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STRUCTURE OF AN ALKALI-SOLUBLE POLYSACCHARIDE FROM THE HYPHAL WALL OF THE BASIDIOMYCETE *Coprinus macrorhizus* VAR. *microsporus*

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ABSTRACT

A unique, alkali-soluble polysaccharide has been isolated from the cell walls of the basidiomycete *Coprinus macrorhizus microsporus*. The polysaccharide, which is primarily a glucan, contains a large proportion of α-(1→4)-linked D-glucose residues and a smaller amount of β-(1→3) and (1→6) linkages, as suggested by methylation, partial acid hydrolysis, periodate oxidation, and enzymic studies. Hydrolysis of the methylated polysaccharide gave equimolar amounts of 2,4-di- and 2,3-di-*O*-methyl-D-glucose; no 2,6-di-*O*-methyl-D-glucose was identified, indicating the absence of branch points joined through O-1, O-3, and O-4. The isolation and identification of 2-*O*-α-glucopyranosylerythritol from the periodate-oxidized polysaccharide suggests that segments of the α-(1→4)-linked D-glucose residues are joined by single (1→3)-linkages. An extracellular enzyme-preparation from *Sporotrichum dimorphosporum* (QM 806) containing both β-(1→3)- and α-(1→4)-D-glucanohydrolase activity released 76% of the reducing groups from the polysaccharide. The polysaccharide also contains minor proportions of xylose, mannose, 2-amino-2-deoxyglucose, and amino acids.

INTRODUCTION

The mycelial cell-wall of Basidiomycetes is primarily carbohydrate and has been shown to be composed of two or more distinct polysaccharides differing in either monosaccharides present or types of glycosidic linkage, or both. The structure of the alkali-soluble (S-glucan) and alkali-resistant (R-glucan) fractions derived from the cell wall of *Schizophyllum commune* have been extensively investigated[1,2]. S-Glucan is a (1→3)-α-D-glucan having a small proportion of α-D-(1→6) linkages[1]. This fraction also contains xylose and mannose that may originate from distinct polymers. The (alkali-resistant) R-glucan is a (1→3)-β-, (1→6)-β-D-glucan containing chitin and protein. In cell walls of *Polyporus tumulosus*[3], the alkali-soluble fraction contains two polysaccharides, a (1→3)-α-D-glucan and a xylomannan. Information

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on the cell wall of the common mushroom (*Agaricus bisporus*) is largely qualitative, although the alkali-soluble fraction has been assumed to be an α-D-glucan[4]. The cell walls of *Sporotrichum dimorphosporum* (Basidiomycete QM 806) contain a β-D-glucan having (1→3), (1→6), and (1→4)-β-D-linked glucopyranosyl residues[5].

Recently, Schaefer[6] has reported on the structure of an alkali-soluble polysaccharide from the cell walls of the basidiomycete, *Coprinus lagopus* (=*cinereus*). Unlike that from *S. commune*, this polysaccharide is a β-D-glucan primarily (1→3)-linked with (1→6) branch points. Marchant[7] examined the wall composition of monokaryotic and dikaryotic mycelia in *Coprinus cinereus*. Dikaryotic cell-walls contained no alkali-soluble fraction and significantly less chitin. The alkali-insoluble polysaccharide from monokaryotic cell-walls was assumed to be a (1→3)-β-(1→6)-β-D-glucan, as reported by Schaefer.

Developmental aspects of the growth of *Coprinus macrorhizus microsporus* have been documented[8-15], but the structure of its cell-wall polysaccharides is unknown. This work reports on the structure of a unique, alkali-soluble polysaccharide from the hyphal wall of this organism.

RESULTS

Polysaccharide isolation. — The cell walls of *Coprinus macrorhizus microsporus* (5377) were prepared by using a combination of techniques: freeze-thawing, homogenization, and sonic oscillation. Sodium dodecyl sulfate was added during sonication to remove cytoplasm and non-wall protein[16]. Extraction of the lipid-free walls with super-heated water was performed to remove any intracellular glycogen and soluble protein[17] that may have adhered to the cell-wall preparation. Fractionation with M alkali at three temperatures (0, 21, and 60°) for 1.5, 18, and 1.5 h, respectively, gave three alkali-soluble fractions (I, II, III) constituting 26% of the original wall-weight. Fraction II, representing 12% of the original wall-weight, is the subject of this communication. Fractions I and III have not been characterized, although they gave glucose on hydrolysis. It is assumed that these fractions resemble fraction II in constitution and structure. The purified polysaccharide was only partially soluble in water.

Composition. — Paper electrophoresis of fraction II gave a single spot (indicating homogeneity) that migrated 1.2 cm toward the cathode; glucose migrated 4.0 cm toward the anode under the same conditions. The complete acid hydrolyzate of fraction II contained glucose and mannose plus a trace of 2-amino-2-deoxyglucose. The hydrolyzate contained other ninhydrin-positive spots that were shown to be amino acids. Products from the complete acid hydrolyzate were also separated by g.l.c. as the butaneboronates[18] of the corresponding alditols. Assuming equivalent molar-response factors[18], 6% of the hydrolyzate was mannose; a trace of 2-amino-2-deoxyglucitol butaneboronate was also detected.

Partial acid hydrolysis. — Partial acid hydrolysis[19] gave (papergram) a mixture of oligosaccharides, three disaccharides, plus glucose and xylose. The disaccharides

were laminarabiose, maltose, and gentiobiose, as identified by comparison with authentic standards using paper chromatography in two solvent systems (*A* and *B*), thus characterizing the linkages as β-(1→3), α-(1→4), and β-(1→6), respectively. Lichenan, which contains β-(1→3) (30%) and β-(1→4) (70%) linkages[20], was hydrolyzed under identical conditions and provided a standard for laminarabiose. Maltose and cellobiose are not readily resolved in most solvent systems, but system B gave sufficient separation for identification. No 2-amino-2-deoxyglucose was detected in the partial acid hydrolyzate.

Linkage configuration. — The optical rotation of fraction II polysaccharide, $[\alpha]_D^{25}$ +85° (~0.7% in M sodium hydroxide), indicates an α-linked glucan; it was different from that of other alkali-soluble glucans from Basidiomycetes. These latter have specific optical rotations on the order of +200° when α-linked[1-3] or 0° when β-linked[6]. The i.r. spectrum of fraction II showed absorptions at 920, 885 (shoulder), 835, and 748 cm^{-1}. The S-glucan from *S. commune* had absorptions at 923, 850, 830, and 775 cm^{-1}, and lichenan gave a single, large peak at 888 cm^{-1}. Barker *et al.*[21] have suggested that α-linked and β-linked polysaccharides exhibit absorptions at 844 ±8 cm^{-1} and 891 ±7 cm^{-1}, respectively. The primarily α-linked nature of fraction II glucan is confirmed by the absorption at 835 cm^{-1}; a shoulder at 885 cm^{-1} indicates some β-linkages may be present. These results support the conclusion that the polysaccharide is a mixed-configurational glucan. Fraction II gave no color with iodine–potassium iodide solution.

Enzymic hydrolysis. — The alkali-soluble polysaccharide underwent extensive hydrolysis by a partially purified enzyme-preparation obtained from the culture filtrate of *S. dimorphosporum* (QM 806); 76% of the reducing groups and 65% of the glucose (Table I) was released. The extent of hydrolysis can only be explained by the action of both α-(1→4)- and β-(1→3)-hydrolase activities present in the enzyme preparation (Table I).

Although *Sporotrichum dimorphosporum* (QM 806) is an excellent source of an exo-(1→3)-β-D-glucanase, the partially purified enzyme-preparation contains significant amounts of (1→4)-α-D-glucanohydrolase activity of unknown specificity. With amylose and glycogen, the amount of glucose released was greater than the reducing sugar liberated (Table I). The stability of the enzyme was indicated by its not undergoing inactivation by boiling water during 5 min, as observed with beta amylase and pullulanase[22]. No β-(1→6) activity and only a trace of cellulolytic activity were detected. The enzymic hydrolyzate of Fraction II polysaccharide contained only glucose and gentiobiose. Small proportions of higher oligosaccharides were also present, and these may be a homologous series of β-(1→6)-linked D-glucose residues, as found in the R-glucan of *S. commune*[2].

Methylation analysis. — The glucan underwent complete methylation by the method of Hakomori[23], as indicated by the absence of i.r. absorption in the region 3400–3500 cm^{-1} and by the detection of only a trace of a monomethyl ether of glucose. Hydrolysis of the permethylated glucan gave five methylated sugars, identified by t.l.c. on silica gel as 2,3,4,6-tetra-, 2,4,6-tri-, 2,3,4-tri-, 2,3,6-tri-, and 2,3-di-*O*-

TABLE I

ENZYMIC HYDROLYSIS BY *Sporotrichum dimorphosporum* (QM 806) PREPARATION

Substrate glucan type	*Reducing sugar released (%) total by weight*	*Glucose released (%) total by weight*
Alkali-soluble glucan *(C. macrorhizus microsporus)*	76	65
S-glucan *(S. commune)* [(1→3)-α-, (1→6)-α-D-glucan]	4.2	3.0
Lichenan [(1→3)-β-, (1→4)-β-D-glucan]	13	3.7
Laminaran [(1→3)-β-D-glucan]	89	84
Pustulan [(1→6)-β-D-glucan]	0	0
Cellulose (Solka-Floc) [(1→4)-β-D-glucan]	0.2	0.1
O-(Carboxymethyl)cellulose [(1→4)-β-D-glucan]	0.3	0.2
Soluble starch [(1→4)-α-, (1→6)-α-D-glucan]	9.0	9.0
Amylose [(1→4)-α-D-glucan]	53	57[a]
Glycogen [(1→4)-α-, (1→6)-α-D-glucan]	90	95[a]
Dextran [(1→6)-α-D-glucan]	11	11

[a]Enzyme was not completely inactivated by heating for 5 min at 100°.

methyl-D-glucose. Their identities were confirmed by g.l.c. as the alditol acetates[24]. The two dimethyl ethers of glucose, 2,3-dimethyl and 2,4-dimethyl, which apparently are not separated by t.l.c. in the solvent system employed, are separated by g.l.c. Retention times and mass spectra of the unknowns matched those of authentic standards. Mol percentages of the methylated sugars obtained on hydrolysis are reported in Table II. Two minor peaks were also observed and were tentatively identified as 2,3,4-tri-*O*-methyl-D-xylose and 2,6-di-*O*-methyl-D-mannose, respectively, based on their relative retention-times and comparison with literature values[24]. The mass spectrum of one unknown, methylated sugar substantiated its identification as 2,3,4-tri-*O*-methyl-D-xylose.

Periodate oxidation and Smith degradation. — The alkali-soluble glucan consumed 0.95 mol of periodate and released 0.19 mol of formic acid per mol of glucose residues. These results are in near-perfect agreement with the methylation results. The expected consumption of periodate based on methylation results would be 0.97 mol per mol of glucose residues, and the expected release of formic acid would be 0.22 mol. The oxidized polysaccharide was reduced with sodium borohydride and the resultant polyalcohol subjected to mild acid hydrolysis at room temperature. The

TABLE II

METHYLATION ANALYSIS

O-Methylated sugar (as alditol acetate)	*Mode of linkage*	*Mole percentage*	T_R[a]
Unknown 1		1.7	0.65
2,3,4,6-Tetra-*O*-methyl-D-glucose	Glc*p*-(1→	13.5	1.00
2,4,6-Tri-*O*-methyl-D-glucose	→3)-Glc*p*-(1→	13.3	1.70
2,3,4-Tri-*O*-methyl-D-glucose	→6)-Glc*p*-(1→	9.1	1.98
2,3,6-Tri-*O*-methyl-D-glucose	→4)-Glc*p*-(1→	47.6	2.07
Unknown 2		1.7	2.41
2,4-Di-*O*-methyl-D-glucose	→6)-Glc*p*-(1→ 3 ↑	6.6	3.01
2,3-Di-*O*-methyl-D-glucose	→6)-Glc*p*-(1→ 4 ↑	6.6	3.12

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

hydrolyzate was evaporated at 40°, deionized, and examined by t.l.c. on cellulose. Erythritol and glycerol were detected and identified by co-chromatography on t.l.c. plates with standards. The identities of these products were confirmed by g.l.c. of their acetates on a 3% ECNSS-M column. Retention times of the unknowns were identical with those of standards acetylated by the same procedure. Quantitation by g.l.c. indicated a 2.1 : 1 mole ratio of erythritol to glycerol. This result is in agreement with methylation results, as erythritol is derived from glucose residues linked either through positions 1 and 4 or 1, 4, and 6, whereas glycerol would be expected from end-groups, glucose residues linked through either positions 1 and 6, or 1, 2, and 6. The alditol acetates were separated by t.l.c. on silica gel and detected by the ferric hydroxamate reaction. The presence of erythritol tetraacetate (R_F 0.58), glycerol triacetate (R_F 0.66), and an unknown acetate (R_F 0.23) was established. As the glucan contains (1→3), (1→4), and (1→6) linkages, it was suspected that the unidentified product was 2-*O*-glucopyranosylerythritol or 3-*O*-glucopyranosylglycerol arising from (1→3)-linked glucose residues flanked by (1→4) or (1→6) linkages, respectively. These glycosides do not react with reducing reagents. The proportion of the unknown product, as indicated by the intensities of the acetate spots on silica gel thin-layer chromatograms, suggested the former. 2-*O*-α-Glucopyranosylerythritol was synthesized from maltose[25]. The configuration of the linkage was supported by enzymic and partial acid hydrolysis. The unknown product was identified as 2-*O*-α-D-glucopyranosylerythritol from equivalent mobilities of the acetates on silica gel thin-layer chromatograms.

Amino acid analysis. — Ninhydrin-positive components other than the 2-amino-2-deoxyglucose were found in the hydrolyzates of fraction II. The amino acid composition of the polysaccharide (Table III) shows relatively high proportions

TABLE III

AMINO ACID ANALYSIS

Amino acid	*μMol amino acid/mmol*	*Amino acid*	*μMol amino acid/mmol*
Aspartic acid	104	Isoleucine	42.5
Threonine	50.1	Leucine	89.4
Serine	70.6	Tyrosine	37.5
Glutamic acid	121	Phenylalanine	47.1
Proline	17.6	Histidine	24.0
Glycine	118	Lysine	69.1
Alanine	107	Arginine	27.9
Valine	74.2		

(> 10%, w/w) of alanine, glycine, aspartic acid, and glutamic acid; the total protein-content based on the amino acid analysis was 1.2% of the dry weight.

DISCUSSION

The alkali-soluble polysaccharide from the cell walls of *Coprinus macrorhizus microsporus* differs from those reported for other Basidiomycetes[1-5] and a generically related species[6]. The presence of (1→4) linkages in the alkali-soluble fraction is substantiated by the detection of erythritol on hydrolysis of the reduced, periodate-oxidized glucan, hydrolysis by an α-(1→4)-D-glucanohydrolase, and the identification of maltose in a partial acid-hydrolyzate. The occurrence of (1→4) linkages in Basidiomycetes is unusual and has been reported in only one other member[5], *S. dimorphosporum*. In *S. dimorphosporum*, the configuration of the (1→4) linkage was assumed, based on optical rotation, to be β. No partial acid hydrolysis was conducted. Enzymic hydrolysis of the polysaccharide from *S. dimorphosporum* with an exo-(1→3)-β-D-glucanase isolated from the same organism released 38% of the reducing groups. Further analysis by methylation of the enzyme-resistant fraction suggested that the (1→3)-β-D-glucanase hydrolyzes (1→4) linkages as well as β-(1→3) linkages. The enzyme was not able to hydrolyze the β-(1→3),(1→6) branch points. No hydrolytic activity was detected against cellulose or *O*-(carboxymethyl)cellulose, but the preparation was not assayed against α-D-glucans. It is possible that the preparation of Bush and Horisberger[5] contained α-(1→4) hydrolase activity, and they may have observed the hydrolysis of α-(1→4) linkages.

The fraction II polysaccharide appears to be a highly branched glucan having branch points at O-6 on both (1→4)- and (1→3)-linked residues. The proportion of end groups is nearly equal to the sum of branch points, and hence little or no crosslinking occurs between the glucan chains. As no 2,6-di-*O*-methyl-D-glucose was detected by methylation analysis and cross-linking could be ruled out, it was apparent that the attachment of (1→4) linkages was via an occasional (1→3) link, as borne out by the identification of 2-*O*-α-glucopyranosylerythritol. Branching at

O-6 occurs in one glucose residue for every eight (1→4)-linked residues. A branch point at O-6 occurs at one glucose residue for three (1→3)-linked residues. Preliminary methylation studies on the glucan of the alkali-insoluble fraction of *C. macrorhizus* cell-wall indicated a very similar structure to that of the alkali-soluble polysaccharide (fraction II).

Xylose and mannose are present in the alkali-soluble polysaccharide from *C. macrorhizus microsporus*. Only glucose was observed (papergram) in the acid hydrolyzate. G.l.c. separation of the hydrolyzate gave indication of some mannose as well as glucose. The small proportion of mannose was probably masked by glucose on the papergram, as these epimers are not well-resolved. Xylose was present in the partial acid hydrolyzate, but was absent from the hydrochloric acid hydrolyzate. It was assumed that these stronger, hydrolytic conditions resulted in almost complete decomposition of the xylose. This behavior has also been observed with the xylose in the cell walls of *P. tumulosus*[3]. In *P. tumulosus*, the xylose was derived from a water-insoluble xylomannan, isolated as a copper complex from the alkali-soluble fraction of the cell walls[3]. The structural characteristics of the xylomannan were similar to those of one isolated from the hyphal wall of *Armillaria mellea*, a Basidiomycete[26]. However the *Armillaria* xylomannan does not form a copper complex and is soluble in water. The alkali-soluble glucan from *S. commune* also contains mannose and xylose[1,2]. Siehr[1] could not precipitate a xylomannan copper-complex from the alkali-soluble fraction of *S. commune*.

The xylomannan of *P. tumulosus* cell walls is an α-(1→3)-linked mannan to which xylose residues are attached[3]. Permethylation, followed by hydrolysis, gave 2,3,4,6-tetra-*O*-methyl mannose, 2,3,4-tri-*O*-methylxylose, 2,4,6-tri-*O*-methylmannose, and a dimethylmannose tentatively characterized as 2,6-dimethylmannose. The trimethylxylose, trimethylmannose, and dimethyl mannose were present in approximately equal proportions. Methylation of the alkali-soluble polysaccharide of *C. macrorhizus microsporus* cell-walls gave equimolar amounts of methylated sugars tentatively characterized as 2,3,4-tri-*O*-methylxylose and 2,6-di-*O*-methylmannose by their relative retention times[24] and mass spectroscopy. Other methylmannoses may have eluted with the methylglucose analogs, as it is known that they have comparable retention-times[24]. Xylose and mannose occur in the alkali-soluble, cell-wall fraction of *C. macrorhizus microsporus*, perhaps as a xylomannan having a structure similar to the xylomannan of *P. tumulosus*. These xylomannans may have a structural role in filamentous fungi[3].

Many fungal cell-wall preparations contain protein as a structural component[27]. The alkali-soluble fraction II of *C. macrorhizus microsporus* contained protein, as do other alkali-soluble, cell-wall fractions of Basidiomycetes. Siehr[1] reported amino acids in the S-glucan of *S. commune*, although no specific identification was carried out. Sietsma and Wessels[2] reported 2.2% (wt.) of amino acids in their S-glucan preparation, but the acids were not identified. In addition, they reported a small proportion of 2-amino-2-deoxyglucose (0.2% by wt.), which probably occurs as 2-acetamido-2-deoxyglucose in the original polysaccharide. A small amount of

2-amino-2-deoxyglucose was detected in fraction II. The protein found in fraction II contains a high proportion of acidic amino acids (Table II). The carbohydrate and protein of yeast mannan are covalently linked, primarily through asparagine, by way of di-*N*-acetylchitobiose and through *O*-glycosidic bonds via hydroxyamino acids[28]. These linkages are characteristic of glycoproteins. Although the information available is scanty, the carbohydrate component of fraction II of *C. macrorhizus microsporus* may be linked covalently to asparagine or glutamine residues in the protein through 2-acetamido-2-deoxyglucose or its dimer.

The cell-wall structure of fungi that are morphologically closely related would not be expected to differ drastically[29]. Structurally, the alkali-soluble polysaccharide from *C. macrorhizus microsporus* cell-walls is substantially different from those reported for other Basidiomycetes. These results suggest a diversity in the cell-wall structure of related fungi that may be species- or perhaps strain-specific. Generalizations on the structural features of cell walls from related organisms should be closely scrutinized.

EXPERIMENTAL

General methods. — Total carbohydrate was determined by the anthrone method[30], reducing sugars by the dinitrosalicylic method[31], and D-glucose specifically by the D-glucose oxidase method as modified by Lloyd and Whelan[32]. Descending chromatography on Whatman No. 1 paper was accomplished by using 12:5:4 (v/v) ethyl acetate–pyridine–water (solvent *A*) or 2:1:1 (v/v) 1-butanol–pyridine–water (solvent *B*)[33]. Reducing sugars were detected with alkaline silver nitrate[33]. 2-Amino-2-deoxyglucose was detected with the ninhydrin reagent[33]. Alditols were separated by t.l.c. on cellulose F (E. Merck) with 7:1:2 (v/v) 1-propanol–ethyl acetate–water[34]. Detection was with alkaline silver nitrate[34]. Sugar and alditol acetates were separated by t.l.c. on plates of silica gel G (E. Merck) developed with 4% methanol in benzene (v/v)[35]. The acetates were detected by the ferric hydroxamate reaction[35]. Methylated sugars obtained on hydrolysis of the permethylated polysaccharide were separated on plates of silica gel G (E. Merck) by using 9:1 (v/v) dichloromethane–methanol for development[36]. I.r. spectra of polysaccharides were measured in a potassium bromide matrix by using a Perkin–Elmer Model 180 IR spectrophotometer at 40°. Optical rotations were measured in M sodium hydroxide with a Kern polarimeter. High-voltage electrophoresis was performed on Whatman No. 1 paper for 1 h with 0.01M borate buffer, pH 9.85. Detection was made with periodate–benzidine[33] or alkaline permanganate[37].

Organisms. — *Coprinus macrorhizus* var. *microsporus* (5377) was kindly provided by Dr. Philip G. Miles (Dept. of Biology, SUNY, Buffalo, NY) who obtained the culture from Dr. Tsuneo Takemaru (Dept. of Biology, Okayama University, Okayama, Japan). *Sporotrichum dimorphosporum* (Basidiomycete QM 806) was from the culture collection of the University of Massachusetts, formerly the collection of the US Army Natick Laboratories, Natick, MA.

Media and cultural conditions. — *C. macrorhizus microsporus* was cultured on complete medium[38] supplemented with 0.05% (w/v) yeast extract. Macerated mycelia from a 10-day-old agar plate maintained at 25° was used to inoculate 1 L of liquid medium contained in 2.8-L Fernbach flasks. The culture was incubated with shaking (90 rotations/min) for 11 days. *S. dimorphosporum* was cultured on liquid medium as described by Peterson and Kirkwood[39].

Preparation of cell walls. — The cells (four 1-L cultures) were recovered by vacuum filtration on two layers of cheesecloth, washed extensively with distilled water, and immediately frozen. Thawed cells (66 g wet wt.) were washed with distilled water by vacuum filtration and suspended in 400 mL of 0.8% sodium chloride (w/v). These were homogenized in a Waring blender at high speed for one min. The homogenate was centrifuged at 16,000 *g* (15 min).

The recovered residue was washed twice with distilled water and the mycelial fragments collected by centrifugation at 10,000*g* (15 min) and stored frozen at 20°. Thawed fragments were suspended in 1% sodium dodecyl sulfate (w/v) and sonicated at maximum output for 2 min at 30-sec intervals. The cell walls were collected by centrifugation at 10,000*g* (30 min) and washed twice with distilled water by centrifugation. The cell walls were then suspended in distilled water, dispersed in a Waring blender, and sonicated at maximum output for 1 min at 30-sec intervals. Recovery was by centrifugation at 10,000*g* (15 min) and the residue was washed twice with distilled water by centrifugation. Sonication (1 min at 30-sec intervals) of the residue was repeated in the presence of 0.8% sodium chloride, followed by centrifugation at 8000*g* (15 min). This residue was frozen, thawed, suspended in water, and dispersed in a Waring blender at high speed. Cell walls were recovered by centrifugation at 10,000*g* (15 min), washed with water, collected by centrifugation at 8000*g* (15 min), and this was followed by a second wash and collection by centrifugation at 5000*g* (15 min). Examination of the final residue with a light microscope and Methylene Blue stain indicated nearly complete cell-breakage and no cytoplasmic contamination. The cell walls were lyophilized, (yield 4.50 g) and stored at 20°. Freeze-dried cell walls were extracted in a Soxhlet apparatus with 2:1 (v/v) chloroform–methanol for 66 h. Lipid-free cell walls were dried to constant weight in an oven at 80° (yield 4.05 g).

Cell-wall fractionation. — Lipid-free cell walls (3.85 g) were autoclaved in distilled water (150 mL) for 1 h at 122°, cooled, and recovered by centrifugation at 10,000*g* (15 min). The cell walls were washed twice by centrifugation and dialyzed against distilled water for 2 days at 4°. Total carbohydrate (208 mg) and free glucose (0 mg) extracted into hot water were measured by the anthrone and glucose oxidase methods, respectively. The hot-water-soluble carbohydrate was not precipitable with cold ethanol. The cell walls were then extracted with M sodium hydroxide (150 mL) at 0° with stirring under nitrogen for 1.5 h. The suspension was centrifuged at 10,000*g* (15 min) and the residue washed twice with distilled water by centrifugation. The combined supernatants were made neutral with acetic acid and the polysaccharide was precipitated with cold ethanol (2 vol). The 0°-alkali-soluble polysaccharide

(fraction I) was recovered by centrifugation and washed twice with cold ethanol. The recovered polysaccharide was again suspended in water, dialyzed for 2 days at 4° against several changes of distilled water, and finally freeze-dried (yield 132 mg). No further analysis was carried out with this fraction. The residue (fraction I-R), after alkali-extraction, was suspended in water, dispersed in a Waring blender at high speed, made neutral with acetic acid, dialyzed, and freeze-dried (yield 2.74 g). Fraction I-R (2.56 g) was extracted with M sodium hydroxide (150 mL) at 21° with stirring under nitrogen for 18 h. The polysaccharide (fraction II, yield 419 mg) was recovered and treated as described for fraction I. The insoluble residue, (fraction II-R, yield 1.02 g) was carried through another extraction by alkali with M sodium hydroxide at 60°, with stirring under nitrogen for 1.5 h. The recovered polysaccharide (fraction III, yield 83 mg) was not further characterized.

Polysaccharide hydrolysis and analysis. — Polysaccharide samples were hydrolyzed in 2M hydrochloric acid for 2 h at 100° in a sealed ampoule. Hydrogen chloride was removed by evaporation *in vacuo* at 40° with repeated additions of water. The hydrolyzate was stored over potassium hydroxide *in vacuo* to remove residual traces of acid. Hydrolyzates were examined by paper and gas–liquid chromatography. The butaneboronic acid esters of the reduced monosaccharides obtained by acid hydrolysis were prepared as described by Eisenberg[18]. Separation was effected on a 3% OV-17 (Chromosorb W-HP, 80–100 mesh) 2 m × 2 mm (i.d.) glass column. The column temperature was 220°; the inlet and transfer temperatures were 240°. Helium was used as carrier gas at a flow rate of 40 mL/min. Peak areas were measured by electronic integration.

Partial hydrolysis was carried out according to the method of Johnston[19]. The polysaccharide (20 mg) was suspended in 88% formic acid (0.5 mL) and heated for 10 min at 100°. Sulfuric acid (0.2M, 4 mL) was added and heating continued for 1 h. The solution was made neutral (barium carbonate) and evaporated under diminished pressure. The hydrolyzate was examined by paper chromatography with solvent systems *A* and *B*.

Methylation analysis. — The polysaccharide was twice methylated according to the procedure of Hakomori[23] as modified by Sandford and Conrad[40]. The sample was dried *in vacuo* for 4 h at 80° prior to methylation. Hydrolysis of the methylated glucan was conducted by the formic–sulfuric acid method[24]. An i.r. spectrum was taken as a thin film evaporated from a chloroform solution on sodium chloride plates. The methylated sugars were separated by t.l.c. on silica gel. Identification of the methylated sugars was confirmed by g.l.c. as their alditol acetates[24]. Separation was accomplished on a glass column (2 m × 2 mm, i.d.) packed with 3% ECNSS-M on Gas-Chrom Q (100–200 mesh), using helium at 40 mL/min as the carrier gas. The inlet and transfer temperatures were 250°, with temperature programming from 140–180° at 1°/min. This technique allowed resolution of the alditol acetates of 2,3,4-tri- and 2,3,6-tri-*O*-methyl-D-glucose. Methylated glucose derivatives were identified by comparison with authentic samples. Peak areas were measured by electronic integration and the relative concentration of methylated sugars calculated

by assuming equal molar response-factors for all derivatives[24]. The alditol acetates of the *O*-methyl sugars were separated and identified by g.l.c.–m.s. by using a Varian 2700 gas chromatograph interfaced to a JEOL-D-100 mass spectrometer equipped with a dedicated TI 980 computer. A glass column [2 m × 2 mm (i.d.)] packed with 3% OV-225 (Chromosorb W-HP, 80–100 mesh), programmed from 165 to 230° at 2° per min, was used for g.l.c.–m.s. The ionization potential for mass spectra was 35 eV. Identification was made by comparison with retention times and fragmentation patterns of authentic standards, or by comparison of major mass-spectral ions with data in the literature[24].

Periodate oxidation and Smith degradation. — The glucan (100 mg) was oxidized by 25mM sodium metaperiodate (100 mL) in the dark at 4°. The consumption of periodate was determined at various time-intervals by the Malaprade method as outlined by Dryhurst[41]. Production of formic acid was monitored by titration with standard alkali, using Bromocresol Purple as indicator[41]. Appropriate blanks were used in each determination. Oxidation was considered complete after 5 days. The oxidized glucan was reduced with sodium borohydride and hydrolyzed under mild acidic conditions[42]. Glycerol and erythritol were identified by t.l.c. on cellulose and as the acetates by g.l.c. on 3% ECNSS-M. G.l.c. was conducted isothermally at 160° with all other conditions identical to those used for methylated sugars. The acetylated derivatives of the Smith products were also separated by chromatography on silica gel. 2-*O*-α-D-Glucopyranosylerythritol was synthesized according to the procedure of Charlson *et al.*[25], acetylated with 1:1 (v/v) acetic anhydride–pyridine, and used as a standard.

Enzymic hydrolysis. — An enzyme mixture containing both β-(1→3) and α-(1→4) hydrolase activities was recovered as the 25–50% ammonium sulfate fraction from the culture medium of *Sporotrichum dimorphosporum*. The precipitate was dissolved in 48mM citrate–phosphate buffer, pH 4.5, and assayed against various substrates: S-glucan from *S commune*, lichenan (Koch–Light) from *Cetraria islandica*, laminaran (Koch–Light) from *Laminaria hyperborea*, pustulan from *Parmelia papullosa* (Calbiochem), cellulose (Solka-Floc, Brown Co.), *O*-(carboxymethyl)-cellulose (Eastman Kodak Co.), soluble starch (Fisher), amylose from potato (Sigma), glycogen from shellfish (Sigma), and dextran (Sigma). The enzyme preparation contained both exo-(1→3)-β- and (1→4)-α-D-glucanohydrolase activities. One L of culture medium yielded 245 units[43] when assayed against laminaran. Hydrolysis of the various substrates was performed in 48mM citrate–phosphate buffer, pH 4.5 for 12 h at 40°. Toluene was added as a bacterial growth-inhibitor. The amount of enzyme added in each case was 0.06 units per mg of substrate. The reaction was stopped by heating for 5 min in a boiling-water bath. Insoluble substrate, if present, was removed by centrifugation prior to the determination of reducing sugar[31] and D-glucose[32].

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