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# PROTEASES OF CLOSTRIDIUM BOTULINUM

## I. CLASSIFICATION OF PROTEASES AND LITERATURE SURVEY

By

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TJABERG, TORE BJARNE: *Proteases of Clostridium botulinum. I. Classification of proteases and literature survey.* Acta vet. scand. 1973, *14*, 184—192. — Proteolytic activity has long been regarded as an important characteristic for distinguishing between different types of Clostridium botulinum. While all strains of Clostridium botulinum type A examined so far possess proteolytic activity, the types B and F have both proteolytic and non-proteolytic varieties. Clostridium botulinum types C, D and E were generally regarded as non-proteolytic, but different investigators have shown proteolytic activity in certain strains of these types. A summary of the classification of proteolytic enzymes in general is given and further, investigations are reviewed on the proteolytic activity in Clostridium botulinum.

Clostridium botulinum; proteases.

### *Classification of proteolytic enzymes**

The classification of proteolytic enzymes has, for a long time, been a matter of discussion. The first approach, made by *Grassmann & Schneider* (1936), classified the enzymes according to the size of the molecules of the substrates utilized. The classification generally accepted in 1942 (*Bergmann* 1942) is shown in Table 1.

Proteolytic enzymes that attacked proteins were called proteinases and those attacking peptides, peptidases. However, *Bergmann & Fruton* (1941) and *Bergmann* (1942) recognized that all proteolytic enzymes attack peptide bonds. They discovered that the specificity of an enzyme depends on its affinity for

* Proteolytic enzyme or protease are the terms used throughout this study. However, in this particular article the terms used by the respective investigators are applied.

Table 1. Classification of proteolytic enzymes according to *Bergmann.*

| | Enzyme | Substrate | Specificity |
|---|---|---|---|
| Peptidases | dipeptidase | dipeptides | dipeptides |
| | aminopolypeptidase | | $\alpha$-$NH_2$ group end of the protein |
| | catheptic carboxy-polypeptidase | polypeptides | $\alpha$-COOH group end of the protein |
| Proteinases | pepsin<br>trypsin<br>papain and cathepsin | high molecular proteins and peptones | cations<br>anions<br>zwitterions |

certain peptide bonds characterized by the constituent amino-acids involved. Bergmann's discovery opened new ways for the classification of proteolytic enzymes, and the classification made by *Smith* (1960) is based on the early discoveries by Bergmann. Smith divided the proteolytic enzymes into two distinct groups:

The endopeptidases (proteinases) act on internal peptide bonds of proteins, or specially substituted peptides, whilst the exopeptidases (peptidases) act on peptide bonds adjacent to the terminal amino acids. The exopeptidases are further subdivided into carboxy- and aminopeptidases according to their ability to hydrolyze peptide bonds adjacent to terminal carboxyl or amino groups. This classification is applicable mainly to animal proteases (*Hagihara* 1960) but has been shown to be inadequate for bacterial proteases.

*Hartley* (1960) suggested another classification based on mechanisms of action, rather than origin, specificity or physiological action. He divided his classification into four groups:

1. *Serine proteinases*
   Chymotrypsin, trypsin, elastase, thrombin, plasmin, subtilisin.
2. *Thiol proteinases*
   Papain, ficin, bromelain and possibly some cathepsins.
3. *Acid proteinases*
   Pepsin, rennin.

4. *Metal proteinases*
Carboxypeptidases, aminopeptidases, dipeptidases.

This classification takes into account the groups involved in the active sites of the enzyme, the enzymes' ability to resist changes to low pH, and the content of metals in the enzymes. This classification has been shown to be of value and is used, to some extent, together with the classification described by *Smith.*

Several of the well-characterized proteases of bacterial and mould origin have been shown to be able to hydrolyze both proteins and many oligo-peptides (*Hagihara*). All well-known microbial proteases have very broad sidechain specificities. A Streptococcus griseus protease was shown to hydrolyze 87 % of the total peptide bonds in egg-albumin. Numerous other examples have been reported (*Hagihara*).

The proteases from bacteria have been increasingly studied since the Second World War. The surveys of *Hagihara* and of *Sandvik* (1962) review the development. The aerobic sporeformers have been extensively studied, as these bacteria are probably the richest, and most convenient, sources of bacterial proteases for industrial use. Various types of Streptococci, Pseudomonas, Clostridium, Proteus and Serratio (*Hagihara, Sandvik*) have been shown to produce proteolytic enzymes.

*Proteolytic enzymes of Clostridium botulinum*

Proteolytic activity has long been regarded as an important characteristic for distinguishing between the different types of Clostridium botulinum. In 1925 *Wagner et al.* found a gelatinase in cultures of Clostridium botulinum. This gelatinase was found to be influenced by the medium in which Clostridium botulinum was grown. While working on the purification of botulinum toxin *Snipe et al.* (1928) found that a gelatinase, a peptidase and lipase were precipitated with the toxin by acidification procedures using the supernatant liquid from cultures. *Elberg & Meyer* (1939) studied the extracellular proteolytic system in relation to toxin production.

Various types of Clostridium botulinum showed no correlation between their extracellular proteolytic enzymes and toxin production. They found that Clostridium botulinum type A secreted

Table 2. Proteolytic activity in different bacteria according to *Maschmann.*

| Organism* | Proteases | | | |
|---|---|---|---|---|
| | oxygen stable | | oxygen labile | |
| | specific gelatinases | proteinases inhibited by normal serum | proteinases inhibited by normal serum | peptidases |
| *B. prodigiosus* | — | + | — | + |
| *B. fluorescence* | — | + | — | + |
| *B. pyocyaneus* | — | + | — | + |
| *B. botulinus* | — | + | + | + |
| *B. chaveaui* | + | — | — | + |
| *B. histolyticus* | + | + | + | + |
| *B. welchi* | + | — | + | + |
| *Vibrio septicus* | + | — | + | + |
| *B. sporogenes* | — | + | + | + |
| *B. tetani* | — | + | 0 | 0 |

* Maschmann's nomenclature.

0: Not tested.

three different types of enzymes in the supernatant, namely a proteinase, a polypeptidase acting on Witte's peptone and an aminopeptidase hydrolyzing leucylglycylglycine at pH 7.0—8.0.

*Maschmann* (1937, 1938) also studied Clostridium botulinum, and the proteases produced by the organisms which Maschmann studied are divided in four groups as shown in Table 2.

*Maschmann* (1943) later on questioned the presence of two proteases; altogether he found three different enzymes from Clostridium botulinum. He worked on types A and B simultaneously and did not differentiate between them. One of the enzymes was a proteinase, and the other two were peptidases. The proteinase attacked ovalbumin, casein, gelatine, and peptone. Its pH-optimum seemed to be pH 7. As a crude material this proteinase was only partially active, although full activity was obtained when cysteine or SH-glutathione was added. HCN also seemed to activate this proteinase. It was observed that normal serum inhibited this protein to some extent.

In 1956 *Millonigg* isolated an aminopeptidase from Clostridium botulinum type B. The material was purified by precipitation from the culture supernatant with ammonium sulphate. Optimum conditions were found to be 1 M sodium chloride at pH 6.0 and an ammonium sulphate saturation value of 0.3.

His solubility measurements and ultracentrifuge analysis on the purified preparation indicated the presence of one component. This proteinase was activated by $Fe^{++}$ cysteine and was shown to be an aminopeptidase capable of splitting tripeptides but not dipeptides.

*Kodama* (1961) investigated the proteolytic activity in culture supernatants of Clostridium botulinum types A and B. He found that both type A and type B culture supernatants exhibited proteolytic activity with a pH-optimum of 7. No activators were needed. Normal sera, EDTA and copper sulphate inhibited the activity, whereas DFP or soybean trypsin inhibitor had no effect. The author claims that these observations show that the proteolytic activities of types A and B Clostridium botulinum are different from that of trypsin, although the proteases are classified as so-called trypsin-type.

Kodama partially purified the proteolytic enzyme fraction from Clostridium botulinum type A supernatant by ammonium sulphate fractionation, followed by acetone precipitation. The specific activity of the enzyme increased approx. 80 to 85 times after this purification, as compared to the culture supernatant.

The optimum pH and temperature of this fraction was 7.0 and 55°C. The activity of this fraction was also inhibited by normal sera, EDTA and copper sulphate although the degree of inhibition was somewhat different from that for the culture supernatant.

Kodama investigated Clostridium botulinum type E-strains for proteolytic activity, but the culture supernatants from the strains investigated did not attack gelatine and casein under the conditions used.

*Ashmarin & Vorontsov* (1963) investigated some properties of highly purified proteinase from Clostridium botulinum type B. The extracellular proteinase of Clostridium botulinum causes the most extensive degradation of gelatine and casein at pH 6.5—8.0. At pH below 4.5 it undergoes irreversible inactivation. The isoelectric point for this proteinase lies within the range pH 7.4—8.05. Mg- and Ca-ions increased the stability of the proteinase. The authors investigated a method for rabbit immunization, and the sera obtained possessed high antiproteinase titres.

In 1964 *Skulberg* made a comparison of the proteolytic activity in toxigenic and non-toxigenic cultures of Clostridium botu-

linum types A and B. The results indicated that the hypotoxigenic variants of Clostridium botulinum types A and B generally have more proteolytic activity than toxigenic forms of the same organisms. He examined the proteolytic activity using Sandvik's method (*Sandvik*) for estimating proteolytic enzymes. This casein precipitating method is considered applicable to quantitative determinations of bacterial proteinases. Skulberg also investigated the production and purification of proteinases produced by toxigenic strains of Clostridium botulinum types A and B. He found sterile skim milk the most suitable medium for the production of casein-precipitating enzymes from Clostridium botulinum types A and B. Concentration of the enzymes was carried out by precipitation of the culture filtrates with ammonium sulphate to 70 % saturation followed by chromatography on DEAE-cellulose to purify and separate the enzymes. The elution of the enzymes took place at 4—5°C with a combined acetate buffer and sodium chloride gradient. The number of peaks with proteolytic activity varied from one experiment to another when using concentrated preparations originating from different cultures of the same organism. Two or three peaks were usually found. Skulberg suggested that further investigations in connection with the fractionation of the casein-precipitating enzymes of Clostridium botulinum may be necessary.

A protease produced by Clostridium botulinum type B (strain Lamanna) was isolated and characterized by *DasGupta & Sugiyama* (1972). Purification steps were precipitation with ammonium sulphate, chromatography on QAE-Sephadex at pH 5.8, gel filtration through Sephadex G-100, and chromatography on SE-Sephadex at pH 5.0. Molecular weight of the enzyme by sedimentation equilibrium is 34,400 and by gel filtration 40,000; isoelectric pH is 4.62. Optimum pH for amidase activity is 6.2 and for esterase activity 6.2—7.0. The enzyme acts only on bonds formed by the carboxyl group of arginine and lysine residues. It is not inhibited by trypsin inhibitor from soy bean, lima bean, or ovomucoid. The enzyme is active only when in the reduced state and is more stable when $Ca^{2+}$ is present.

*Hausken* (1967) used an immunoelectrophoretic method (*Sandvik*) for the serological differentiation of extracellular proteolytic enzymes in order to study the serological relationship between species of the genus Clostridium. This technique is an immunoelectrophoretic procedure, in which the proteolytic activ-

ity of the enzymes is neutralized by specific antiproteinases. They are produced in rabbits after subcutaneous injection of the crude proteinase together with Freund's adjuvant. With this method the author found a close relationship between proteolytic strains of Clostridium perfringens, Clostridium sporogenes and Clostridium botulinum. He also observed cross-reactions between enzymes of Clostridium perfringens and Clostridium botulinum types A, B and E. The author did not investigate whether any serological relationship between the proteolytic enzymes of the different types of Clostridium botulinum existed.

The immunological relationships between the various strains of Clostridium botulinum and related organisms, using their somatic antigens, have been investigated by the *Division of Microbiology, Bureau of Science, FDA* (1969). Antisera were produced in rabbits, and the antigens consisted of overnight broth cultures heated for one hr. at 100°C. The methods of investigation included tube agglutination, agglutinin absorption and indirect fluorescent antibody techniques. The results indicated a system which classifies the various types of Clostridium botulinum according to their proteolytic activities, rather than by the specific toxins produced by each type.Thus Clostridium botulinum type E and its non-toxigenic variants share common somatic antigens with non-proteolytic strains of types B and F. Absorption of one of the antisera with antigen of the other types concerned, removes the antibody of all three types. Three atypical strains also cross-reacted with these antisera, but OS and TP variants did not, nor did type A, type C, Clostridium perfringens, Clostridium sporogenes, Clostridium bifermentans or Clostridium sordelli. Another serological group incorporated Clostridium botulinum type A and the proteolytic strains of types B and F. Clostridium sporogenes strain 3679 also agglutinates with antisera of this group, but with a lower titre. These results are in agreement with DNA-homology studies reported by *Lee & Riemann* (1970).

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SAMMENDRAG

*Clostridium botulinum proteases. I. Klassifisering av proteaser og litteraturoversigt.*

Proteolytisk aktivitet betraktes som et viktig taksonomisk kriterium for Clostridium botulinum. Alle undersøkte Clostridium botulinum type A-stammer har proteolytisk aktivitet, mens Clostridium botulinum type B og F har både proteolytiske og ikke-proteolytiske stammer. Clostridium botulinum type C og D ble tidligere betraktet som ikke proteolytiske, men senere undersøkelser har vist at svakt toksiske stammer har proteolytisk aktivitet. Det er delte meninger om hvorvidt Clostridium botulinum type E-stammer har proteolytisk aktivitet. I denne artikkel gis en kort oversikt om klassifisering av proteolytiske enzymer og videre en oversikt over utførte undersøkelser av proteolytisk aktivitet hos forskjellige Clostridium botulinum stammer.

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