type E strain was weakly toxic without any activation. For type B (non-proteolytic) activation by botulinum proteases could be seen more clearly since toxicity was not found either in the control or the culture with inhibitors added. For type E both the culture with inhibitors and the control had nearly the same toxicity as the cultures activated by botulinum proteases. The results obtained show a certain relationship between non-proteolytic type B and type E. Such relationship has also been found in DNA homology studies (*Lee & Riemann* 1970), and the present investigation shows that, as with type E strains, activation seems to be necessary in order to obtain

toxic effect. Clostridium botulinum type E is known to be non-proteolytic and *Duff et al.* (1956) demonstrated that addition of trypsin to culture supernatants raised the toxin titre considerably. In the present investigation the highest toxicity in the cultures was reached on introducing proteases into the culture after 48 hrs. of incubation at 30°C. This indicates that a certain time is needed for the formation or liberation of progenitor toxin and that, when proteolytic enzymes are introduced into the culture at the start of the incubation period, a combination of activation and inactivation may take place. When introducing proteases into type E cultures after 24 and 48 hrs. of incubation the rise in toxicity was followed by a distinct fall, while this was not seen on adding the proteolytic enzymes at zero hours. The type E strain seems to produce progenitor toxin which is activated by the proteases present, but at the same time the proteases are responsible for degradation of the toxic molecule, so that the total level of toxicity found in the culture at various times is dependant on both activation and degradation of toxin, and as seen in Fig. 1 this gives a steady increase in toxicity with time. When introducing proteolytic enzymes into the type E-culture after 24 and 48 hrs. the progenitor toxin is already activated to a certain degree and the proteases degrade this toxin while activation occurs to a lesser extent, which results in a total decrease in toxicity as seen in Fig. 4.

Skulberg (1964) found that certain strains of Clostridium botulinum type C developed non-proteolytic and proteolytic mutants and that the most proteolytic strains did not produce any toxin at all. In the present investigation it was of particular interest to investigate the proteolytic strains further to see whether proteases or protease inhibitors would influence the toxin formation during growth. The proteolytic type C strain tested was found not to possess any toxicity at all, and trypsin and botulinum proteases did not lead to any toxin production. Introducing bovine serum into the culture medium decreased the proteolytic activity, but no toxicity could be found. Activation of type C-toxin produced by non-proteolytic type C strains was reported for the first time by *Eklund & Poysky* (1972). In their investigation they found two toxins (C_1 and C_2) to be produced by certain strains of Clostridium botulinum type C and also two toxins (C_2 and D) to be produced by certain strains of type D. The C_2-toxin required trypsin for activation, while C_1 and D did

not. They found that unless C_1 and D-toxins were specifically neutralized, increases in C_2 toxicity by trypsin activation would be masked and go undetected. The strain of Clostridium botulinum type C used in the present investigation was strain Cid, which is known to produce C_1 and C_2 toxins (*Eklund & Poysky*). In this investigation toxicity was not found at all. The reason for this could be a mutation in the culture used.

Different biological inhibitors and chemicals have been shown to inhibit proteolytic enzymes produced by different types of Clostridium botulinum (*Tjaberg & Fossum* 1973), and one of those complexes, the bovine serum inhibitors, was tested in this investigation. The fact that bovine serum inhibited proteolytic activity completely in all tests, but did not cause any decrease in toxicity compared with the control in any of the experiments, is of particular interest. This observation may indicate that some activation of the toxin may take place even in the absence of extracellular CP-activity. On the other hand this need not exclude an intracellular protease activation of the protoxin as the serum inhibitors are probably unable to pass through the cell membranes of the organisms. Very weak proteolysis could occur intracellularly and cause toxin formation to reach the same level as the control. The liberation of toxin might then be caused by autolysis of vegetative cells. Another explanation could be that the progenitor toxin present is broken down to smaller toxic units by physical forces and without any help from a protease. In this connection it was of particular interest to investigate the combined effects of proteases and protease inhibitors on the development of toxin in Clostridium botulinum, and Clostridium botulinum type B was selected for this purpose. When adding inhibitors at zero hours the cultures generally had the same toxicity as the control after 24 and 48 hrs., respectively, and addition of proteolytic enzymes at these intervals caused a large activation of toxicity with trypsin as the best activator. However, a subsequent fall in toxicity could be seen, which indicated that also a degradation of toxin had occurred possibly due to cleavage of peptide bonds by trypsin and botulinum proteases. When adding proteolytic enzymes at zero hours an increase in toxicity could be seen and trypsin was the best activator. Introducing inhibitors did not influence the maximum toxicity, but it seems that the protease inhibitors have some stabilizing effect on the already found toxins. The results obtained in this part of the investi-

gation clearly point out the differences obtained on adding protease before inhibitors or vice versa. It is difficult to explain that proteolytic activity could exist in cultures already containing inhibitors (Fig. 5), but these inhibitors may possibly be bound to other proteinaceous material and thereby inactivated. By adding protease and protease inhibitors to a proteolytic type B culture simultaneously at zero time and after 24 hrs. of incubation it was found that the toxicity of this culture followed that of the control throughout the experiment. The results obtained in this investigation show that proteases from Clostridium botulinum types A, B, C and F influence the formation and activation of toxin in cultures of Clostridium botulinum types A and B in a similar manner, and that these proteases can activate progenitor toxin produced by Clostridium botulinum type E and non-proteolytic type B.

It is important to note that protease-treated preparations of impure toxins can show an increase in toxicity for different reasons. A conversion of non-toxic protein to toxic protein may take place, secondly an increase in toxicity could be due to release of trapped toxic molecules from an aggregate including extraneous material, thirdly an increase in toxicity of an already toxic molecule by a change in size or shape could occur, and finally an increase in toxicity by proteases could be caused by destruction of a neutralizing or antidotal substance.

The greater enhancement of toxicity in cultures of Clostridium botulinum types A, B and E by trypsin than by proteases from Clostridium botulinum types A, B and F is interesting. In these studies the CP-activity of trypsin in the cultures was adjusted to the same range as for the different proteases of Clostridium botulinum, while measured by the method of Kunitz (*Tjaberg* 1973 b) trypsin had nearly eight times less activity than the botulinum proteases used. This point is of considerable interest and shows that various proteolytic enzymes react differently according to the assay system being used. Trypsin's activity in increasing toxicity compared with Clostridium botulinum proteases may be caused by cleavage of specific bonds in the proteins or influencing active sites. The sequence in which peptide bonds are cleaved may determine the toxicity obtained. It has been shown that trypsin (*DasGupta & Sugiyama* 1972) and a protease isolated from Clostridium botulinum type B (strain Lamanna) both acted on peptide bonds formed by the

carboxyl group of only arginyl and lysyl residues, but in contrast to trypsin the protease from type B has a preference for arginyl compared to lysyl residues. This circumstance could partly explain the difference in activation for the two enzymes (*DasGupta & Sugiyama*).

From the present investigation it has been shown that botulinal proteases and trypsin influence the formation and activation of toxin for the botulinum types commonly involved in outbreaks of botulism in humans.

In certain outbreaks of botulism the vehicle involved has not been found due to small amounts of toxin, and it has been difficult to explain the severity of the disease without finding any implicated food. Proteolytic enzymes and especially trypsin are present in the digestive tract, and some activation most probably occurs. Activation of food or cultures thought to contain type E with trypsin has been commonly employed for some years. The results of this work indicate that trypsinization could be valuable for the detection of Clostridium botulinum types A and B as well. However, it is important to point out that the activation periods should not exceed 1 hr., because prolonged periods of activation could cause degradation of toxin.

There seems to be a complex relationship between the development of toxin and the manner in which proteases and possibly inhibitors are introduced into the cultures containing protoxin, progenitor toxin or toxin as such. It is important to be aware of this fact since the possibility for producing toxin, or finding toxin, could depend on special circumstances in cultures or in foods. Under certain conditions proteolytic enzymes could cause an increase in toxicity in a commodity, while under others the same enzymes could destroy the toxin.

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SAMMENDRAG

Clostridium botulinum proteaser. VI. Virkning av proteaser og proteaseinhibitorer på utvikling av toksisitet i voksende kulturer av Clostridium botulinum.

Det er utført undersøkelser over virkning av proteaser (trypsin og Clostridium botulinum proteaser) og protease inhibitorer på toksindannelsen hos voksende kulturer av Clostridium botulinum A, B, C og E. Trypsin økte toksisiteten i kulturer av Clostridium botulinum type A, B og E mer enn hva Clostridium botulinum proteasene gjorde. Protease inhibitorer hindret ikke toksindannelsen hos Clostridium botulinum sammenlignet med kontroll. Den proteolytiske type C stam-

men som ble undersøkt dannet ikke toksin hverken med eller uten nærvær av protease eller protease inhibitorer. Videre undersøkte man den kombinerte effekt av proteaser og protease inhibitorer ved å tilsette disse til den samme kultur, men ved forskjellige tidspunkter. Proteaseinhibitorene hindret ikke toksinutvikling sammenlignet med kontroll, og proteaser tilsatt kulturer hvor inhibitorer var til stede økte toksisiteten i løpet av 2—3 timer, med et derpå følgende fall. Dersom man tilsatte proteaser ved starten av forsøket, økte dette toksisiteten i forhold til kontroll, og protease inhibitorer tilsatt denne kultur etter 24 og 48 timer hemmet ikke den videre toksinutvikling selv om proteolytisk aktivitet i kulturen ble hemmet fullstendig. På den annen side synes det som om inhibitorerne hemmet degradering av toksinet. I det hele tatt synes undersøkelsene å vise et komplekst forhold mellom protoksin og toksin og hvordan proteolytiske enzymer og inhibitorer influerer disse. Resultatene indikerer at Clostridium botulinum proteaser og trypsin influerer dannelse og/eller aktivering av toksin hos Clostridium botulinum type A og proteolytisk type B. Når det gjelder aktivering av progenitor toksin hos Clostridium botulinum type E og ikke proteolytisk type B, så økte toksisiteten etter tilsetting av proteaser fra Clostridium botulinum. Trypsin hadde imidlertid en bedre aktiverende effekt. Ut fra de foreliggende data er det grunn til å påpeke at trypsin-tilsetting, som er vanlig å benytte til næringsmidler eller kulturer som mistenkes å inneholde type E, også bør forsøkes til kulturer eller næringsmidler som mistenkes å inneholde Clostridium botulinum type A og B. Tilsetting av protease inhibitor hemmet ikke toksindannelsen hos Clostridium botulinum type A og B.

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