STRUCTURE OF A β-D-GLUCAN FROM THE MYCELIAL WALL OF *Basidiomycete QM 806*

D. A. BUSH AND M. HORISBERGER
Nestlé's Products Technical Assistance Co. Ltd., Vevey (Switzerland)
(Received June 14, 1971; accepted in revised form November 22, 1971)

ABSTRACT

A water-soluble β-D-glucan has been isolated from the mycelial wall of *Basidiomycete QM 806*. The structure of this glucan was investigated by methylation, periodate, and enzymic studies. Hydrolysis of the methylated glucan gave 2,3,4,6-tetra-, 2,4,6-, 2,3,4- and 2,3,6-tri-, and 2,4-di-*O*-methyl-D-glucose in the following molar proportions: 1.0:1.0:0.8:1.2:1.0. Periodate oxidation of the glucan followed by reduction and mild acid hydrolysis gave glycerol, erythritol, and D-glucose in the molar proportions, 2.1, 1.0, and 2.0, respectively. The glucan was degraded to the extent of 38% by an exo-β-(1→3)-glucanase isolated from the same organism, though the branch points (joined through O-1, O-3, and O-6) appeared to be resistant to the enzyme, whereas the (1→4) linkages were not. On the basis of these findings, the structure of the glucan and the possible role of the glucanase are discussed.

INTRODUCTION

Although the walls of fungal mycelium have been studied far less than bacterial cell walls, they are known to contain a large proportion of carbohydrate material. The nature of the carbohydrates in the mycelial wall is often related to the taxonomic position of the fungus; most of the typical septate, mycelial fungi have a wall consisting of glucan and chitin[1]. The structure of fungal glucans has been extensively reviewed[2], and both β-(1→3) and β-(1→6) linkages were shown to occur frequently. The arrangement of such polysaccharides in the fungal wall has been studied in three fungi: *Neurospora crassa*, *Schizophyllum commune*, and *Phytophthora parasitica*[3]. The glucans appeared to form the outer layers of the hyphal wall, with chitin and some protein forming a core.

A conidial *Basidiomycete* (species QM 806 of Reese and Mandels[4]), which has not been fully classified, is a copious producer of an extracellular exo-β-(1→3)-glucanase. This enzyme, which has been purified and studied[5,6], is of great interest for its use in the production of protoplasts from yeast and in the analysis of yeast cell-wall carbohydrates. It has also been used extensively in the determination of the structure of microbial glucans[7–11]. As the enzyme is produced constitutively at the end of the growth period, it was suggested[4] that its purpose was the "intracellular hydrolysis (and synthesis) of reserve material containing β-D-(1→3) glucosidic linkages". In

view of this hypothesis and the reports[2] of β-(1 → 3) linkages in fungal-wall glucans, it was decided to examine the carbohydrate composition of the mycelial walls of *Basidiomycete QM 806*, in order to ascertain the structure and importance of β-D-(1 → 3)-linked glucan in this organism.

RESULTS AND DISCUSSION

Preliminary analysis of the carbohydrate components of the mycelial wall of *Basidiomycete QM 806* (consisting of 76% carbohydrate, measured as anhydroglucose) showed a large part of them (67%) to be soluble in hot 5M potassium hydroxide. Only glucose and glucosamine were found by paper chromatography in the hydrolyzates of the wall, suggesting the presence of a glucan and chitin. The alkali-extracted material contained only D-glucose on hydrolysis, whereas the residue (26% of the carbohydrate) contained both D-glucose and glucosamine, indicating the possible presence of two types of glucans.

The alkali-extractable glucan was purified, whereupon it was found to be soluble in water. It was shown to be free of low-molecular weight material by precipitation with 3 vol. of ethanol and by passage through Sephadex G-200 and Sepharose 4B, where it was eluted very near the solvent front, as a single peak indicating a high-molecular weight. However, measurement of reducing terminal units indicated a molecular weight of only 35,000 (DP 216). This anomaly remains to be explained.

The glucan was methylated twice by the liquid ammonia procedure[12]. On acid hydrolysis of the methylated product, 2,4-di-, 2,3,6-tri-, 2,3,4-tri-, 2,4,6-tri- and 2,3,4,6 tetra-*O*-methyl-D-glucose were detected by g.l.c. in the molar ratios 1.0:1.2:0.8:1.0:1.0. The presence of these five methyl ethers was confirmed by paper chromatography, although 2,3,4-tri- and 2,4,6-tri-*O*-methyl-D-glucose overlapped partially. The structural features indicated by the presence of these methylated D-glucose compounds are shown in Table I. The glucan thus contains a mixture of (1 → 4)-

TABLE I

HYDROLYSIS OF THE METHYLATED GLUCAN

Product of hydrolysis	*Mode of linkage*	*Molar proportions*	*G.l.c. (T)*[a]	
			Lit.[13] *values*	*Found*
O-Methyl-D-glucose				
2,3,4,6-Tetra-	Gp-(1→	1.0	1.0	1.0
2,4,6-Tri-	→3)-G*p*-(1→	1.0	1.95	1.97
2,3,4-Tri-	→6)-G*p* (1→	0.8	2.49	2.51
2,3,6-Tri-	→4)-G*p* (1→	1.2	2.50	2.55
2,4-Di-	→6)-G*p* (1→ 3 ↑	1.0	5.10	5.24

[a]Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

(1.2 moles) and (1 → 6)-linked (0.8 mole) D-glucopyranosyl residues, together with (1 → 3)-linked D-glucopyranosyl residues (2.0 moles) having branches at O-6 (1.0 mole). As 2,4-di-*O*-methyl-D-glucose was the only di-*O*-methyl-D-glucose detected by g.l.c. or by paper chromatography, the presence of other branch points was thus excluded. Equimolar quantities of 2,3,4,6-tetra- and 2,4-di-*O*-methyl-D-glucose were detected in proportions that indicated a branch point for every five D-glucose residues.

The presence of non reducing terminal or (1 → 6)-linked, (1 → 4)-linked and (1 → 3) or (1 → 3, 1 → 6)-linked D-glucose residues was confirmed by the detection of glycerol (2.1 moles), erythritol (1.0 mole), and D-glucose (2.0 moles) in the hydrolyzate of the periodate-oxidized glucan, degraded by the Smith procedure. The values expected from the methylation data are 1.8 moles for glycerol, 1.2 moles for erythritol and 2.0 moles for glucose, which are in good agreement with the observed values.

From the specific optical rotation, $[\alpha]_D^{25}$ −1.2° in water, it was deduced that the glucan was β-D-linked. Upon treatment of the glucan with the exo-β-(1 → 3)-glucanase of *Basidiomycete QM 806*, reducing groups were liberated (36–38 molar proportions per cent) with the production of D-glucose and gentiobiose in the molar ratio of 10 to 1. Such results could be explained if most of the β-(1 → 3) linkages, including the branch points, were hydrolyzed by the glucanase. However, passage of the enzyme-degraded glucan through a Sephadex G-25 column and subsequent methylation of the two resultant high-molecular weight fractions A and B, suggested that this was not so. These fractions, making up 35% by weight of the original glucan, were methylated twice by a modified Hakamori method[14]. On acid hydrolysis of the methylated fractions, 2,4-di-, 2,3,4-tri-, 2,4,6-tri- and 2,3,4,6-tetra-*O*-methyl-D-glucose were detected by g.l.c. in the molar ratios 1.1:0.8:0.2:1.0. The structural features suggested by the results of the methylation procedure are shown in Table II. Thus, the enzyme-resistant fractions contained (1 → 6)-linked D-glucopyranosyl residues (0.8 mole) together with

TABLE II

HYDROLYSIS OF METHYLATED FRACTIONS A AND B OBTAINED FROM THE SEPHADEX G-25 COLUMN

Product of hydrolysis	*Mode of linkage*	*Molar proportions*		*G.l.c. (T)*[a]		
		A	*B*	*Lit.*[13] *values*	*Found*	
					A	*B*
O-Methyl-D-glucose						
2,3,4,6-Tetra-	*Gp*-(1→	1.0	1.0	1.0	1.0	1.0
2,4,6-Tri-	→3)-*Gp*-(1→	0.2	0.2	1.95	1.96	1.96
2,3,4-Tri-	→6)-*Gp*-(1→	0.8	0.8	2.49	2.49	2.49
2,3,6-Tri	→4)-*Gp*-(1→	none	trace	2.50		2.54
2,4-Di	→6)-*Gp*-(1→ 3 ↑	1.1	1.2	5.10	5.32	5.32

[a]Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

(1→3)-linked D-glucopyranosyl residues (1.3 moles) having branches at O-6 (1.1 moles). Equimolar quantities of 2,3,4,6-tetra-and 2,4-di-*O*-methyl-D-glucose were detected in proportions that indicated one branch point for every three D-glucose residues. The presence of a trace of 2,3,6-tri-*O*-methyl-D-glucose indicated that an occasional (1→4) linkage was present in fraction B.

It thus appears that the *Basidiomycete QM 806* β-(1→3)-glucanase is able to degrade β-(1→4) linkages as well as β-(1→3) linkages, but is not able to hydrolyse the β-(1→3, 1→6)-branch points. It should be pointed out, however, that no reducing groups were produced when the enzyme was allowed to act on cellulose or carboxymethylcellulose for 23 h, under the conditions that were used for the digestion of the glucan. Parrish and Perlin[15], and Parish *et al.*[16] have described the hydrolysis of β-(1→4)-glycosidic linkages by a specific β-(1→3)-glucanase, and it may be that similar conditions of specificity exist for the *Basidiomycete QM 806* β-(1→3)-glucanase. It is our intention to investigate this point further by use of glucans of known composition. The apparent resistance of the β-(1→3, 1→6)-branch points to the *QM 806* glucanase supports the findings of Nakajima *et al.*[11], who found that the degradation of a β-glucan from the walls of *Piricularia oryzae* with *QM 806* glucanase produced, among the degradation products, laminaribiose and 3-*O*-β-gentiobiosyl-D-glucose.

In conclusion, it may be stated that the *Basidiomycete QM 806* mycelial wall contains a branched β-D-glucan, the glucose units of which are joined by a mixture of (1→6), (1→4) and (1→3) linkages. Approximately half of the β-(1→3)-linked D-glucose residues have branches at O-6. This glucan is partially (36–38%) degraded by the exo-β-(1→3)-glucanase of the same organism, thus affording support to the theory that the glucanase may be involved in the degradation and synthesis of cellular polysaccharides. The results suggested also that the *Basidiomycete QM 806* exo-β-(1→3)-glucanase was capable of cleaving certain (1→4) linkages, and it is hoped that further work will clarify this point.

EXPERIMENTAL

General methods. — Reducing groups were determined by the Nelson–Somogyi method[17], total carbohydrate by the phenol–sulfuric acid method[18] with D-glucose as standard, glycerol and erythritol by formaldehyde determination after periodate oxidation[19], and D-glucose specifically by the D-glucose oxidase method[20]. The degree of polymerisation of the glucan was measured by a modified Park–Johnson method[21]. Sugars were separated by chromatography on Whatman No. 1 or No. 3 paper in pyridine–ethyl acetate–water (2:5:7, upper phase, solvent A)[22]. The chromatograms were developed with ammoniacal silver nitrate[23]. Glucosamine was identified by heating the paper after treatment with 0.5M sodium hydroxide in ethanol–butanol (2:3) and examining it under a u.v. lamp[24]. Methyl sugars were separated on Whatman No. 1 paper in 2-butanone–water azeotrope (solvent B)[25] and were detected with a *p*-aminobenzoic acid reagent[26]. The methoxyl content of the methylated glucan was determined by the Zeisel method[27].

Organism. — *Basidiomycete QM 806*[4], obtained through the courtesy of Dr. E. T. Reese (U. S. Army Natick Laboratories, Massachusetts), was grown[28] on a D-glucose (4%), yeast extract (Difco 1%), KH_2PO_4 (0.3%) medium adjusted to pH 4.8 with phosphoric acid, in a Chemap 15-liter fermenter for 4 days at 30°. The mycelium was obtained from the culture by filtration and washing with water.

Preparation of mycelial walls. — Mycelium (10 g dry wt.) was washed with acetate buffer (50mM, pH 5.0) and ground in liquid nitrogen. It was then suspended in buffer and ground in a Braun homogenizer (3 min) using glass beads (0.1 mm diam.). After removal of the glass beads by filtration, the homogenate was centrifuged at 6000 *g*. The residue was then suspended in 0.8M mannitol buffer[29], shaken, and centrifuged at 6000 *g* (20 min). The residue was resuspended in mannitol buffer and the supernatant discarded. The process was repeated 5 times after which the residue was washed 4 times with water. It was free of cytoplasmic material, as shown by microscopic examination. The walls were freeze-dried (yield, 700 mg).

Analysis of wall carbohydrates. — Total carbohydrate was measured by the phenol–sulfuric acid method after hydrolysis of the walls by the 72%–8% sulfuric acid method[30]. The carbohydrates of the mycelial wall were fractionated according to the following procedure. A sample (20 mg) of mycelial wall was shaken for 1 h with water (5 ml). The residue was filtered off on a sintered glass filter, and the filtrate was analyzed for carbohydrate. The residue was dissolved in 5M potassium hydroxide (6 ml), heated for 0.5 h at 100°, and then filtered off as before. The residue was washed twice with hot water (5 ml), and the filtrate and washings were combined and analyzed for total carbohydrate. The residue was dissolved in 72% sulfuric acid (2 ml) and analyzed for total carbohydrate.

Preparation of mycelial glucan. — Washed mycelium (50 g dry wt.) was autoclaved in water (1 l) for 1 h at 120°. The mycelium was then filtered off and washed with hot water. The filtrate and washings were discarded. The residue was extracted with 3% sodium hydroxide (1 l) at 90° for 1 h, after which it was cooled and centrifuged off. The procedure was repeated and the residue was washed. The supernatants and washings were combined and the pH brought to 5.0 with acetic acid, the slight precipitate that formed being discarded. The supernatant was concentrated, dialyzed for 24 h against running tap water, and treated with Dowex resins (H^+ and OH^-) to remove the remaining salts. The polysaccharide was precipitated with ethanol (3 vol), washed twice with ethanol and acetone, and then dried in *vacuo*; yield 3.5 g, $[\alpha]_D^{25}$ −1.2° (*c* 1.2, water). On hydrolysis with sulfuric acid, the polysaccharide (10 mg) gave D-glucose (9.9 mg) as the only identifiable sugar.

Methylation analysis of the glucan. — The dried glucan (400 mg) was methylated twice by the sodium–liquid ammonia procedure[12]; yield, 145 mg (36%); $[\alpha]_D^{25}$ +48° (*c* 0.5 chloroform); OMe, 45.0%. The methylated glucan (40 mg) was hydrolyzed by the formic–sulfuric acid method[30]. The hydrolyzate was neutralized by addition of barium carbonate, and concentrated to a small volume. Examination of the sirup by paper chromatography (solvent B) showed the presence of five components corresponding to 2,3,4,6-tetra-, 2,3,6-, 2,4,6- and 2,3,4-tri-, and 2,4-di-*O*-methyl-D-

glucose. The methyl sugars were further identified and determined as alditol acetates by g.l.c. with the method of Björndal *et al.*[13]. A glass column (200 × 0.4 mm) packed with 3% ECNSS-M (a nitrile silicone polymer supplied precoated by Applied Science Labs., State College, Pennsylvania 16801) on Chromosorb Q (100–120 mesh) was flushed with 40 ml of nitrogen per min at 180°. The chromatograph was a Hewlett–Packard research model No. 5750 with a flame ionisation detector. The flash-heater temperature was 270° and the detector temperature was 235°. The relative concentration of individual sugars was calculated by assuming that the response factor is proportional to the molecular weight of the acetylated methyl alditol[31]. Positive identification of the methyl glucoses was obtained by g.l.c. of authentic samples.

Periodate oxidation of the glucan. — The dried glucan (400 mg) was oxidized with 80mM sodium metaperiodate (100 ml) at 4° in the dark, and the consumption of periodate and the formation of formic acid were measured[32]. The oxidation (complete after 3 days) was continued over 5 days, and the periodate consumption was calculated by extrapolation to time zero. The oxidized polysaccharide was degraded according to the Smith procedure[33].

Degradation of the glucan with exo-β-(1→3)-glucanase. — The exo-β-(1→3)-glucanase, produced after 14 days of growth of *Basidiomycete QM 806* in shaken culture, was purified as described by Huotari *et al.*[5]. The enzyme (200 units[5]) was allowed to act on the glucan (70 mg) in the presence of acetate buffer (50mM, pH 5.0) for 15 h. At the end of the reaction time, the reducing groups and the D-glucose liberated were measured by the Nelson–Somogyi[17] and D-glucose oxidase[20] methods, respectively. The reaction mixture was concentrated, applied to a Sephadex G-25 column, and eluted with water. Three peaks were obtained: Fraction A 18.5 mg, DP 50, $[\alpha]_D^{25}$ −36° (*c* 1.0, water); Fraction B (6.75 mg, DP 39), and Fraction C (22.2 mg, DP 1.3). Paper chromatography of each peak (solvent A) indicated non-migrating material in Fractions A and B and D-glucose, gentiobiose, and a small amount of unidentified material in Fraction C. The molar ratio of D-glucose to gentiobiose was found to be 10:1. Fractions A and B were methylated twice by a modified Hakamori method[14] and then hydrolyzed by the formic–sulfuric acid method[30]. The resulting methyl sugars were analyzed, as their corresponding acetylated methyl alditols, by g.l.c.[13] with authentic samples of methyl D-glucoses as standards.

ACKNOWLEDGEMENTS

The authors thank Dr. D. Reymond for the critical review of the text, and Miss E. Wespi and Mr. J. Kundert for technical assistance.

REFERENCES

1 S. BARTNICKI-GARCIA, *Ann. Rev. Microbiol.*, 22 (1968) 87.
2 P. A. J. GORIN AND J. F. T. SPENCER, *Advan. Carbohyd. Chem.*, 23 (1968) 367.
3 D. HUNSLEY AND J. H. BURNETT, *J. Gen. Microbiol.*, 62 (1970) 203.
4 E. T. REESE AND M. MANDELS, *Can. J. Microbiol.*, 5 (1959) 173.
5 F. I. HUOTARI, T. E. NELSON, F. SMITH, AND S. KIRKWOOD, *J. Biol. Chem.*, 243 (1968) 952.

6 T. E. NELSON, J. V. SCALETTI, F. SMITH, AND S. KIRKWOOD, *Can. J. Chem.*, 41 (1963) 1671.
7 H. O. BOUVENG, H. KIESSLING, B. LINDBERG, AND J. MCKAY, *Acta Chem. Scand.*, 17 (1963) 1351.
8 L. L. WALLEN, R. A. RHODES, AND H. R. SHULKE, *Appl. Microbiol.*, 13 (1965) 272.
9 K. W. BUCK, A. W. CHEN, A. G. DICKERSON, AND E. B. CHAIN, *J. Gen. Microbiol.*, 51 (1968) 337.
10 K. K. BATRA, J. H. NORDIN, AND S. KIRKWOOD, *Carbohyd. Res.* 9 (1969) 221.
11 T. NAKAJIMA, K. TAMARI, K. MATSUDA, H. TANAKA, AND N. OGASAWARA, *Agr. Biol. Chem.*, 34 (1970) 553.
12 E. L. HIRST AND E. PERCIVAL, *Methods Carbohyd. Chem.*, 5 (1965) 287.
13 H. BJÖRNDAL, B. LINDBERG, AND S. SVENSSON, *Acta Chem. Scand.*, 21 (1967) 1801.
14 P. A. SANDFORD AND H. E. CONRAD, *Biochemistry*, 5 (1966) 1508.
15 F. W. PARRISH AND A. S. PERLIN, *Nature*, 187 (1960) 1110.
16 F. W. PARRISH, A. S. PERLIN, AND E. T. REESE, *Can. J. Chem.*, 38 (1960) 2094.
17 N. NELSON, *J. Biol. Chem.*, 153 (1944) 375.
18 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350.
19 G. W. HAY, B. A. LEWIS, AND F. SMITH, *Methods Carbohyd. Chem.*, 5 (1965) 377.
20 A. ST. G. HUGGETT AND D. A. NIXON, *Biochem. J.*, 66 (1957) 12P.
21 J. S. THOMPSON AND G. D. SHOCKMAN, *Anal. Biochem.*, 22 (1968) 260.
22 E. F. MCFARREN, K. BRAND, AND H. R. RUTKOWSKI, *Anal. Chem.*, 23 (1951) 1146.
23 F. SMITH AND R. MONTGOMERY, *Chemistry of Plant Gums and Mucilages*, Reinhold., New York, 1959, p. 88.
24 N. SHARON AND S. SEIFTER, *J. Biol. Chem.*, 239 (1964) 2398PC.
25 L. BOGGS, L. S. CUENDET, I. EHRENTHAL, R. KOCH, AND F. SMITH, *Nature*, 166 (1950) 520.
26 A. S. SAINI, *J. Chromatogr.*, 24 (1966) 484.
27 F. J. BATES, *Polarimetry, Saccharimetry and the Sugars*, Circular of the National Bureau of Standards, U. S. A., 1942, p. 509.
28 D. PETERSON, private communication.
29 P. J. MILL, *J. Gen. Microbiol.*, 44 (1966) 329.
30 H. O. BOUVENG AND B. LINDBERG, *Methods Carbohyd. Chem.*, 5 (1965) 296.
31 C. G. HELLERQUIST, B. LINDBERG, S. SVENSSON, T. HOLME, AND A. A. LINDBERG, *Carbohyd. Res.*, 8 (1968) 43.
32 G. W. HAY, B. A. LEWIS, AND F. SMITH, *Methods Carbohyd. Chem.*, 5 (1965) 357.
33 I. J. GOLDSTEIN, G. W. HAY, B. A. LEWIS, AND F. SMITH, *Methods Carbohyd. Chem.*, 5 (1965) 361.