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Polysaccharides of *Pleurotus ostreatus*: Isolation and structure of pleuran, an alkali-insoluble β-D-glucan

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An alkali-insoluble skeletal β-D-glucan has been isolated from the fruit bodies of *Pleurotus ostreatus*. The polysaccharide has a branched structure consisting of a backbone of (1 → 3)-linked β-D-glucopyranosyl residues, every fourth being substituted at 0–6 with single D-glucopyranosyl groups. The polysaccharide contained a small proportion (7%) of (1 → 6)- and (1 → 4)-linked interior residues. The β-D-glucan (pleuran) demonstrated efficacy in promoting the survival of mice susceptible to bacterial infections.

INTRODUCTION

A number of fungi including various strains of *Basidiomycetes* have been reported to contain antitumor-active (1 → 3)-β-D-glucans, e.g. lentinan obtained from *Lentinus edodes* (Sasaki & Takasuka, 1976; Chihara *et al.*, 1970), schizophyllan from *Schizopyllum commune* (Kikumoto *et al.*, 1971; Komatsu *et al.*, 1969), and scleroglucan from *Sclerotium glucanicum* (Johnson *et al.*, 1963; Singh *et al.*, 1974), which differ in their structural features and physicochemical properties. Recently a hot-water soluble polysaccharide isolated from the edible mushroom *Pleurotus ostreatus* (Fr.) Quel. has been shown to exhibit high antitumor activity against Sarkoma 180 ascyte cells (Yoshioka *et al.*, 1985). This polysaccharide, a (1 → 3)-β-D-glucan highly branched at 0–6 (d.b. 1/4), was characterized by methylation analysis, periodate oxidation, NMR spectrometry, and bioassay.

In our investigation of structure–activity relationships in fungal polysaccharides we focused our attention on the alkali-insoluble polysaccharides of *Pleurotus ostreatus* fruit bodies. Characterization of the structure of this material is now reported.

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MATERIAL AND METHODS

General

All evaporations were conducted under diminished pressure at 40°C. Gas–liquid chromatography (GLC) was performed on a Hewlett-Packard Model 5711 A instrument equipped with a column (200 × 0·3 cm) of 3% of SP-2340 on Chromosorb WAW-DMCS (80–100 mesh). Elution was for 4 min at 180°C, increasing to 210°C at 2°C/min with a nitrogen flow of 30 ml/min.

Gas-liquid chromatography-mass spectrometry (GLC-MS) analysis was carried out on a Hewlett-Packard HP 5940 instrument under the following conditions: capillary glass-column SP-2330 (30 m × 0·25 mm, 0·2 μm), carrier gas helium, flow rate 20 ml/min; column temperature program used for mono- and diglucosyl alditols was 80°C (0·5 min), 7·5°C min^{-1} to → 95°C, then 5·8°C/min to → 240°C, and isothermally at 240°C for 30 min.

Fast-atom bombardment-mass spectrometry (FAB-MS) spectra were recorded with a JMS SX/SX 102 A mass spectrometer operating at an accelerating voltage of 10 kV. The samples were dissolved in water (10 μg/μl) and a 1 μl aliquot was mixed with a drop of glycerol or

thioglycerol on the target, and subjected to xenon (6 kV) bombardment.

Descending paper chromatography (PC) was performed on a Whatman No. 1 paper using 1-butanol–ethanol–water–ammonia (4:1:5:trace, v/v) and detection with aniline hydrogen phthalate. The mobilities R_{Glc} are expressed relative to that of 2,3,4,6-tetra-O-methyl-glucose.

High-performance liquid chromatography (HPLC) was carried out on a Hewlett-Packard 1050 instrument equipped with a column (250 × 4 mm i.d.) of Separon SGX C 18 (5 μm) using water as mobile phase at ambient temperature.

Proton-decoupled ^{13}C-NMR spectrum was recorded on an FT-NMR spectrometer Bruker AM-300 at 75·468 MHz in D_2O at 25°C, methanol was used as the internal standard [50·15 ppm relative to $(CH_3)_4Si$].

For analysis of the component sugars, a sample (~3 mg) of polysaccharide was suspended in aqueous 72% (w/w) sulfuric acid (0·2 ml) for 1 h at 30°C. The mixture was then diluted with water (5·6 ml) heated for 6 h at 100°C, cooled, neutralized ($BaCO_3$), filtered, treated with Dowex 50W (H^+) resin, and concentrated. The sugars were determined as alditol acetates by GLC.

The absolute configuration (D or L) of the glycosyl residues was determined as described by Gerwig *et al.* (1979).

D-Glucan

Isolation

The polysaccharide was isolated from *P. ostreatus* according to the procedure developed by Kuniak *et al.* (1991). Fruit bodies (100 g, 11% dry weight) were disintegrated with a Turmix blender in 0·15 M aqueous sodium hydroxide solution (500 ml) at ambient temperature, then stirred for 2 h at 95°C. The residue was collected by centrifugation (1500 × *g* for 10 min) washed with water until neutral, then suspended in 0·06% aqueous sodium chlorite solution, adjusted to pH 4·5 with acetic acid, stirred for 6 h at 50°C and centrifuged. The residue was washed successively with water and acetone and then dried *in vacuo* at 60°C. The yield was 505 mg (found: nitrogen 0, ash 0·2%, IR 890 cm^{-1}). Complete hydrolysis followed by GLC analysis revealed only the presence of glucose.

The heterogeneous etherification of the particulate D-glucan with monochloroacetic acid in alkaline medium gave the sodium salt of the water-soluble O-(carboxymethyl) glucan derivative with the degree of substitution 0·5. The yield was 98% (Kuniak *et al.*, 1987).

Partial acid hydrolysis

The D-glucan (1·5 g) was treated twice with 0·4 M sulfuric acid (30 ml) for 8 h at 100°C. The hydrolysate was cooled after each treatment and the part to be hydrolysed further was collected by centrifugation. The combined hydrolysates were neutralized ($BaCO_3$) filtered and freeze-dried. The low molecular-weight fragments (135 mg) after fractionation on a column (1·3 × 200 cm) of Bio-Gel P-2 with water, gave glucose $[\alpha]_D$ + 52° (c1, water) and a mixture of gluco-oligosaccharides (d.p. 2–5) which were further purified by HPLC.

The oligosaccharides were reduced ($NaBD_4$) and deionized with Dowex 50W (H^+) resin. The oligosaccharide alditols were methylated (Hakomori, 1964), then purified using Sep-Pak C_{18} cartridge (Waters Assoc.) and analysed by GLC-MS (e.i.) to produce fragment ions of the A, J, and alditol series (Kochetkov & Chizhov, 1966). The samples were hydrolysed (0·1 M hydrochloric acid, 2 h, 100°C) and the resulting sugars were converted into their alditol ($NaBH_4$) acetates and identified by GLC-MS (Jansson *et al.*, 1976).

Of the methylated disaccharide alditols (1 and 2), 1 (R_t 26·22 min) gave fragment ions at m/z 296 (abJ_1), 236 (bA_1), 219 and 187 (aA_1 and aA_2) and ions of the alditol series at m/z 337, 178, and 146; whereas 2 (R_t 29·05 min) gave ions at m/z 338 and 133, indicating 1 to be D-Glc*p*-(1 → 6)-D-Glc-ol-1-d and 2 to be D-Glc*p*-(1 → 3)-D-Glc-ol-1-d (Kärkkäinen, 1970).

The MS (e.i.) spectrum of the methylated trisaccharide alditol 3 (R_t 50·2 min) contained ions due to bcJ_1, cA_1, aA_2 and H_1 (base peak) at m/z 296, 236, 187 and 88, which characterized the (1 → 6) linkage between units a and b (Kärkkäinen, 1971). Methylation analysis showed that it was composed of terminal non-reducing glucosyl, 6-linked glucosyl and 3-linked glucitol residues. Thus, 3 was identified as D-Glc*p*-(1 → 6)-D-Glc*p*-(1 → 3)-D-Glc-ol-1-d.

The mass spectrum of methylated 4 (R_t 54·17 min) differed from that of 3 by the absence of an ion peak at m/z 296 (bcJ_1) and the presence of ion peaks at m/z 187 (base peak) and at m/z 159 which proved the (1 → 3)-linkage position between units a and b (Kärkkäinen, 1971). Methylation analysis showed that it was composed of terminal non-reducing glucosyl, 3-linked glucosyl, and 3-linked glucitol. Thus, 4 was identified as D-Glc*p*-(1 → 3)-D-Glc*p*-(1 → 3)-D-Glc-ol-1-d.

The FAB-MS spectra of compounds 5 and 6 contained characteristic peaks of pseudomolecular $[M + H]^+$ ions at m/z 670 and 832 which were subjected to MS/MS fragmentation measurements. Two fragmentation routes (Kováčik *et al.*, 1992, 1993) resulting in sequence ions giving peaks at m/z 163, 325, and 487 (A-type ions) and peaks of protonated ions at m/z 508, 346, and 184 (B-type ions) identified 5 as a straight-chain hexose containing tetrasaccharide alditol.

The MS/MS spectrum of 6 contained, in comparison with that of 5, an additional pair of peaks at m/z 649 and 670, which proved 6 to be a straight-chain hexose containing pentasaccharide alditol. Methylation analysis showed that 5 and 6 were composed of terminal non-

Table 1. Glycosyl linkage compositions of β-D-glucans from *Pleurotus ostreatus*

O-Methyl-D-glucose	$T^{\dagger}$	Molar ratios		Linkage β-D-indicated
		Pleuran	HA glucan[§]	
2,3,4,6-Tetra-	1·00	1·0	1·0	D-Glc*p*-(1→
2,4,6-Tri-	1·31	2·7	3·0	→3)-D-Glc*p*-(1→
2,3,6-Tri-	1·34	0·2		→4)-D-Glc*p*-(1→
2,3,4-Tri-	1·36	0·2		→6)-D-Glc*p*-(1→
2,4-Di-	1·68	1·0	1·0	→3,6)-D-Glc*p*-(1→
4,6-Di-	1·58	Tr[‡]		→2,3)-D-Glc*p*-(1→

[†]Retention times of the corresponding alditol acetates with respect to that of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol.
[‡]Tr = trace.
[§]According to Yoshioka *et al.* (1985).

reducing glucosyl, 3- and 6-linked glucosyl and 3-linked glucitol in the molar ratios (based on peak areas) 0·9:1·1:1·0:1·1 and 0·9:1·9:1·0:1·1, respectively.

Methylation analysis

A portion (40 mg) of D-glucan sample was methylated first by the Haworth (1915) method, followed by the Hakomori (1964) method, then twice by the Purdie procedure (Hirst & Percival, 1965) to give a product (40 mg) showing no IR absorption for hydroxyl (found: methoxyl, 45·2%). The methylated polysaccharide (10 mg) was hydrolysed with aqueous 90% (w/w) formic acid (1 ml) for 1 h at 100°C followed by 2 M hydrochloric acid for 8 h at 100°C. PC revealed di-, tri-, and tetra-O-methylglucose with R_{Glc} 0·6, 0·82, 0·87, and 1·0 which were converted into their alditol acetates. Analysis by GLC-MS gave the results shown in Table 1.

Smith degradation

A glucan sample (100 mg) was stirred with 0·12 M sodium metaperiodate for 96 h (40 ml) in the dark. The oxidized polysaccharide was reduced ($NaBH_4$) for 96 h, acetic acid was added and the solution was dialysed. A portion of glucan polyol (4 mg) was hydrolysed with 2 M trifluoroacetic acid for 2 h at 90°C. GLC showed the presence of glycerol and glucose in the molar proportions 1·0:3·4 and small amounts of erythritol. Smith degradation (Goldstein *et al.*, 1965) was complete after mild hydrolysis with 1 M trifluoroacetic acid for 48 h at ambient temperature. The reaction mixture was made neutral with $NaHCO_3$, dialysed, and the degraded glucan was recovered by lyophilization (yield 60 mg).

RESULTS AND DISCUSSION

Fruit bodies of *Pleurotus ostreatus* (*Basidiomycetes*) were harvested locally, disintegrated and extracted with methanol and acetone. Successive extraction with

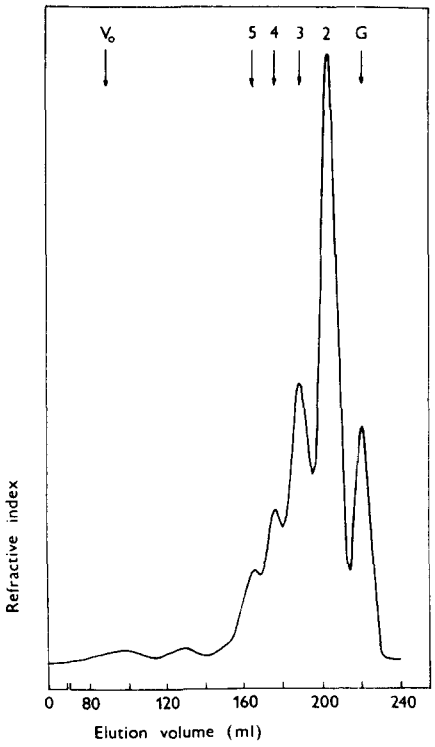
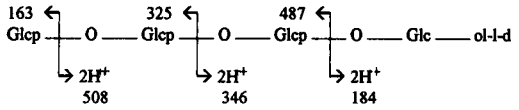


Fig. 1. Gel filtration on Bio-Gel column (1·3 × 200 cm) of the oligosaccharides from pleuran hydrolysate. V_0, void volume: 2–5 standard compounds of gluco-oligosaccharides d.p. 2–5: G, D-glucose.

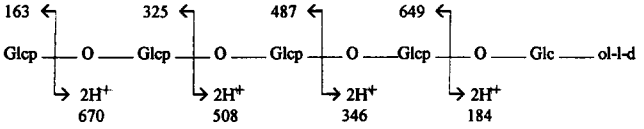
aqueous alkaline solutions followed by sodium chlorite treatment provided a white, alkali-insoluble core material (recovery ~50% of the dry weight of the sample) containing glucose as the only component monosaccharide and was not contaminated with proteins (Kuniak *et al.*, 1991). On the basis of GLC analysis of the per-O-(trimethylsilyl)ated (−)-2-butyl glycoside the absolute configuration of glucose was shown to be D. The ^{13}C-NMR spectrum of the O-(carboxymethyl)glucan derivative contained a signal in the anomeric region at 103·9 ppm attributable to the β-D-glucopyranosyl residues (Agrawal, 1992).

Partial acid-hydrolysis of D-glucan gave glucose and gluco-oligosaccharides (d.p. 2–5) that were resolved on Bio-Gel P-2 (Fig. 1) and then reduced to their corresponding alditols. The structures of di- and trisaccharide alditols 1–4 were established by mass spectrometry and methylation analysis to be D-Glc*p*-(1 → 6)-glucitol (1), D-Glc*p*-(1 → 3)-glucitol (2), D-Glc*p*-(1 → 6)-D-Glc*p*- (1 → 3)-glucitol (3) and, D-Glc*p*-(1 → 3)-D-Glc*p*-(1 → 3)-glucitol (4). Compounds (5) and (6) were characterized by FAB-MS and MS/MS measurements (Kováčik *et al.*, 1992, 1993) and methylation analysis as straight-chain glucotetraose (5) and glucopentaose alditols (6) containing (1 → 6) and (1 → 3) linkages.

The partially methylated monosaccharides obtained by hydrolysis of the methylated D-glucan were identified as the corresponding alditol acetates by GLC-MS



(5)



(6)

(Jansson *et al.*, 1976). The results are listed in Table 1.

Methylation analysis of the D-glucan revealed the presence of 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-O-methyl-glucose together with small portions of 2,3,4- and 2,3,6-tri-O-methyl-glucose (molar proportions 1·0:2·7:1·0:0·2:0·2) (Table 1). This finding was further supported by the products obtained by periodate oxidation, borohydride reduction and acid hydrolysis of the glucan. GLC analysis showed the presence of glycerol and glucose in a molar proportion 1·0:3·4, consistent with the data obtained by the methylation analysis (1·0:3·2). Detection of trace amounts of erythritol indicated the presence of (1 → 4)-linked glucopyranosyl residues. The periodate-resistant, degraded glucan (Smith degradation) on methylation gave 2,4,6-tri-O-methyl-glucose and only trace amounts of 2,3,4,6-tetra- and 2,4-di-O-methyl-glucoses.

From the structures of 1–4 and the results of methylation analysis before and after Smith degradation (Goldstein *et al.*, 1965) it follows that the D-glucan has a highly branched structure consisting of a backbone chain of (1 → 3)-linked glucopyranosyl residues with one of every two or three residues substituted at 0–6 with single glucosyl groups. In addition, the glucan contains small proportions of (1 → 6)- and (1 → 4)-linked interior residues. Although the precise location of these interior linkages was not determined, identification of 3, 5, and 6 as well as isolation of the water-insoluble (1 → 3)-linked linear glucan obtained after Smith degradation indicated their location in side chains.

The (1 → 3)-β-D-glucans so far isolated from fungi and other sources have essentially the same structural features. There are differences in the size and shape of the macromolecules associated with side chains, e.g. degree of branching, distribution and length of branches, etc. (Kishida *et al.*, 1989; Sone *et al.*, 1985; Shida *et al.*, 1981). The primary structure of pleuran from *Pleurotus ostreatus* is similar to that of β-D-glucans commonly found in other *Basidiomycetes* and *Ascomycetes*, e.g. lentinan, scleroglucan, schizophyllan; these (1-3)-β-D-glucans have two branches for every five D-glucopyranosyl residues (lentinan), one for every three or four (schizophyllan) or one for every three residues (scleroglucan) at 0–6. While there seem to exist no significant differences between D-glucans as far as the basic structure is concerned, their solubility properties appear to be distinctly different. In contrast to water- and alkali-soluble D-glucans, pleuran was found to be insoluble in alkali. This may be due to different higher order structure rather than higher molecular mass of the polysaccharide. With respect to degree of branching pleuran appears to be closely related to the water-soluble (1 → 3)-β-D-glucan isolated from the same fungus (Yoshioka *et al.*, 1985) (Table 1). The only difference between the two polysaccharides appears to be the occurrence of (1 → 6)- and (1 → 4)-linked interior residues and the higher molecular weight of the alkali-insoluble type. These characteristics might explain the differences in solubility and extraction behavior of these polymers.

Pleuran was tested for its immunomodulatory activity. It promoted survival of mice susceptible to systematic *Listeria monocytogeneses* infection when administered prophylactically by the intraperitoneal route. The doses above 10 mg/kg given 72 and 24 h before infection were maximally effective. Similarly, an apparent dose-dependent promotion of survival of mice susceptible to pulmonary *Haemophilus influenzae* infections occurred when pleuran was administered intraperitoneally 72 and 24 h prior to infection. The optimal dose was 3 mg/kg. However, in the latter model, some efficacy was observed when the compound was administered orally, with an optimal dose of 3 mg/kg (Kunz & O'Reilly, 1991).

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