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PROTEASES OF CLOSTRIDIUM BOTULINUM

II. THE RELATIONSHIP BETWEEN GROWTH MEDIUM AND THE PRODUCTION OF PROTEASES BY CLOSTRIDIUM BOTULINUM TYPES A, B, C, D, E AND F

By

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TJABERG, TORE BJARNE: *Proteases of Clostridium botulinum. II. The relationship between growth medium and the production of proteases by Clostridium botulinum types A, B, C, D, E and F.* Acta vet. scand. 1973, *14*, 193—200. — The present investigation was carried out in order to find a suitable medium for the production of proteolytic enzymes from different types of Clostridium botulinum. Proteolytic activity was found in Clostridium botulinum types A, B, C, D and F, while supernatants of Clostridium botulinum type E did not possess any proteolytic activity at all.

Skim milk medium possessed the greatest ability for the production of proteolytic enzymes from the different cultures of Clostridium botulinum tested, while Robertson's meat broth produced lowest amounts. Highest titres were usually found after 4—5 days of incubation and, after this period, the level of proteolytic activity decreased.

Clostridium botulinum; proteases.

Many different media have been used for cultivating bacteria with the aim of producing proteolytic enzymes. The medium giving optimal growth of the bacterium to be investigated has usually been used without taking into consideration the possibility of modifying the medium to give an increased yield of proteolytic enzymes. Skim milk has often been used as a suitable medium for the production of proteolytic enzymes from different bacteria (*Sandvik* 1962, *Skulberg* 1964). *Sandvik* (1962) usually used milk agar based on skim milk or diluted skim milk. The use of both types of media permitted the selection of the culture giving the highest yield of enzymes. With certain strains of Staphylococci, a semi-solid agar has been utilized. A number of

other media and the addition of various salts were investigated, but without any improvement on the media already described. *Skulberg* made a comparison of different media for use in the production of proteolytic enzymes from Clostridium botulinum types A and B. Trypticase-yeast extract-medium, Robertson's broth medium, corn steep medium, peptone water and skim milk medium were compared. Skim milk proved to be the best and gave maximum proteolytic activity after 9 days of incubation for Clostridium botulinum type A, after 7 days of incubation for Clostridium botulinum type B.

Hampson et al. (1963) used a specially prepared casein medium (*Gladstone & Fildes* 1940) for producing a protease from Proteus mirabilis. The casein was hydrolyzed at 37°C for 2 weeks, thereby reducing the formation of insoluble material. This crude hydrolysate contained coloured substances which interfered with the isolation procedures, and the medium was treated with charcoal. Complete removal of the coloured components from the medium considerably reduced the formation of this protease.

Niven (1944) used a casein hydrolysate medium for the cultivation of Streptococcus lactis. *Williamson et al.* (1964) used a modification of this medium where the casein hydrolysate was replaced by non-hydrolyzed casein at a concentration of 2 mg/ml medium, for production of proteolytic enzymes from Streptococcus lactis. *Bleiveis & Zimmermann* (1964) used N-Z casein synthetic medium for the production of protease from Streptococcus faecalis var. liquefaciens.

Before further investigations of the proteolytic enzymes from different types of Clostridium botulinum could be carried out it was necessary to find a suitable medium which would allow the different types to grow and produce fairly good yields of proteolytic enzymes. This investigation was carried out with the aim of studying the proteolytic activity of different types of Clostridium botulinum, and to test their ability to produce proteolytic enzymes in different media.

MATERIALS AND METHODS

Bacterial cultures

The bacterial cultures used in the present investigation were Clostridium botulinum type A (strain Hall), Clostridium botuli-

num type B (strain Beans), Clostridium botulinum type C (strain Cid proteolytic), Clostridium botulinum type D (strain DIR), Clostridium botulinum type F (strain Langeland) and Clostridium botulinum type E (strain Hazen, strain Fredriksberg, strain 8-OT, strain E-mink, strain E rakefisk V/N, strain 1576/617, strain 1537/62, strain 3226/601, strain 1663/61, strain 1957, strain 16/63). The various types and strains were obtained from the collections of Dr. Anton Skulberg, the Norwegian Food Research Institute, Ås, Norway and Dr. Hans Riemann, Department of Epidemiology and Preventive Medicine, University of California, Davis, USA.

Culture media

The organisms were cultivated on Robertson's meat broth (*Sandvik* 1960), plated onto blood agar (3 % agar) and incubated under anaerobic conditions for 20 hrs. at 35°C. From this medium one colony was selected and cultured on Robertson's meat broth. Three ml of this final culture broth was added to the experimental media. The media used in the present investigations were:

1. *Robertson's meat broth* (R) (*Sandvik* 1960)
2. *Skim milk medium* (SM)
   skim milk — pH = 6.8 — 100 %
3. *Skim milk with yeast extract* (SMY)
   yeast extract (Difco) — 1 % w/v
   skim milk — to 100 %
   pH = 7.0
4. *Proteosepeptone, trypticase, yeast extract, glucosemedium* (PTYG)
   proteosepeptone (Difco) — 2 % w/v
   trypticase (N-Z-amin type B, Sheffield chemicals, New York) — 2 % w/v
   yeast extract (Difco) — 2 % w/v
   glucose (Merck) — 0.5 % w/v
   distilled water — to 100 %
   pH = 7.0

The inoculated media were incubated at 35°C and samples were withdrawn after 1, 2, 3, 4, 5, 6, 7 and 12 days.

For Clostridium botulinum type E incubated at 30°C, samples

were also taken after 14, 21 and 28 days. Bacterial counts were made on all samples in a Petroff-Hauser counting chamber, and the samples were adjusted to the same bacterial count with distilled water before further dilution.

Protease determination

The determination of casein precipitating activity was performed according to *Sandvik* (1962). The culture broths were used in these assays, and a double dilution technique was employed with distilled water as diluent. The CP-titre was determined according to *Sandvik* (1962).

Determination of protease inhibitors

The crosswise casein precipitation inhibition (CPI) test was performed on culture supernatants of Clostridium botulinum types A, B and E according to a method described by *Fossum* (1970).

RESULTS

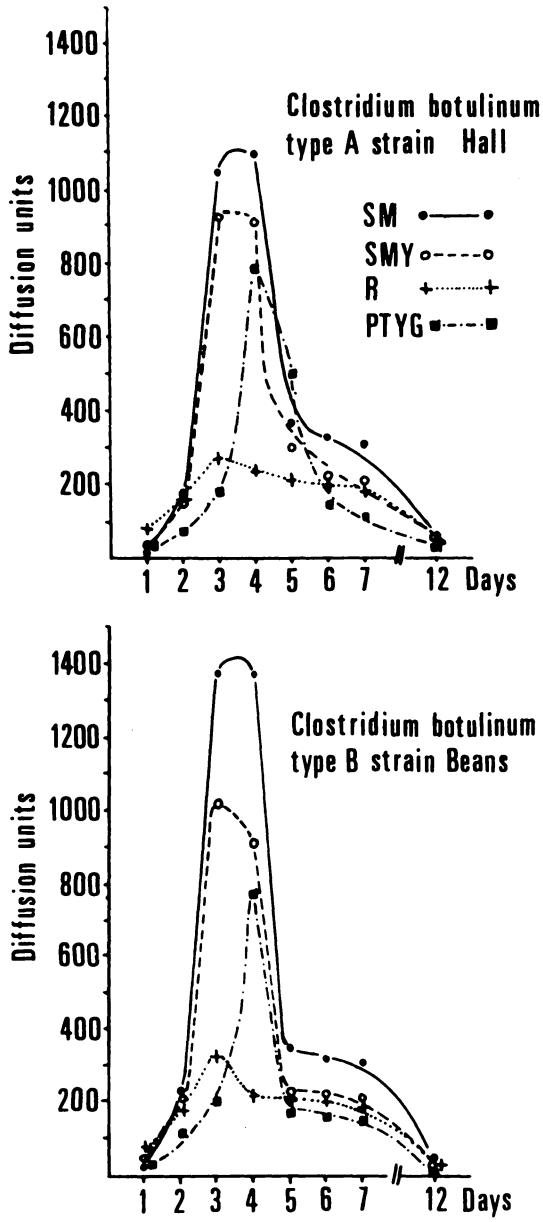
Figs. 1—5 give the proteolytic activities of Clostridium botulinum types A, B, C, D and F in different media.

It was not possible to find any proteolytic activity in the culture broths from the strains of Clostridium botulinum type E tested. Supernatants from the strains of Clostridium botulinum type E tested did not inhibit the proteolytic activity of trypsin on casein agar.

Trypsin inhibitors were not found in supernatants from any of the cultures tested.

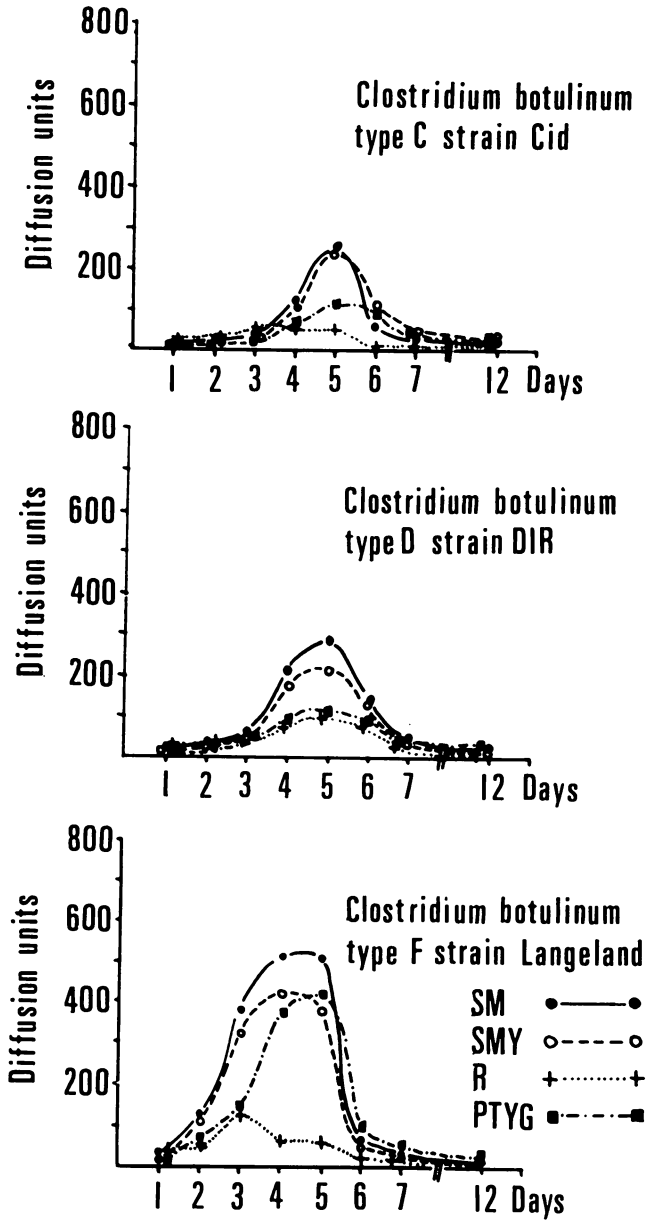
DISCUSSION

Clostridium botulinum types A, B, C, D and F all possessed proteolytic activity while no proteolytic activity could be found in the strains of Clostridium botulinum type E tested. Proteolytic activity in Clostridium botulinum type E has been described by *Dolman* (1957), *Skulberg* (1964) and *Hausken* (1967). The strains used by *Skulberg* and *Hausken* were tested in the four media without finding any proteolytic activity after 28 days of incubation. *Høyem & Skulberg* (1962) described inhibitors against trypsin in cultures of Clostridium botulinum types A, B and E. The possible occurrence of inhibitors was therefore



Figures 1 and 2. The proteolytic activities in different media obtained from Clostridium botulinum type A (strain Hall) and Clostridium botulinum type B (strain Beans).

●———● SM (Skim milk); o-----o SMY (Skim milk with yeast extract); +·····+ R (Robertson's meat broth); ■·-·-·■ PTYG (Proteosepeptone, trypticase, yeast extract, glucose medium).



Figures 3, 4 and 5. The proteolytic activities in different media obtained from Clostridium botulinum type C (strain Cid proteolytic), Clostridium botulinum type D (strain DIR) and Clostridium botulinum type F (strain Langeland).

●———● SM (Skim milk); o----o SMY (Skim milk with yeast extract); +·····+ R (Robertson's meat broth); ■·-·-·■ PTYG (Proteosepeptone, trypticase, yeast extract, glucose medium).

considered as one of the main reasons for not finding proteolytic activity in Clostridium botulinum type E, but no inhibitory action against trypsin was found in the supernatants of the 11 strains of Clostridium botulinum type E tested in this investigation. Trypsin inhibitors were not found in cultures of Clostridium botulinum type A (strain Hall) and Clostridium botulinum type B (strain Beans). This is in disagreement with results obtained by *Høyem & Skulberg.*

Skim milk medium seemed to possess greatest ability for the production of proteolytic enzymes from the cultures of Clostridium botulinum tested, while Robertson's meat broth produced the lowest amount. After 1 day of incubation, however, it was generally noted that the cultures cultivated on Robertson's meat broth had the highest proteolytic activity, but later in the incubation period the proteolytic activity decreased to a lower level than for the other media. These results led to the choice of skim milk medium as the medium for producing proteolytic enzymes from Clostridium botulinum.

Highest titres were usually found after 4—5 days of incubation and, after this period, the proteolytic activity decreased. Tests for toxicity gave the same pattern for Clostridium botulinum types A, B, and F. The cultures of Clostridium botulinum type C and Clostridium botulinum type D did not possess any toxic activity. This is consistent with previous studies (*Skulberg*) which showed that proteolytic strains of Clostridium botulinum types C and D are weakly toxic, if at all.

Under the present circumstances Clostridium botulinum types A, B and F produced the greatest amounts of proteolytic enzymes, while Clostridium botulinum types C and D gave the lowest amounts. This is in close agreement with the fact that Clostridium botulinum types C and D are regarded as non-proteolytic, and only few hypotoxigenic forms are proteolytically active.

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SAMMENDRAG

Clostridium botulinum proteaser. II. Sammenheng mellem vekstmedium og produksjon av proteaser hos Clostridium botulinum type A, B, C, D, E og F.

Undersøkelsen ble utført få å finne et egnet medium til produksjon av proteolytiske enzymer fra forskjellige stammer av Clostridium botulinum. Man fant proteolytiske enzymer i supernatant fra kulturer av Clostridium botulinum type A, B, C, D og F, mens det ikke ble funnet proteaser hos noen av de kulturer av Clostridium botulinum type E som ble undersøkt. I tidligere undersøkelser er det funnet inhibitorer overfor trypsin i supernatant hos kulturer av Clostridium botulinum type B og E. Det ble ikke funnet inhibitorer overfor trypsin i noen av de undersøkte kulturer av Clostridium botulinum.

Skummet melk medium ga best produksjon av proteolytiske enzymer fra alle stammer av Clostridium botulinum som på forhånd var funnet å være proteolytiske. Høyest titer fant man regelmessig etter 4—5 dager med en derpå følgende senkning av titeret mot 0 etter 10—12 dager.

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