ISOLATION OF A TYPE E SPECIFIC SPORE ANTIGEN OF *CLOSTRIDIUM BOTULINUM*

by

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ABSTRACT

Spores of strains of Clostridium botulinum type E, from trypticase peptone glucose broth cultures, were disrupted in a Bronwill homogenizer and the extracts assayed for serological activity by immunodiffusion. The spore extracts of the Beluga, 070 and PM-15 strains yielded six, three, four precipitin lines with their homologous antisera. Immunodiffusion tests with spore antisera of the heterologous strains indicated that some of the precipitating antigens were strain specific and others were shared by several strains, and at least one spore antigen was common to the seven toxigenic and one nontoxigenic type E strains. Extracts of the spores of C. botulinum types A, B and C. bifermentans did not show precipitin lines with type E spore antisera.

The isolation of the 'common' antigen from spores of the Beluga strain was achieved by means of DEAE-cellulose column chromatography, ammonium sulphate precipitation, gel filtration on Sephadex G-200, and electrofocusing between pH 3-6. The yield from 250 mg of lyophilized spore extract was 1.5 mg of antigen. The homogeneity and purity of the antigen was established by disc electrophoresis. The purified antigen was water soluble and heat stable with an isoelectric point of pI 2.1, and appeared as a pale bluish powder in the dry state. Preliminary biochemical analyses showed that protein, carbohydrate and hexosamine were present in a ratio of 9:3:1.

Immunodiffusion tests showed that the purified spore antigen was common to and specific for the type E strains. The findings indicate that immunochemical studies of the purified 'common' antigen would elucidate the nature of the antigenic determinant of type E specificity. In addition, the preparation of a monospecific type E fluorescein labelled antiserum would facilitate the precise identification of the strains for epidemiological studies and for laboratory diagnosis.

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INTRODUCTION

The identification of the serotypes of Clostridium botulinum, based on the characterization of the type specific toxins, is no longer tenable because of the large numbers of nontoxigenic type E strains which have been recognized (15, 27, 64). Serological tests based on more stable structural components, spore antigens, in particular, which are widely distributed among both the toxigenic and the nontoxigenic strains (19, 66), should provide a more reliable approach. The presence of a multiplicity and diversity of spore antigens of type E strains has been recently demonstrated by immunodiffusion tests (40), and in accord with the findings of the direct fluorescent antibody test (19), at least one of the antigens was common to the type E strains.

The present study describes the isolation and partial characterization of a spore antigen common to and specific for type E strains.

HISTORICAL

Russian workers were the first to isolate, from sturgeon, a type E strain of Clostridium botulinum which had been implicated in an outbreak of botulism in 1937 (24). Since then, other sources of E strains have included marine and aquatic materials from various regions of the northern hemisphere: Japan, the United States, Canada, Sweden, Denmark and the Soviet Union (16).

Of the methods advocated for detection of C. botulinum type E, the fluorescent antibody technique (FAT) has offered the greatest potential for the screening of environmental materials. The FAT was originally developed by Coons (10) for demonstrating the presence of pneumococcal antigens in tissue (11), and has been used by Kalitina (83) to identify serotypes of C. botulinum by staining with fluorescein conjugates of antisera prepared against flagella antigens. The differentiation of type E strains from other serotypes of C. botulinum was achieved by Walker and Batty (76, 77) with fluorescein labelled antisera prepared against vegetative cells of an E strain which specifically stained type E strains. Another immunofluorescent study by Georgala and Boothroyd (21) showed that only 50% of the E strains were specifically stained, and suggested that this was due to a multiplicity of antigens present in vegetative cells of the isolates. These workers also illustrated the non-identity of spore and of vegetative cell antigens, and reported that the spore antigens appeared

to be much more widely distributed than vegetative antigens. Fluorescein conjugates of spore antisera were recently used to stain the spores of a variety of E strains (19).

Antigenicity of bacterial spores was first demonstrated by Defalle (14) in 1902, and no further progress was made until Howie and Cruickshank (28) demonstrated the non-identity of spore and whole cell antigens of C. sporogenes by agglutinin absorption tests. Vennes and Gerhardt (71), Tomcsik and Baumann-Grace (69) have shown that intact or broken spore coats contain antigens which are not present at the surface layer or in other structures of the vegetative cells. The differences between antigens of the spore walls and those of the vegetative cells may be readily understood in terms of their chemical composition (61, 67). In a recent serological analysis of C. botulinum by agglutinin absorption tests, Solomon et al (66) have confirmed the type specificity of the spore antigens.

Three precipitating antigens have been identified in spore extracts of Bacillus subtilis by immunoelectrophoresis (8), and a comparable study of the antigens of B. cereus showed that nine of the twelve antigens were spore specific (3). Immunodiffusion analysis of spore extracts by Walker et al (75) showed that spores of C. sporogenes contained at least four antigens. Recently, Law and Hawirko (40) reported that spore extracts of C. botulinum type E strains contained a multiplicity and diversity of antigens, some of which appeared to be strain specific, whereas others were shared by several

of the strains. In accord with the findings of FAT (19), at least one of the antigens was common to spores of the eight E strains tested.

The hot acid method of extraction of soluble antigens from bacterial cells and spores was introduced by Lancefield for the serological grouping of the hemolytic streptococci (38, 39), and has been applied to the isolation of a type specific carbohydrate from staphylococci (32), precipitating antigens from spores of B. subtilis and B. vulgatus (37). Formamide has been used to extract antigens from the cells of Bacillus spp. (48) and C. perfringens (30) and the spores of C. sporogenes (75). Mechanical devices have also proved effective in extracting soluble antigens from bacterial cells and spores (29, 81). The disruption of spores of Clostridium spp. (40) has recently been accomplished with a Bronwill homogenizer.

In the last decade, column chromatography has been extensively used for the isolation and purification of antigens, enzymes and other complex mixtures. A common type of chromatography is the ion-exchange chromatography using cellulose ion-exchangers developed by Peterson and Sober (56). Haukenes (26) isolated a polysaccharide antigen from Staphylococcus aureus by chromatography on DEAE-cellulose and Dowex-1 ion-exchange columns. A similar procedure was used by Losengrad and Oeding (42) to obtain a polysaccharide antigen from distrupted cells of strains of Staph. epidermidis. Keeler and

Pier (33) used DEAE-cellulose column chromatography for the isolation of antigenic material from Nocardia asteroides. Willers et al (80) separated group F antigen from streptococci on DEAE-cellulose using distilled water as the eluant. In 1967, Michel and Krause (45) separated the group F carbohydrate on a column of powdered DEAE-cellulose. Another type of chromatography, which may be used in combination with cellulose ion-exchangers, is the partition chromatography in a non-ionic dextran gel (Sephadex) developed by Porath and Flodin (57). This technique has been used to separate antigens of Corynebacterium hofmanni (4), C. xerosis (50), Bacillus M (82), Entamoeba histolytica (2), mammary tumor virus (49), rabies virus (46) and human aorta (34). The degree of purification of antigens may be assessed by the gel diffusion method, originally described by Bechhold (6), and used by Oudin (52) as single diffusion in one dimension. A double diffusion technique, developed by Ouchterlony (51) and Elek (17), has largely replaced the single diffusion method in antigenic analyses.

Although the general concept of isoelectric fractionation or electrofocusing has been known for some time (68), its application to the separation of biological materials has only recently been introduced (23, 41, 58, 73, 78). Electrofocusing refers to the fractionation of large molecular weight ampholytes, such as proteins, according to their isoelectric points by exposure to an applied voltage in a natural pH

gradient, and is generally carried out in a sucrose density gradient contained in a double-jacketed column, or in polyacrylamide gel by gel electrofocusing (55). In 1969, Valmet (70) described a zone convection electrofocusing process which appears to offer a much simpler technique for preparative purposes.

The main obstacles to the development and application of electrofocusing had been technical, primarily related to the prevention of convection in the electrolyte system and the maintenance of a stable pH gradient. In 1954, Kolin (35) succeeded in preventing convective disturbances in a liquid column by using a density gradient which consisted of a sucrose solution with a gradually decreasing concentration from the bottom to the top of the column. A major advance has been the synthesis of a series of ampholytes which proved suitable for producing an equilibrated pH gradient (73). Vesterberg and Svensson called such a pH gradient a natural one, since until the current is applied, the pH is constant throughout the solution. The pH gradient in equilibrium occurs after the current has transported each low molecular ampholyte to its isoelectric point. The state of equilibrium provides a stable, natural pH gradient which allows the isoelectric fractionation of high molecular ampholytes, such as proteins and polypeptides.

It is primarily due to the fundamental work of Vesterberg and Svensson that electrofocusing has become a technique of

major importance comparable to the classical procedures of electrophoresis, gel filtration and ion-exchange chromatography. Electrofocusing has been applied to studies of cytochrome c from beef heart (20), bovine metmyoglobin (62), Myxine glutinosa L. hemoglobins (58), human erythrocuprein (5), insulin (54) and clotting factors II (prothrombin) and IX (plasma thromboplastin component) (53), and to fractionations of yeast invertase (72), cellulases (1), fungal laccase (31), α,-acid glycoprotein from chimpanzee plasma (41) and enzymes and toxins from Staphylococcus aureus (74). Recently, Ali Khan and Meerovitch (2) used electrofocusing in 5% polyacrylamide gel with 2% ampholine (pH 3-10) in the physiochemical studies of the antigens of Entamoeba histolytica.

Polyacrylamide gel electrophoresis, which was first utilized by Raymond and Weintraub (59), has been used to determine the purity and homogeneity of human serum proteins (13), protein components and esterases of the various strains of group D streptococci (44) and antigens isolated from Nocardia asteroides (33), Bacillus M (82), Corynebacterium hofmanni (4) and C. xerosis (50).

MATERIALS AND METHODS

Cultures

Clostridium botulinum type E toxigenic strains 070, 5191, 5192, 715 and the nontoxigenic PM-15 strain were received from Food and Drug Administration, Washington, D.C. The toxigenic VH-O from Food and Drug Directorate, Ottawa; ATCC 9564 from the American Type Culture Collection; the Beluga strain and *C. botulinum* types A and B and *C. bifermentans* G301 from the Laboratory of Hygiene, Ottawa.

Buffer

Sodium phosphate buffer 0.02 M, pH 7.2, was used throughout these studies.

Growth and Preparation of Spores

Sporulation was induced by a modified pseudosynchronous growth technique (40). Colonies of type E strains, grown on egg yolk agar for two days at 30 C, were transferred to trypticase peptone glucose (TPG) broth containing 5% trypticase (Baltimore Biological Laboratory), 0.5% proteose peptone (Difco), 0.4% glucose and 0.2% mercaptoacetic acid (Matheson, Coleman and Bell). After incubation for two to four days at 30 C, the degree of sporulation was estimated by phase contrast microscopy. Cultures showing a high degree of sporulation, with endospores completely released, were transferred to a series of 20 tubes of TPG using a 10% inoculum. After incubation, an

inoculum from the culture tube showing the highest degree of sporulation was transferred to another series of tubes, and then repeated three or four times until >90% sporulation was achieved. Sporulation in large volumes was induced with a 10% inoculum in 1400 ml TPG.

Twenty grams of spores were obtained from 18 liters TPG cultures showing ≥90% sporulation, and harvested by centrifugation in a Sorvall RC2-B at 10,000 x g for 15 min. The spore mass was washed in distilled water and homogenized with a minimum amount of phosphate buffer in a Bellco tissue grinder. The homogenate was digested with trypsin (Nutritional Biochemicals Corporation), 100 µg/ml and lysozyme (Mann Research Laboratory), 200 µg/ml, for 2 hr at 37 C. The cellular debris was removed by differential centrifugation at 1000 x g for 20 min, 4000 x g for 10 min, 10,000 x g for 5 min. The cleaned spores were washed for at least four times with phosphate buffer before storage at -20 C.

Preparation of Spore Extracts

The method described by Law and Hawirko (40) was used with minor modifications. Approximately 10 g lots of cleaned spores of Clostridium spp. were suspended in a minimum amount of buffer and five volumes of 0.25 - 0.30 mm glass beads. The spores were disrupted in a Bronwill homogenizer for five 90 sec periods. The spore coats were removed by centrifugation at 18,000 x g for 20 min, and the supernatant was dialyzed

against water overnight at 4 C, lyophilized, and stored at -20 C.

Acid extracts of whole spores were prepared for control studies. Two grams wet mass of the Beluga strain was treated with four volumes of 0.0625 N HCl, held in a boiling water bath for 10 min, cooled rapidly on ice, adjusted to pH 7.2, and centrifuged at 20,000 x g for 15 min. The supernatant was dialyzed against four liters of phosphate buffer at 4 C for 24 hr, lyophilized, and stored at -20 C.

Fractionation of Spore Extract

(a) DEAE-cellulose

Diethylaminoethyl (DEAE)-cellulose (Sigma, fine mesh), prepared as described by Peterson and Sober (56), was washed once in distilled water, twice in 0.5 N NaOH/0.5 N NaCl, several times in distilled water, and finally in phosphate buffer until pH 7.2 was obtained. A column (2.5 x 45 cm) was packed by pouring the slurry into a reservoir mounted on the column, and equilibrated by passing through 500 ml of phosphate buffer with a pressure of about 100 cm water. Approximately 250 mg of lyophilized extract of the Beluga strain was dissolved in 10 ml of the buffer, absorbed onto the DEAE column, and eluted using a continuous linear gradient up to 0.5 M KCl in the buffer at 4 C. The flow rate was regulated to about 50 ml per hr and 200 fractions of 4 ml were collected. The protein content of each fraction was determined by UV absorption at 280 nm on a Beckman spectrophotometer.

The fractions from each of the absorption peaks were pooled, concentrated by dialysis against air, and subsequently against two liters of buffer for 24 hr at 4 C.

(b) Ammonium Sulphate Precipitation

The proteins of the pooled fractions of the first DEAE-cellulose absorption peak were precipitated by ammonium sulphate added dropwise with constant stirring until the concentration was 70% saturation. The precipitate was collected by centrifugation at 15,000 x g for 20 min, redissolved in 5 ml of phosphate buffer, and dialysed for 24 hr against the buffer.

(c) Sephadex G-200

The procedures were carried out at 4 C. The dry beads of Sephadex G-200 (Pharmacia) were hydrated in a solution of phosphate buffer, allowed to swell for five days, and decanted to remove the smallest particles. The gel was suspended in an excess of the buffer and aspirated until bubbling ceased. A chromatographic column (1.6 x 90 cm) was filled with the buffer, and the slurry was poured down a glass rod into the reservoir mounted on the top of the column. To regulate the operating pressure, the outlet tubing of the column was positioned just below the liquid level in the reservoir, and opened to allow the solvent to flow out slowly. The column outlet tubing was gradually lowered until a pressure of 90 mm was reached, and the column was equilibrated for two

to three days with the same buffer solution. Most of the eluant above the gel surface was removed by suction and by opening the column outlet, the remaining eluant was drained away. The outlet was closed while the sample was pipetted on top of the bed, and then opened to allow the sample to drain into the bed. After the surface was washed with a small amount of the buffer, the column was layered with buffer and eluted at a flow rate of four ml per hr. Column effluents were collected in three ml fractions, and the optical density of each was determined at 280 nm in a Beckman Spectrophotometer. The fractions from each of the absorption peaks were pooled, dialysed against two liters distilled water, and lyophilized.

(d) Electrofocusing

The isoelectric fractionation apparatus was assembled as described by Vesterberg and Svensson (73). An LKB column (LKB 8100-10, vol 110 ml) and pH 3-10 ampholine carrier ampholytes (LKB Produkter AB, Stockholm-Bromma 1, Sweden) were used. The dense electrode solution contained 12.0 g sucrose, 14 ml H_2O and 0.2 ml concd H_2SO_4; the light electrode solution, 0.1 g NaOH and 10 ml H_2O; the heavy gradient solution, 28 g sucrose, 42 ml H_2O and 0.9 ml carrier ampholytes; the light gradient solution, 60 ml H_2O and 1.6 ml carrier ampholytes.

The dense electrode solution was poured into the central tube of column with the valve in open position using a funnel and tubing. A sample, containing 15 mg lyophilized fraction from the second Sephadex G-200 absorption peak, was dissolved in the light gradient solution and poured into one of the vessels of a gradient mixer. After the heavy gradient was placed in the second vessel, the connecting valve was opened to allow gradient mixing. The gradient solution was run down smoothly along the inner wall of the electrofocusing compartment by means of gravity feed. The electrofocusing compartment was filled with the light electrode solution with a funnel and tubing, and electrolysis was conducted at 200 v for 48 hr at 4 C. After electrolysis, the valve was closed and the column emptied at a flow rate of 1.5 ml per min to deliver one ml aliquots. The absorbance of each aliquot was determined at 280 nm in a Beckman Spectrophotometer and the pH in the Bach-Simpson pH meter. The fractions from each major absorption peak were pooled, dialysed against distilled water for 96 hr to remove the ampholytes and sucrose, and lyophilized. The experiment was repeated with the lyophilized sample from the absorption peak at pI 2.1 using pH 3-6 ampholine carrier ampholytes, and a potential of 400 v applied to the column. The fractions from the absorption peak were pooled, dialysed against distilled water for 96 hr, lyophilized, and stored at -20 C.

Polyacrylamide Gel Electrophoresis

Acrylamide gel electrophoresis was performed as described by Davis (13) using the Canalco Disc Electrophoresis Trial Kit (CIC, Bethesda, Maryland).

Samples containing 100 µg dry wt of purified and crude extracts of Beluga spores were mixed with equal amounts of 40% sucrose in a total volume of 0.20 ml. Electrophoresis was carried out in Tris-glycine buffer, pH 9.5, for 60 to 90 min at 1.25 ma per column (initial current) and 2.5 ma per column (after the dye had entered the stacking gel) using the Canalco Model 150 v power supply. After completion of the run, the gel columns (5 x 75 mm) were removed, stained for 90 min with 0.025% coomasie blue for proteins, and destained with 7% acetic acid.

Preparation of Antisera

Adult albino rabbits were immunized with spore suspensions and with spore extracts of the type E 070, Beluga and PM-15 strains.

Cleaned spores were suspended in 0.2% formol saline, heated for 15 min at 100 C, standardised to a density equal to McFarland tube no. 3, and stored at room temperature for 72 hr prior to intravenous inoculations at the rate of three per week for four weeks. A second course of inoculations was carried out after a rest period of one week, and the sera were collected five to six days after the final injection.

Extracts of cleaned spores containing 10 mg solid per ml saline were sterilised by Millipore filtration. Various routes of inoculations, spaced three days apart, were applied according to the procedure of Kwapinsky (36), 0.3 ml subcutaneous, 0.4 ml intramuscular, 0.4 ml foot pad, 0.4 ml another foot pad, 0.4 ml intravenous, 0.5 ml subcutaneous. Sera were collected one week after the final injection.

Absorption of Antisera

An equal volume of vegetative cells from an 18 hr culture was mixed with the spore antiserum, and left at room temperature for 2 hr with occasional shaking.

Immunodiffusion Tests

A modification of Ouchterlony's agar plate method (51) was used. The bottom of a smooth glass Petri dish (inner diam 9 cm) was coated with a film of 2.0% Noble agar (Difco), and then layered with 20 ml of 0.85% Noble agar containing 1% sodium azide and 0.88% sodium chloride, and stored at 4 C for 24 hr. A horizontal central trough (2 x 50 mm) was cut, and filled with undiluted antiserum. Antigen wells (diam 4 mm), placed 4 mm from the trough, were punched with a no. 1 cork borer, and filled with 0.03 ml of antigens. Radial immunodiffusion of the purified antigen was carried out with the antiserum placed in a central well of 7 mm and dilutions of antigen in four circumferential wells, 4 mm in diam, cut equal distances apart so that the distance from the edge of

the center well was about 10 mm. The plates were kept in a moist chamber at room temperature, and observed daily for precipitin lines for a period of four days.

Immunodiffusion was also performed on cellulose acetate membrane using NIL-Saravis Immunodiffusion Kit. The template, support, and base were washed in soap, rinsed thoroughly and dried. A cellotate membrane was soaked in saline, and then 'rolled' onto the bottom surface of the template. A piece of parafilm M 3 1/16 in. x 3 7/8 in. was wet with saline, and placed over the cellotate membrane on the bottom of the template. The template and film assembly were inverted and placed carefully onto the support and base. The template was fastened to the base with nylon bolts, and tightened with the torque wrench to approximately five lb. After excess saline was expelled from the wells, the test antigens were deposited in the wells using a Takatsky micropipette, and the channels were slowly filled with antiserum. The assembly was incubated in a moist chamber at room temperature for three days, and the wells and channels were flushed with saline before the unit was dissembled. The membrane was washed in a few changes of saline for 60 min, stained for five min with 0.2% ponceau S dye, and destained for 1 hr in four changes of 5% acetic acid.

Protein Determination

Protein content was assayed by the Folin method of Lowry *et al* (43) using crystalline bovine albumin as standard.

Carbohydrate Determination

Carbohydrate was estimated by a modification of the Anthrone method of Scott and Melvin (65) using dextrose as standard.

Hexosamine Determination

The modified Elson-Morgan method as outlined in Kabat and Mayer (18) was followed using glucosamine hydrochloride as standard.

RESULTS

Strains of C. botulinum types E, A and B, and C. bifermentans, on initial transfer from egg yolk agar into TPG broth, showed sporulation ranging from 0 to 20%. After the second transfer of a 20% sporulating inoculum, up to 60% was observed in some tubes, and >90% was attained after three to five subsequent transfers of selected inocula. Sporulating cells first appeared after 15 hr growth, increased to 60% after 24 hr, >90% after 60 hr, and the spores were released from their sporangia after 72 hr. Transfer of selected inocula, repeated for as many as 20 times, consistently showed a spore population of >90%.

Subcultures of strains of the Clostridium spp. showed <50% sporulation in thioglycollate broth, Brewer modified (BBL), <70% sporulation in trypticase peptone glucose biphasic medium and >90% sporulation with abundant growth in standard TPG broth.

Phase contrast microscopic examination of the spore mass showed that spores, cleaned by digestion with lysozyme and trypsin, and repeated washing and differential centrifugation, were free of vegetative cells and debris. The yield from 18 liters of TPG cultures was approximately 16 g of clean spore mass.

Immunodiffusion tests of the Beluga spore antiserum showed six precipitin lines with Beluga spore extracts, three

lines with spore extracts of the 070 strain, two lines with spore extracts of PM-15, VH-0, 5192, 715, 9564 and one line with the 5191 strain. Immunodiffusion tests of the 070 spore antiserum showed three lines with spore extracts of the 070 strain, two lines with spore extracts of 5192, 715, 9564, Beluga and one line with PM-15, VH-O and 5191 strains. Immunodiffusion tests of the PM-15 spore antiserum showed four lines with PM-15 spore extracts, three lines with spore extracts of 715, 9564, two lines with Beluga, 070, 5192 and one line with 5191 and VH-O strains (Table 1).

A line of identity or 'common' antigen was observed with extracts of 7/7 toxigenic and 1/1 nontoxigenic strains of type E spores and with a hot acid extract of whole spores of the Beluga strain (Figs. 8, 9, 10, 11a, 11b, 12a). Absorption of the spore antisera with young vegetative cells did not eliminate the precipitin line of identity. Spore extracts of C. botulinum types A and B and of C. bifermentans did not form precipitin lines with the type E spore antisera.

Spore extracts of the Beluga strain were separated into six absorption peaks by DEAE-cellulose column chromatography (Fig. 1). Immunodiffusion tests of the Beluga spore antiserum with samples of the pooled fractions of the first, second and fourth absorption peaks gave three, two, and two precipitin lines respectively. The first peak was shown to contain the 'common' antigen (Figs. 7, 11a, 12b). Precipitin lines were not obtained from the samples of the fractions of the third, fifth and sixth peaks.

Fractions from the first absorption peak, eluted on a Sephadex G-200 column after precipitation with ammonium sulphate, were separated into two absorption peaks (Fig. 2). Immunodiffusion tests showed that the 'common' antigen was present in the second Sephadex peak (Fig. 12b).

Lyophilized pooled fractions containing the 'common' antigen, electrolysed in a LKB electrofocusing column using pH 3-10 ampholine carrier ampholytes, were separated into two major absorption peaks at pI 2.1 and pI 4.8 (Fig. 3). The peak at pI 2.1 contained the 'common' antigen (Figs. 8, 9, 10, 11b, 12a, 12b). Pooled fractions from this peak were focused at the same pI as a single peak when subjected to electrolysis using pH 3-6 ampholine carrier ampholytes (Fig. 4). The purity of the 'common' antigen was verified by disc electrophoresis on polyacrylamide gel; a single band at a distance of 5 mm behind the tracking band was obtained (Fig. 5). At least 20 bands were observed with the crude extract (Fig. 6), eight with the first DEAE absorption peak, and five with the second Sephadex absorption peak. The single precipitin line to the purified antigen (Figs. 8, 9, 10, 11b, 12a, 12b) appeared closer to the antiserum source than to the antigen even when the latter was diluted ten fold. The precipitin line in the radial immunodiffusion test curved towards the antiserum well.

The yield of the purified 'common' antigen from 250 mg of lyophilized spore extract of the Beluga strain was 1.5 mg.

The antigen was water soluble, heat stable, and appeared as a pale bluish powder in the dry state. Biochemical analyses showed a protein content of 45%, carbohydrate 14% and hexosamine 5%.

TABLE I

Antigens of spores of *Clostridium botulinum* type E strains

Spore extracts	Antiserum		
	Beluga	070	PM-15
	Number of precipitin lines		
C. botulinum, type E			
Toxigenic strains			
Beluga	6	2	2
070	3	3	2
VH-O	2	1	1
5191	1	1	1
5192	2	2	2
715	2	2	3
9564	2	2	3
Nontoxigenic			
PM-15	2	1	4
C. botulinum			
Type A	0	0	0
Type B	0	0	0
C. bifermentans	0	0	0

Fig. 1. Chromatography of crude spore extract of the Beluga strain on DEAE-cellulose.

 precipitation with Beluga spore antiserum

 line of identity

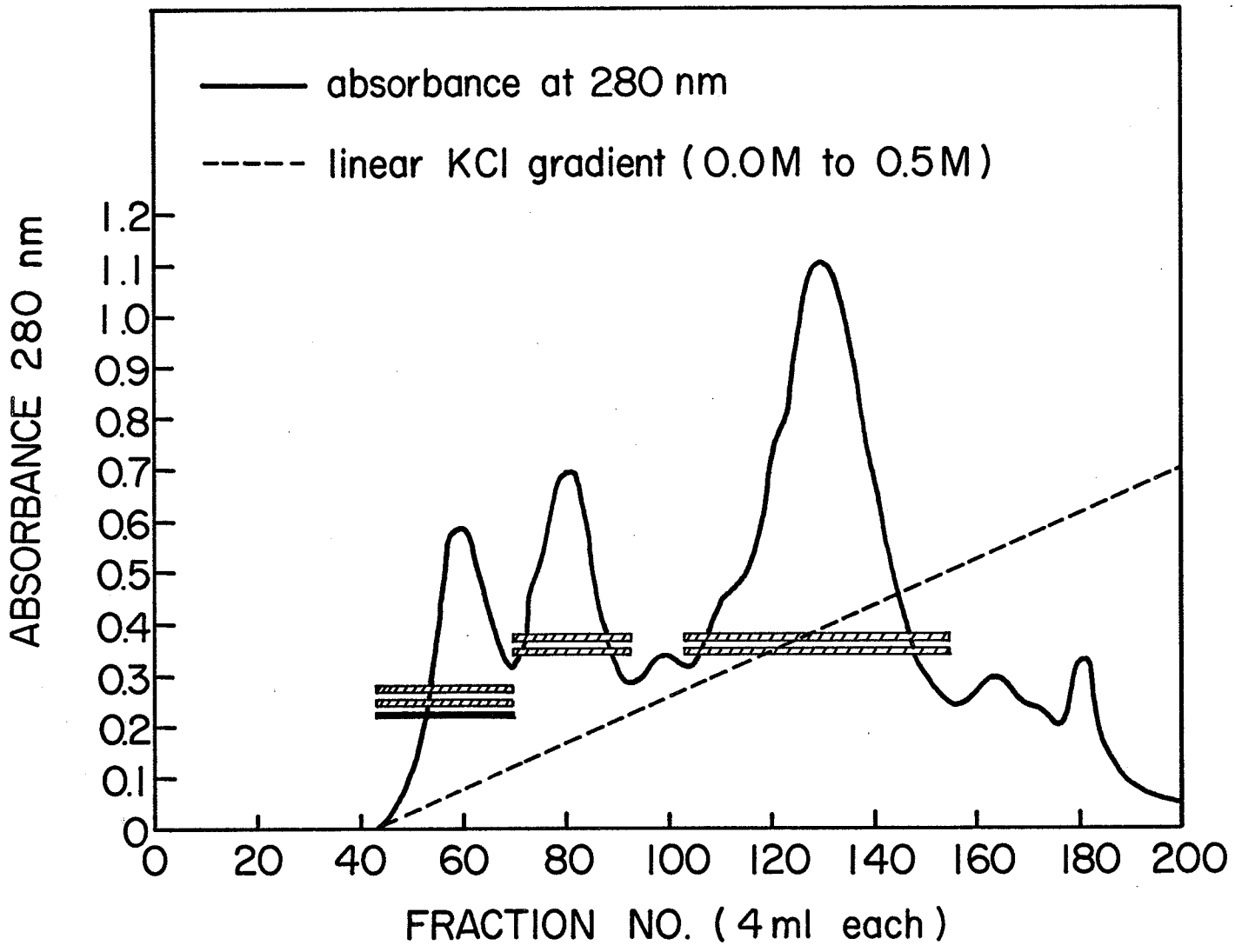

absorbance at 280 nm
linear KCl gradient (0.0M to 0.5M)
ABSORBANCE 280 nm
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
140
160
180
200
FRACTION NO. (4ml each)

Fig. 2. Chromatography of the fractions of the first DEAE-cellulose absorption peak on Sephadex G-200.

 precipitation with PM-15 spore antiserum

 line of identity

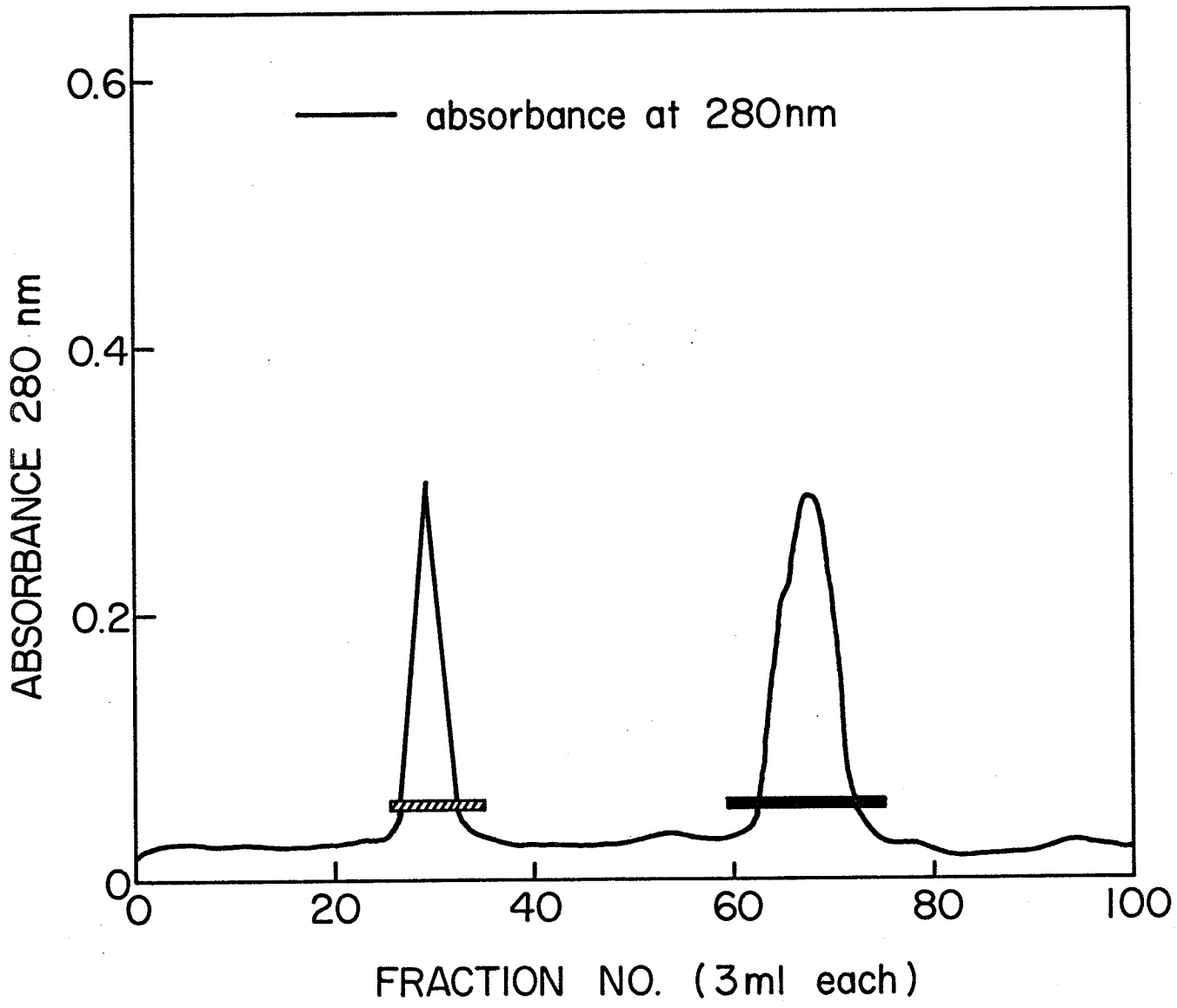

absorbance at 280nm
ABSORBANCE 280 nm
0.6
0.4
0.2
0
0
20
40
60
80
100
FRACTION NO. (3ml each)

Fig. 3. Electrofocusing of the fractions of the second Sephadex G-200 absorption peak in a pH 3-10 gradient.

▬▬ line of identity with PM-15 spore antiserum

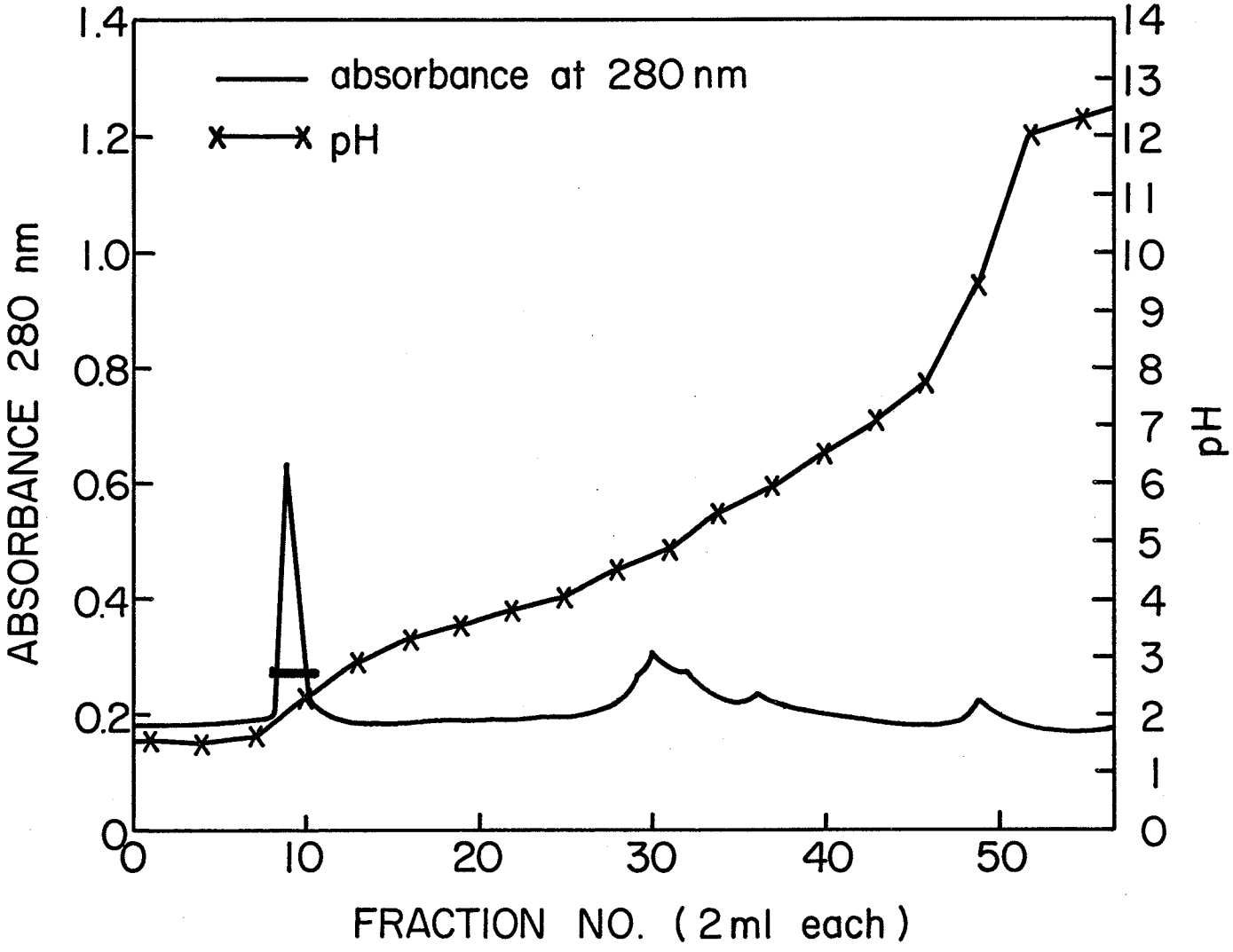
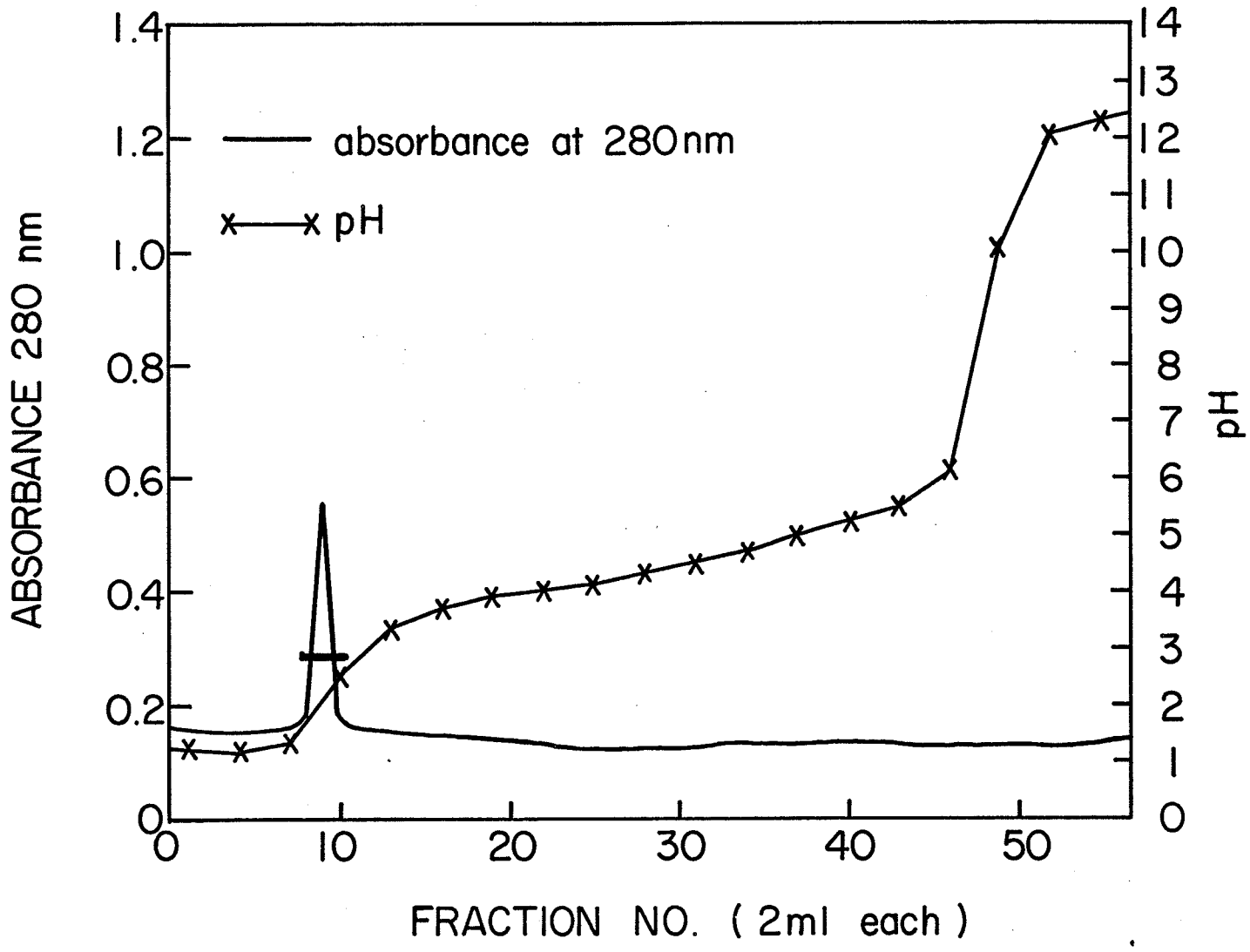

absorbance at 280 nm
pH
ABSORBANCE 280 nm
pH
FRACTION NO. (2 ml each)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
0
10
20
30
40
50

Fig. 4. Reelectrofocusing of the fractions of the first peak in a pH 3-6 gradient.

 line of identity with PM-15 spore antiserum


absorbance at 280nm
pH
ABSORBANCE 280 nm
pH
FRACTION NO. (2ml each)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
0
10
20
30
40
50

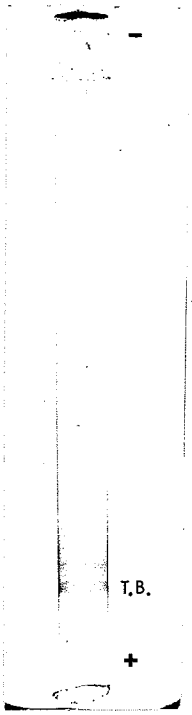


Fig. 5. Polyacrylamide gel disc electrophoresis of the purified 'common' antigen. T.B., tracking band.

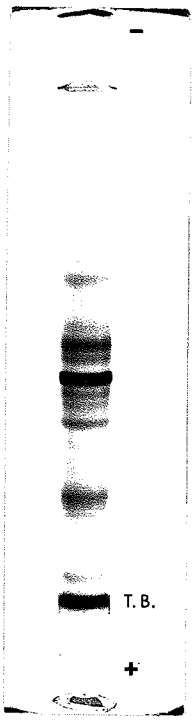


Fig. 6. Polyacrylamide gel disc electrophoresis of crude spore extract of Beluga. T.B., tracking band.

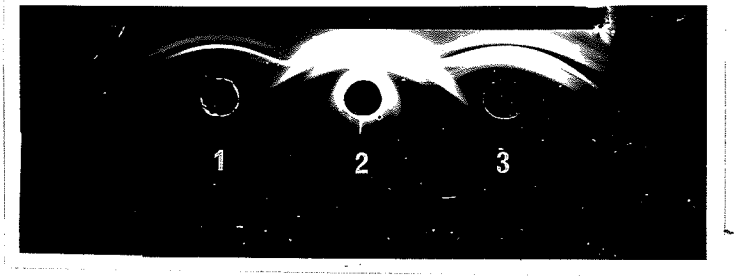


Fig. 7. Agar diffusion with antiserum against spores of type E Beluga strain in horizontal trough.

Well (1) ammonium sulphate precipitate of fractions of the first DEAE-cellulose absorption peak;

(2) spore extract, Beluga;

(3) fractions of the first DEAE-cellulose absorption peak.

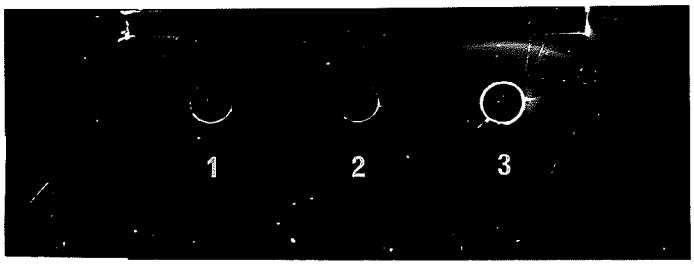


Fig. 8. Agar diffusion with antiserum against spores of type E PM-15 strain in horizontal trough.

Well (1) spore extract, VH-O;

(2) purified spore antigen, Beluga;

(3) spore extract, 9564.

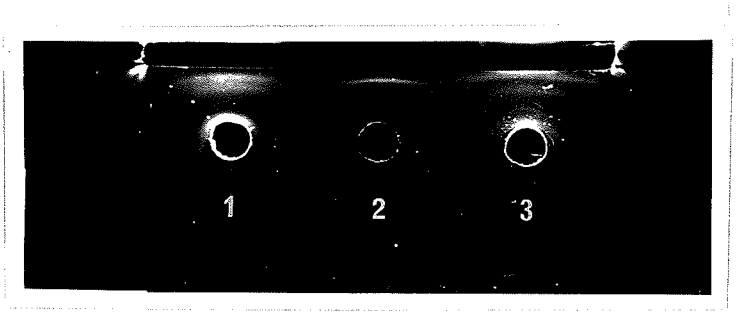


Fig. 9. Agar diffusion with antiserum against spores of type E PM-15 strain in horizontal trough.

Well (1) spore extract, 715;

(2) purified spore antigen, Beluga;

(3) spore extract, 9564.

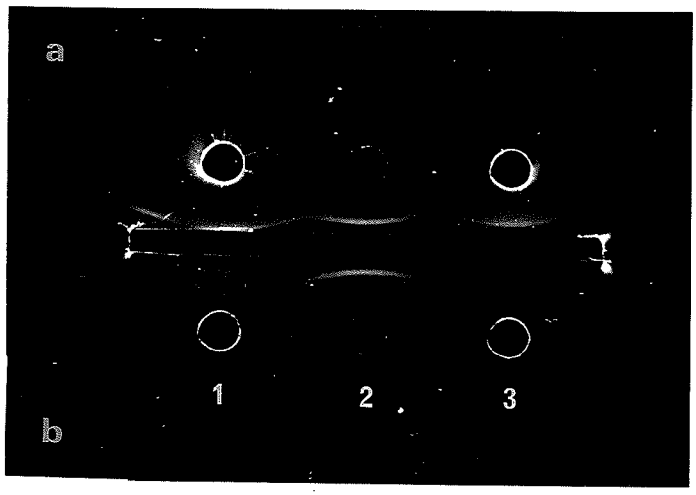


Fig. 10. Agar diffusion with antiserum against spores of type E PM-15 strain in horizontal trough.

(a) Well (1) spore extract, 5192;

(2) purified spore antigen, Beluga;

(3) spore extract, 9564.

(b) Well (1) spore extract, 070;

(2) purified spore antigen, Beluga;

(3) spore extract, 5191.

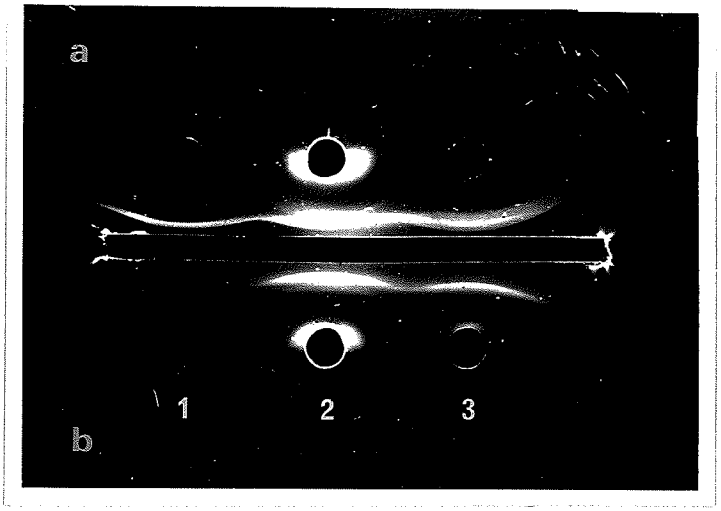


Fig. 11. Agar diffusion with antiserum against spores of type E PM-15 strain in horizontal trough.

(a) Well (1) fractions of the first DEAE-cellulose absorption peak;

(2) spore extract, PM-15;

(3) hot acid extract of whole spores, Beluga.

(b) Well (1) purified spore antigen, Beluga;

(2) spore extract, PM-15;

(3) spore extract, Beluga.

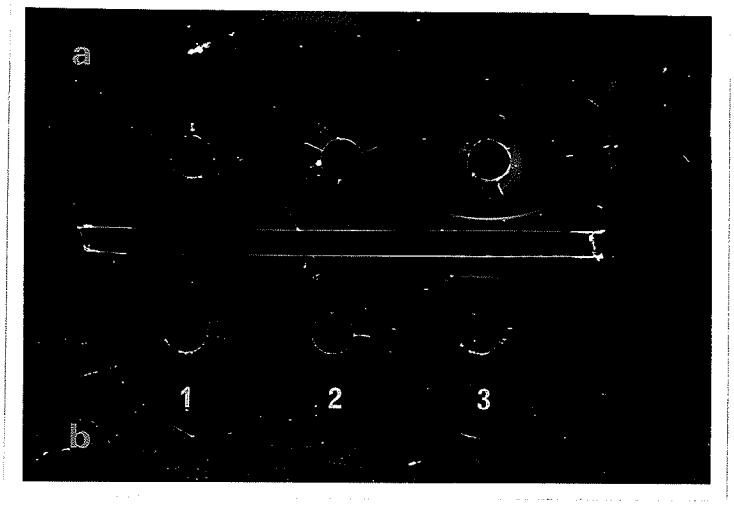


Fig. 12. Agar diffusion with antiserum against spores of type E PM-15 strain in horizontal trough.

(a) Well (1) spore extract, VH-O;

(2) purified spore antigen, Beluga;

(3) spore extract, PM-15.

(b) Well (1) fractions of the second Sephadex G-200 absorption peak;

(2) purified spore antigen, Beluga;

(3) fractions of the first DEAE-cellulose absorption peak.

DISCUSSION

Although type E strains of C. botulinum sporulated readily in the TPG medium described by Schmidt et al (63), the degree of sporulation fluctuated from 10 to 90% even when aliquots of the same inoculum were used. The biphasic culture system, which was recommended for sporulation of type E strains by Bruch et al (7), showed only about 70% sporulation in this study. A stable population sporulating with >90% was only obtained by the pseudosynchronous technique, but even then, a high degree of sporulation did not occur in volumes of media over two liters. Since the acidity of the medium is increased in large volumes (9), the pH may be a controlling factor.

The separation of spores from vegetative cells and their remnants has remained a tedious procedure. The cleaning of clostridial spores is generally accomplished by the aqueous polymer system described by Sacks and Alderton (60), but the technique was not adequate for handling large volumes. In this study, clean suspensions were obtained by lysis of vegetative cells with lysozyme and trypsin, followed by repeated washing and differential centrifugation.

The pattern of agar diffusion in this study differed from that used by Ouchterlony in that the antiserum was placed in a horizontal trough with wells containing spore

extracts placed on either side. The arrangement allowed the pattern of identity to be more easily established. The antisera against spore extracts yielded precipitin lines of greater density than those observed with antisera against the spore suspensions. One explanation may be that, the antisera against spore extracts contained a higher concentration of antibody (22).

The immunocell apparatus for immunodiffusion on cellulose acetate membranes has been recommended (47), but the precipitin lines observed in this study were not as clear, and the formation of the lines could not be followed during the incubation period. The technique, however, is simple requiring less than one hour for preparation and, in addition, provides a permanent record of the results. It would appear to offer advantages for the routine screening of clinical materials.

Immunodiffusion tests of spore extracts with spore antisera against PM-15, 070 and Beluga strains confirmed the presence of a multiplicity and diversity of spore antigens (40). Variations in intensity and in number of precipitin lines observed with spore extracts of the same strain in this study were likely due to a quantitative difference in the various preparations. As observed by Law and Hawirko (40), some of the antigens were strain specific, whereas some were shared by several of the type E strains.

At least one of the antigens was common to and specific for type E strains, since a precipitin line of identity was shared by all the strains tested, and did not form with spore extracts of types A, B and C. bifermentans. Since the antiserum against the nontoxigenic PM-15 strain gave lines of identity with spore extracts of toxigenic strains, type E specificity appears to be unrelated to toxigenicity. Immunodiffusion tests of spore antisera, absorbed with vegetative cells of a homologous strain, still exhibited the precipitin line of identity, thus confirming that the 'common' antigen was a component of the spore body.

The evidence that the line of identity appeared closer to the antiserum source than to the spore extract, even when the latter was diluted ten fold, indicates that the 'common' antigen had a greater diffusion rate than the immunoglobulin, and was very likely a smaller molecule. This was confirmed by the curvature of the precipitin line towards the antibody source when antiserum as well as purified antigen diffused from circular wells (12).

The initial stage of purification of the 'common' antigen was readily accomplished by column chromatography on DEAE-cellulose. Although the antigen was eluted in the first absorption peak, two other antigens were present, and further separation by gel filtration proved difficult even with the Sephadex G-100, G-150 and G-200 in columns of different lengths and diameters. After filtration on Sephadex G-200, the 'common' antigen was eluted in a broad peak which gave

at least five protein bands by disc electrophoresis. Presumably, on the basis of a molecular sieve principle, proteins with similar molecular weights do not readily separate.

The technique of electrofocusing is becoming increasingly popular for the isolation of proteins (23, 41, 58, 73, 78), although, in some instances, it has proved disappointing because of the tendency for proteins to precipitate (25, 53). In this study, electrofocusing in a pH 3-10 gradient worked superbly for the complete isolation of the 'common' antigen from the other proteins. The separation was confirmed by refocusing the antigen in a pH 3-6 gradient, which improved the resolution between proteins of pI values within that range. The line of complete identity of the purified antigen with that of the antigen present in the absorption peaks obtained by column chromatography confirmed that the antigen was unaltered by the separation and purification procedures.

Disc electrophoresis of the crude spore extract on polyacrylamide gel stained by coomasie blue showed at least 20 bands, but only one band was obtained with the purified antigen, indicating that the extraneous proteins had been removed.

Because of the small amount of spores obtained by the methods which are available for use at the present time, the total yield of purified antigen was only about five mg, and insufficient for a more complete characterization. The limited biochemical analyses seemed to indicate that the

antigen is a mucoprotein (79). Since the antigen was also extracted from intact spores with hot HCl, the polymer is most likely a spore wall component. The slightly bluish colour of the antigen is suggestive of the presence of Cu^{++}.

A considerable amount of the purified 'common' antigen is necessary for immunochemical studies in order to elucidate the nature of the antigenic determinant of type E specificity, and for the preparation of a monospecific fluorescein labelled antiserum to identify type E strains in epidemiological studies. A systematic study of antigenic components of spores would provide a new basis for evaluating the taxonomic relationships of the Clostridium spp.

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