AN INTRACELLULAR α-D-GLUCAN FROM *CLOSTRIDIUM BOTULINUM*, TYPE E

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ABSTRACT

An intracellular α-D-glucan has been isolated from cultured cells of *Clostridium botulinum*, type E (Minnesota). The dextrorotatory glucan consumed 1.02 moles of periodate and released 59 mmoles of formic acid per anhydroglucose unit. The fully methylated derivative was composed of 2,3,4,6-tetra-*O*-methyl-D-glucose (5%), 2,3,6-tri-*O*-methyl-D-glucose (90%), and 2,3-di-*O*-methyl-D-glucose (5%). The overall α-D anomeric configuration for the glucosidic linkages in the polymer was assessed by p.m.r. spectroscopy of the methylated derivative. This observation was corroborated by the identification of the partial hydrolysis products as maltose, isomaltose, maltotriose, and maltotetraose by use of g.l.c. and mass spectroscopic analyses of their corresponding permethylated alditols. The results indicate that the α-D-glucan is a ramified molecule composed of branched chains of D-glucopyranosyl residues linked α-(1→4) with α-(1→6) linkages at the branch points and an average of about 17 units between branches which favored an amylopectin rather than a glycogen structure. No linear amylose fraction was isolated from the intracellular polysaccharide of the cultured bacillus.

INTRODUCTION

Clostridium botulinum, type E, is a gram positive anaerobic bacillus frequently associated with botulinum food poisoning in marine products. When cultured in a D-glucose-containing medium, the organism accumulates an intracellular D-glucan which is rapidly depleted as a carbon and energy source during spore maturation[1].

Polysaccharides elaborated by *C. perfringens*[2-4], *C. oedematiens*[5], *C. welchii*[6], and *C. histolyticum*[7], have received only superficial examination. Apart from the enzymic analysis of the D-glucan from *C. butyricum*[8], no structural analysis of a chemical nature has been conducted on the polysaccharides isolated from this genus of bacteria. A structural study was therefore undertaken on the intracellular D-glucan synthesized by *C. botulinum*, type E.

RESULTS AND DISCUSSION

The Minnesota strain of *C. botulinum*, type E, was cultured in a trypticase-peptone media containing 0.4% of D-glucose. When the cells had accumulated a maximum content of D-glucan, they were harvested by centrifugation, washed with water, homogenized, and subsequently extracted with hot water. The combined extracts were concentrated, dialyzed, and freeze-dried to yield a polysaccharide which furnished only D-glucose on hydrolysis.

An aqueous solution of the crude polysaccharide, when saturated with thymol, failed to provide an insoluble amylose-type fraction, and the remaining soluble polymer was subsequently deproteinized by treatment with chloral hydrate, dialyzed, and freeze-dried to afford a pure D-glucan containing only D-glucose (estimated 99.8%), and sedimenting as a single component on ultracentrifugal analysis.

The D-glucan consumed 1.02 moles of sodium metaperiodate per anhydrohexose unit and afforded 59 mmoles of formic acid per anhydrohexose unit when oxidized in buffered solution. Hydrolysis of the reduced polyaldehyde afforded glycerol and erythritol as demonstrated by g.l.c. of their corresponding acetates. Unoxidized D-glucose was not detected, substantiating the periodate consumption figures.

Treatment of the D-glucan with methylsulfinyl carbanion and methyl iodide according to the Hakomori procedure[9] gave a fully methylated polysaccharide which on hydrolysis afforded 2,3,4,6-tetra-, 2,3,6-tri-, and 2,3-di-*O*-methyl-D-glucose in the molar ratio of 1:18:1, as demonstrated by g.l.c. and mass spectroscopic analyses of

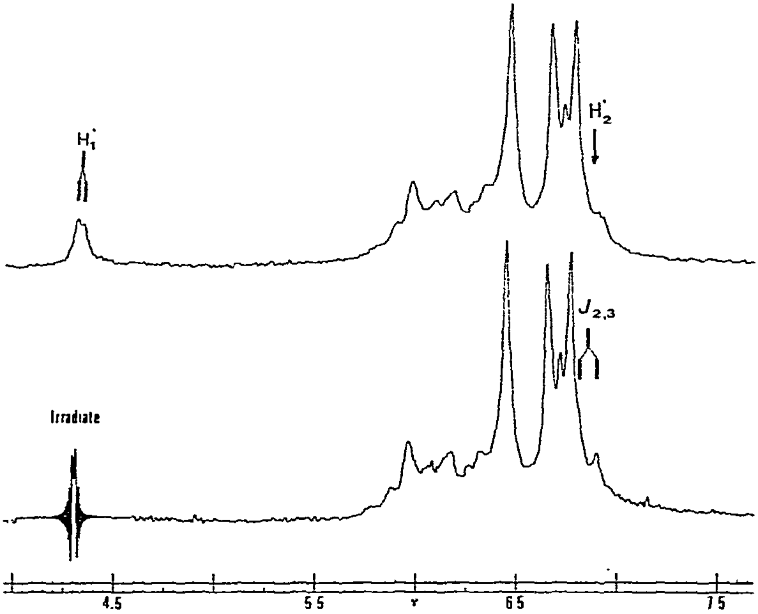

Fig. 1. P.m.r. (100 MHz) in benzene-d_6 of the methylated derivative of the D-glucan isolated from *Clostridium botulinum*, type E.

their derived alditol acetates. These results attested to the presence in the polysaccharide of 1,4- and 1,4,6-linked D-glucopyranose residues.

The methylated D-glucan in benzene-d_6 provided a p.m.r. spectrum (Fig. 1), having a doublet at τ 4.31, $J_{1',2'}$ 3.1 Hz, for the anomeric protons, H-1′, of the α-D-(1→4)-linked residues in the polymer[10]. Irradiation of this signal collapsed the partially obscured quartet signal at τ 6.87 for H-2′ into a doublet $J_{2',3'}$ 9.6 Hz, indicating that the α-D-(1→4)-linked D-glucopyranose residues in this polymer must exist in the *C1* conformation[11]. The anomeric-proton signal for linear (1→6)-linked α-D-glucose residues in the spectrum of permethylated pullulan in benzene-d_6 has been shown[12] to resonate at τ 4.85. However, even in very high gain spectra, no signal was evident for the anomeric protons of the branch point residues in the ramified D-glucan from *C. botulinum*. Previous observations had suggested that the signal derived from the anomeric protons in a permethylated glucan would only be evident in a single-scan p.m.r. spectrum when that anomeric form constituted at least 5–10% of the forms present in the polymer[11]. It was therefore necessary to assess by depolymerisation experiments the anomeric configuration of the 5% of α-D-(1→6)-glucosidic linkages present in the D-glucan, although the overall anomeric configuration of the polysaccharide had been established by the above p.m.r. studies.

Hydrolysis of the D-glucan with oxalic acid provided a mixture of oligosaccharides which was shown, by paper chromatography, to contain maltose, isomaltose, maltotriose, and maltotetraose. To verify these assignments the hydrolyzate was reduced, the aldosylalditols fully methylated, subsequently fractionated by g.l.c., and subjected to mass spectrometry. Four fractions were identified as the derived permethylated alditols of maltose, isomaltose, maltotriose, and maltotetraose in the ratio of 5.6:1:2.6:1.3. The electron-impact fragmentation pattern of permethylated maltitol[13], maltotriitol, and maltotetraitol[14] exhibited identical, major-ion fragments differing only in their relative intensities; the spectrum of the latter compound provided more intense ion fragments derived from cleavage of the hexose units (*m/e* 219, 187, 155, and 115) and less intense ions formed from the ionized alditol residue (*m/e* 235 and 171) than the corresponding ion fragments derived from the maltitol derivative. The mass spectrum furnished by the permethylated isomaltitol fraction exhibited the diagnostic ion at *m/e* 177 (10% base peak at *m/e* 101) formed from primary scission of the C-4 to C-5 bond in the alditol moiety.

The just presented evidence indicates that the intracellular D-glucan from *C. botulinum*, type E, has a branched structure consisting of an average of seventeen glucopyranosyl residues linked α-D-(1→4) for each residue bearing an α-D-linked branch point at C-6. The high average chain length and absorption frequency of the iodine complex[15] favored an amylopectin rather than a glycogen structure. However, the intrinsic viscosity number, 43, for the D-glucan when compared to that observed for potato amylopectin, 150, and for glycogen, 6, suggests that the physical state of this amylopectin may be modified by glycogen-like or by more highly-branched areas within the molecule[16].

The structure of the D-glucan from *C. botulinum* cannot be compared with that isolated from *C. butyricum* since the only structural evidence presented in the latter study[8] was based on the hydrolytic action of β-amylase on the impure D-glucan and suggested a structure composed primarily of α-D-(1→4)-linked glucopyranose residues.

The D-glucan from *C. botulinum*, unlike most land plant starches, contains no linear amylose fraction, and thus this bacillus may be useful in studying the mechanism of the biosynthesis of the amylopectin molecule, by a route other than that involving the 'amylose synthetase' pathway[17].

EXPERIMENTAL

General methods. — Paper chromatography was performed on Whatman No. 1 paper with the irrigants: (A)10:4:3 (v/v) ethyl acetate–pyridine–water, (B) 18:3:1:4 (v/v) ethyl acetate–acetic acid–formic acid–water, (C) 3:1:1 (v/v) butyl alcohol–ethanol–water. Sugars were detected on chromatograms with *p*-anisidine hydrochloride[18] or alkaline silver nitrate[19]. D-Glucose was estimated by the phenol–sulfuric acid method[20]; methoxyl content was determined by heating at reflux with hydriodic acid[21]. A Beckman model L2-65B ultracentrifuge was used at 60,000 r.p.m. for sedimentation analysis. Specific rotations are equilibrium values observed at 18° in the solvent specified. Gas-liquid chromatographic separations were performed on (a) a 1.20 m × 6 mm column containing 0.2% (w/w) poly(glycol adipate), 0.2% (w/w) poly(glycol succinate) and 0.4% XF 1150 on dimethylchlorosilane treated Chromosorb W (60–80 mesh) with a gas flow rate of 60 ml of nitrogen per min and (b) a 1.80 m × 6 mm column containing 10% (w/w) SE 30 on dimethylchlorosilane treated Chromosorb W (60–80 mesh) with a gas flow rate of 80 ml of nitrogen per min. Retention values T_{AMG} are quoted relative to the mobility of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on column (a) at 150° and T_{MM} values relative to the mobility of permethylated maltitol on column (b) at 290°. Proton magnetic resonance (p.m.r.) spectra were obtained with a Varian HA100 spectrometer, τ values are relative to tetramethylsilane as an internal standard in the solvent specified. The mass spectra were obtained with a Nuclide spectrometer.

Preparation of crude polysaccharide. — Stock cultures of *Clostridium botulinum*, type E, strain Minnesota, were maintained in the medium of Follinazzo and Troy[22]. Trypticase-peptone medium[23] containing 0.4% D-glucose (sterilized separately) was employed for growth and polysaccharide production. An FD bench scale fermenter (Fermentation-Design Inc., Edison, N. J.), with conventional stirring and equipped with a 14-liter vessel, was sparged with oxygen-free nitrogen at a flow rate of 0.5 l/min and maintained at 30±0.5°. The inoculum consisted of heat-shocked (13 min at 60°) spores to give an initial concentration of 10^5 spores/ml of growth medium.

The culture was monitored during growth for intracellular D-glucan levels[1], and the cells were harvested at maximum accumulation of D-glucan (approx. 16 h) with a Sharples Centrifuge at 4°. The cells were washed three times in distilled water, resuspended in 100 ml of distilled water, and disrupted in a Braun Model MSK cell homogenizer with Teflon-coated glass beads (0.17–0.18 mm) at 4,000 r.p.m. The homogenate was exhaustively extracted with water at 95°, the combined extracts centrifuged, and the supernatant dialyzed, concentrated, and freeze-dried to yield the crude polysaccharide (520 mg). Paper chromatographic examination of a hydrolyzed portion of this material (0.5M sulfuric acid, 100°, 6 h) revealed glucose as the sole sugar component.

Purification of the D-*glucan.* — The polysaccharide (350 mg) was dissolved in water (50 ml) at 95° in an atmosphere of nitrogen. The solution was cooled to 60°, saturated with powdered thymol, and allowed to cool with stirring to 20°, and then kept for 2 days. The precipitate formed was removed by centrifugation and dried by solvent exchange (40 mg). Chromatographic examination in solvents (A), (B), and (C) of a hydrolyzed portion of this residue revealed only a trace of glucose. The centrifugate was freed from thymol by extraction with ether and treated with chloral hydrate (33%, 60°, 0.5 h). The insoluble residue was centrifuged off and the purified D-glucan (290 mg) isolated from the aqueous solution by freeze-drying after dialysis.

Properties of the D-*glucan.* — The polysaccharide (10% in 0.1M potassium-hydroxide) sedimented as a single component on ultracentrifugal analysis. Observations of the Schlieren pattern during the acceleration period showed that there was no rapidly sedimenting material[24]. The D-glucan had $[\alpha]_D^{18}$ +191° (*c* 0.4, water), contained 99.8% of D-glucose by colorimetric estimation, and gave an iodine complex $\lambda_{max}^{H_2O}$ 538 nm[15]. The intrinsic viscosity of the D-glucan was measured at 20° in an Ostwald viscometer: the limiting viscosity number $[\eta]$ was 43, oyster glycogen 6, and potato amylopectin 150, all measured as solutions in 0.5M sodium hydroxide.

Periodate oxidation. — The D-glucan (9.46 mg) was oxidized with 0.06M sodium metaperiodate (10 ml) at 1°; the consumption followed spectrophotometrically[25] was constant at 1.02 moles per anhydrohexose unit after 40 h. Oxidation of the D-glucan (24.4 mg) with 0.3M sodium metaperiodate (12.5 ml) in aqueous sodium chloride (3%, 12.5 ml) at 1° produced 59 mmoles of formic acid per anhydrohexose unit. A portion of the polyaldehyde was reduced, hydrolyzed, further treated with sodium borohydride, acetylated, and the derived acetates analyzed by g.l.c. on column (a). The acetates of glycerol and erythritol, but not glucitol, were detected.

Methylation studies. — The D-glucan (100 mg) dissolved in dry dimethyl sulfoxide (25 ml) was successively treated with methylsulfinyl carbanion and methyl iodide according to the Hakomori procedure[9]. The reaction mixture was dialyzed and freeze-dried to yield a product which was fractionated by dissolution in boiling mixtures of chloroform–light petroleum. The main fraction (80 mg) showed OCH_3 45.1% and $[\alpha]_D^{18}$ +196° (*c* 1.2, chloroform), and was soluble in 1:4 chloroform–light petroleum. A portion of the methylated polysaccharide (10 mg) was hydrolyzed with 90% formic acid for 1 h at 100°, followed by 0.25M sulfuric acid for 12 h at 100°, subsequently neutralized, and the sugars reduced with sodium borohydride. The resulting, partially methylated alditols were treated with 1:1 acetic anhydride–pyridine and the product chromatographed by g.l.c. on column (a); the fractions obtained by preparative separation were subjected to mass spectrometry. Three fractions having

T_{AMG} 1.0, 2.5, and 4.0 were shown by chromatography and mass spectrometry to be identical with 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol, and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol in the molar proportions of 1:18:1. The p.m.r. spectrum of the methylated polysaccharide in chloroform-*d* (*ca.* 30 mg per 0.5 ml) exhibited signals at τ 4.43 (H-1′) and 6.77 (H-2′), and distinct signals at τ 6.44, 6.49, and 6.61 for the methoxyl groups. The spectrum of the methylated D-glucan in benzene-d_6 is illustrated in Fig. 1.

Partial hydrolysis. — The D-glucan (45 mg) was heated with 0.1M oxalic acid for 7 h at 100° in a sealed tube. Chromatographic examination in solvents (A) and (B) of the neutralized hydrolyzate revealed the presence of D-glucose, maltose, maltotriose, and maltotetraose. The hydrolyzate was treated with sodium borohydride, deionized with Rexyn 101 (H^+), and concentrated, and the boric acid removed by codistillation with methanol. The residue was dissolved in dry dimethyl sulfoxide and methylated with methylsulfinyl carbanion and methyl iodide. The reaction mixture was poured into water and extracted with chloroform, and the extracts were dried and evaporated under high vacuum to remove traces of dimethyl sulfoxide. The methylated aldosylalditols were chromatographed by g.l.c. on column (b) and the fractions separated preparatively and subjected to mass spectrometry. Four fractions having T_{MM} 1.0, 1.23, 6.40, and 37.2 (81 min), in the ratio 5.6:1.0:2.6:1.3, were shown by chromatography and mass spectrometry to be identical with the permethylated derivatives of 4-*O*-α-D-glucopyranosyl-D-glucitol, 6-*O*-α-D-glucopyranosyl-D-glucitol, *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucitol, and *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucitol. The mass spectrum of the latter compound had major peaks at m/e 45 (40%), 59 (15%), 71 (33%), 75 (40%), 88 (88%), 89 (23%), 101 (48%), 111 (49%), 115 (35%), 127 (16%), 133 (7.8%), 145 (10%), 155 (40%), 171 (23%), 187 (100%), 219 (9.4%), 235 (6.2%), 295 (3.9%), 305 (1.6%), 391 (1.2%), 407 (2.3%), 423 (2.7%), 439 (4.7%), and 499 (1.2%).

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