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The structure of a polysaccharide from Fraction-II of an edible mushroom, *Pleurotus florida*

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Abstract—A water-soluble polysaccharide was isolated from Fraction-II of the aqueous extract of the fruit bodies of the mushroom, *Pleurotus florida*. Compositional analysis, methylation analysis, periodate oxidation study, Smith degradation, and NMR studies (^{1}H, ^{13}C, DQF-COSY, TOCSY, NOESY, HSQC, and HMBC) revealed the presence of the following repeating unit in the polysaccharide:

α-D-Glc*p*
1
↓
4
→6)-α-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→
2
↑
1
β-D-Man*p*

Keywords: *Pleurotus florida*; Polysaccharide; Structure; NMR spectroscopy

1. Introduction

Mushrooms are a nutritionally beneficial foodstuff and a source of physiologically beneficial and nontoxic medicines.[1] They have been used in folk medicine throughout the world since ancient times. Attempts have been made in many parts of the world to explore the use of mushrooms and their metabolites for the treatment of a variety of human ailments, and they are now considered to be one of the most useful antitumor agents for clinical uses.[2] *Pleurotus* species are commonly called oyster mushrooms. There are about 40 species of this mushroom. They enjoy worldwide distribution, both in temperate and tropical parts of the world. Oyster mushrooms now rank second among the important cultivated mushrooms in the world.[3] One of the species of the genus *Pleurotus* is *Pleurotus florida*, a delicious edible mushroom with high therapeutic use that is cultivated on a commercial scale in many parts of the world, including India. Studies on the nutritive value of the fruit bodies of *P. florida* have shown that it contains 37.19% protein, 3.72% fat, and 10.98% ash on a dry weight basis.[4] The methanol extract of this mushroom has remarkable capacity to inhibit the growth of solid tumors induced by the EAC cell line in a dose-dependent

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manner in Swiss albino mice.[5] The hot water extract of the fruit bodies of several mushrooms has been reported to show marked host-mediated antitumor activity against sarcoma 180 in a murine model.[6] The hot water extract of the fruit bodies of *P. florida* has been shown to consist of three different polysaccharides. Among them a new (1→3)-, (1→6)-branched glucan,[7] and a (1→6)-α-glucan[8] have been previously reported by us. The structural studies of the rest of the water-soluble polysaccharides were carried out and is reported herein.

2. Results and discussion

Fruit bodies of the mushroom, *P. florida*, were washed with water and boiled with distilled water for 6 h. The whole mixture was then kept overnight at 4 °C and filtered through linen cloth. The filtrate was centrifuged at low temperature. The supernatant was precipitated in 1:5 (v/v) EtOH. The precipitated polysaccharide was collected through centrifugation and dried. The crude polysaccharide was then dissolved in a minimum volume of distilled water and dialyzed through a DEAE cellulose bag against distilled water for 4 h to remove small carbohydrate molecules. The material retained inside the cellulose bag was freeze dried and collected (yield, 2 g). The crude polysaccharide was then allowed to dissolve in 1% NaCl solution to give two fractions, a NaCl-soluble fraction and a NaCl-insoluble fraction. The NaCl-soluble fraction (30 mg) on fractionation through a Sephadex G-75 gel column yielded two polysaccharide fractions, Fraction-I (7 mg) and Fraction-II (12 mg). Fraction-I has been reported as a new (1→3)-, (1→6)-branched glucan.[7] Fraction-II was identified as a heteropolysaccharide composed of D-mannose, D-galactose, and D-glucose. The molecular weight[9] of Fraction-II was estimated from a calibration curve prepared with standard dextrans as $\sim 4.8 \times 10^4$ Da. The polysaccharide showed a specific rotation of $[\alpha]_D$ +80.3 (*c* 0.08, H_2O, 30 °C). We report herein the structural characterization of the polysaccharide of Fraction-II.

The polysaccharide on acid hydrolysis by 2 M TFA showed the presence of D-glucose, D- mannose, and D-galactose that was detected by PC as well as GLC analysis, and they were found to be present in a molar ratio of 1.0:1.0:2.0, respectively.

The total carbohydrate of this fraction was estimated to be 98.4% using the phenol–sulfuric acid method.[10] The absolute configuration[11] of each monosaccharide was determined by GLC examination of (+)-2-butyl 2,3,4,6-tetra-*O*-TMS-glycosides and showed that all have the D configuration.

The mode of linkages of the polysaccharide was determined by methylation analysis using the method of Ciucanu and Kerek,[12] followed by hydrolysis and alditol acetate preparation. The alditol acetates were then analyzed through GLC using columns A, 3% ECNSS-M and B, 1% OV-225 as well as by GLC–MS using an HP-5 fused silica capillary column. The presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol; 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol; 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol, and 1,2,4,5,6-penta-*O*-acetyl-3-*O*-methyl-D-galactitol in a molar ratio of 1.0:1.0:1.0:1.0 was detected. These results indicate that nonreducing-end D-glucopyranosyl, and D-mannopyranosyl, (1→6)-linked D-galactopyranosyl, and (1→2,4,6)-linked D-galactopyranosyl moieties are present in the polysaccharide. Thereafter, a periodate oxidation experiment was carried out with Fraction-II. The periodate-oxidized, reduced material of Fraction-II, upon hydrolysis with TFA followed by GLC analysis, showed the presence of D-galactose only. This indicates that D-glucose and D-mannose are consumed during oxidation. GLC analysis of periodate-oxidized, reduced, and methylated polysaccharide showed that the monomethyl sugar residue, 1,2,4,5,6-penta-*O*-acetyl-3-*O*-methyl-D-galactitol, was retained. This result indicates that both the nonreducing-end D-glucopyranosyl, D-mannopyranosyl, and (1→6)-linked D-galactopyranosyl moieties are consumed during oxidation. A Smith degradation[13,14] experiment was also carried out with the polysaccharide to determine the sequences of sugar residues present in the polysaccharide. GLC analysis of the alditol acetates of the acid-hydrolyzed product from the Smith-degraded polysaccharide showed the presence of D-galactose and D-glycerol in a molar ratio 1.0:1.0. Glycerol is produced from (1→6)-linked D-galactopyranosyl moieties during periodate oxidation followed by Smith degradation. The GLC–MS analysis of the alditol acetates of methylated, reduced Smith-degraded product revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol, which indicates that 1,2,4,6-linked D-galactose is present in the backbone of the polysaccharide, Fraction-II.

The ^{1}H NMR (500 MHz) spectrum (Fig. 1) at 35 °C showed four signals in the anomeric region at δ 5.12, 4.98, 4.80, and 4.51 ppm in a ratio of 1.0:1.0:1.0:1.0. In the ^{13}C NMR spectrum (Fig. 2) at 27 °C, four anomeric signals appeared at δ 103.4, δ 102.1, and δ 98.3 ppm in a ratio of 1.0:2.0:1.0. Since a small $J_{1,2}$ value for the D-mannosyl residue does not give information about the anomeric configuration, a proton-coupled ^{13}C NMR experiment was carried out. All the ^{1}H and ^{13}C signals were assigned using DQF-COSY, TOCSY, HSQC, and HMBC NMR experiments. The four sugar moieties were designated as residues I, II, III, and IV according to their decreasing chemical shifts (Table 1) in the ^{1}H NMR spectrum.

Residue I was assigned to (1→2,4,6)-linked-α-D-galactopyranose. The *galacto* configuration was assigned from the large $^3J_{2,3}$ coupling constant of ~8 Hz and relatively small $^3J_{3,4}$ coupling constant of ~3 Hz. The

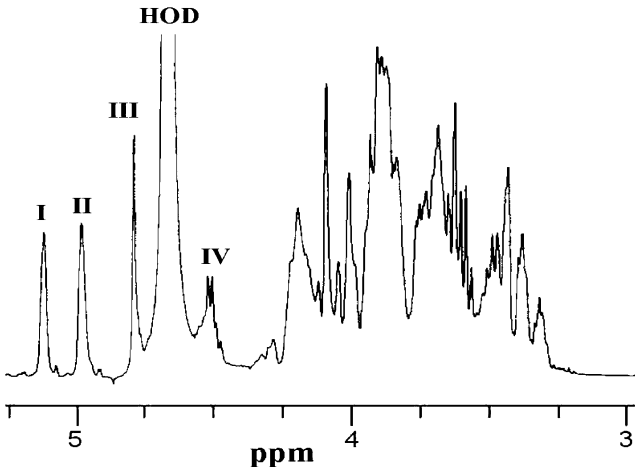

Figure 1. ^{1}H NMR spectrum (500 MHz, D_2O, 35 °C) of the polysaccharide from Fraction-II isolated from *P. florida.*

α-configuration of residue I (δ 5.12) was assigned from $^{3}J_{1,2}$ coupling constant of ~3 Hz and $^{1}J_{C,H}$ of ~170 Hz. The chemical shifts of all the protons (H-1–H-6) were identified from DQF-COSY and TOCSY spectra (Fig. 3, top panel). All carbon signals of residue I were assigned from the HSQC spectrum. The anomeric carbon signal of residue I at 98.3 ppm was confirmed by the presence of a cross-peak IC-1, IVH-6a, and IVH-6b in the HMBC experiment (Fig. 4 and Table 4). The downfield shift of C-2 (δ 76.1), C-4 (δ 76.6), and C-6 (δ 69.3) carbon signals with respect to standard values of methyl glycosides[15,16] indicates that residue I is a (1→2,4,6)-linked D-galactose moiety.

Residue II has an anomeric proton signal at δ 4.98 ppm, and the $^{3}J_{1,2} \sim 3.2$ Hz, $^{2}J_{C,H} \sim 171$ Hz indicate that it is an α-linked moiety. Large coupling constants $^{3}J_{2,3}$ and $^{3}J_{3,4}$ (~10 Hz) were observed from the DQF-COSY spectrum for residue II, supporting that it is a D-glucosyl moiety. The chemical shifts of all the protons (H-1–H-6) were identified from the DQF-COSY and TOCSY spectra. The carbon signals (Table 3) from C-1 to C-6 for residue II were identified from the HSQC spectrum, and these nearly correspond to the standard values of methyl glycosides.[15,16] The C-1 signal of residue II at 102.1 ppm was confirmed by the appearance

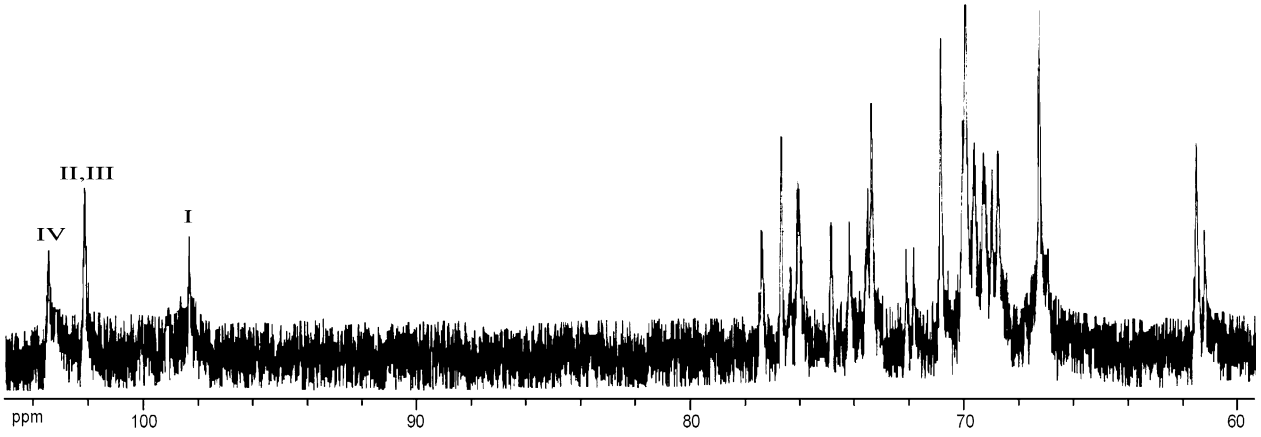

Figure 2. ^{13}C NMR spectrum (125 MHz, D_2O, 27 °C) of the polysaccharide from Fraction-II isolated from *P. florida.*

Table 1. The ^{1}H NMR data at 35 °C for the polysaccharide (Fraction-II) isolated from *P. florida*[a]

Glycosyl residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
→2,4,6)-α-D-Gal*p*-(1→ **I**	5.12	3.93	3.99	4.20	4.05	3.69	3.71
α-D-Glc*p*-(1→ **II**	4.98	3.85	4.01	3.60	3.88	4.22	4.28
β-D-Man*p*-(1→ **III**	4.80	4.09	3.58	3.65	3.38	3.74	3.89
→6)-β-D-Gal*p*-(1→ **IV**	4.51	3.32	3.49	4.09	3.63	3.84	3.91

[a] Values of chemical shifts were recorded with respect to the HOD signal fixed at δ 4.66 ppm at 35 °C.

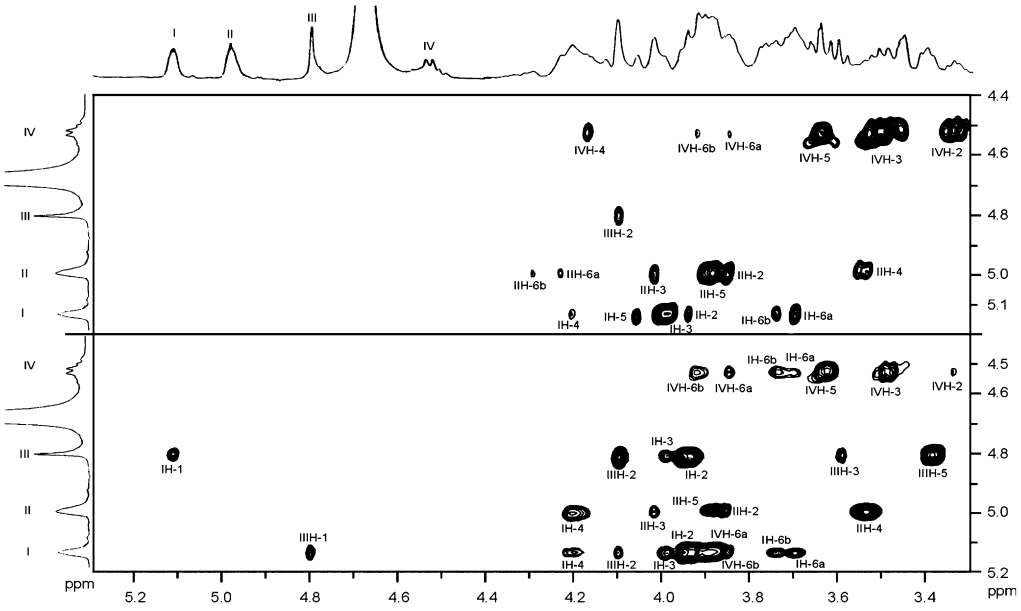

Figure 3. TOCSY (top panel) and NOESY (bottom panel) spectra of the polysaccharide from Fraction-II isolated from *P. florida*. The mixing time for the TOCSY spectrum shown was 150 ms. Complete assignment required several TOCSY experiments having mixing times ranging from 60 to 300 ms. The NOESY mixing time was 300 ms.

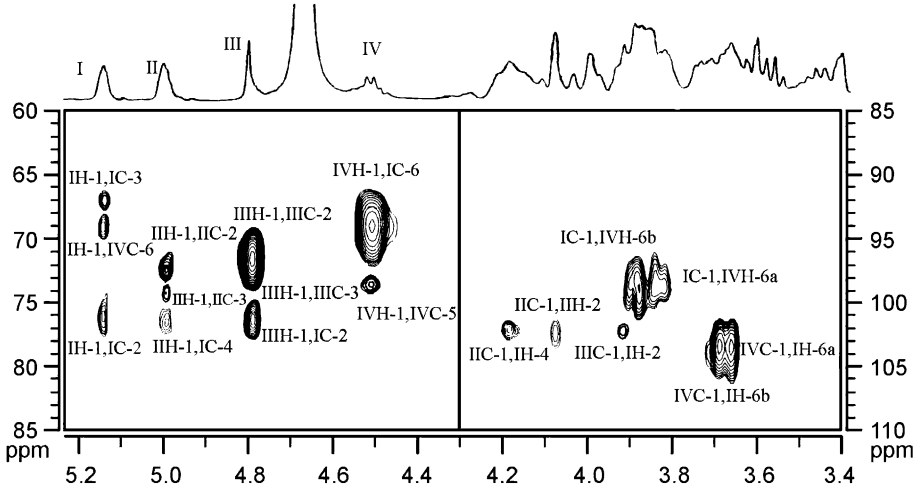

Figure 4. HMBC spectrum (anomeric proton region in the left panel and anomeric carbon region in right panel) of the polysaccharide from Fraction-II isolated from *P. florida*. The delay time in the HMBC experiment was 80 ms.

of a cross-peak IIC-1, IH-4 in the HMBC experiment (Table 4 and Fig. 4).

Residue III was assigned to the nonreducing-end D-mannopyranosyl unit. The manno configuration for residue III was supported from large coupling constants of $^{3}J_{3,4}$ (~7.0 Hz) and $^{3}J_{4,5}$ (~9.0 Hz). The anomeric signal for residue III at δ 4.80 ($^{3}J_{1,2} \sim 1$ Hz, and $^{3}J_{2,3} \sim 3.5$ Hz) and $J_{C,H} \sim 160$ Hz indicate that D-mannose is β-linked. The chemical shifts of all the protons (H-1–H-6) were identified from the DQF-COSY and TOCSY spectra. The carbon chemical shifts from C-1 to C-6 for residue III were assigned from the HSQC spectrum, and these nearly correspond to the standard values of methyl glycosides[15,16] of D-mannose. The C-1 signal of residue III at 102.1 ppm was confirmed by the appearance of a cross-peak IIIC-1, IH-2 in the HMBC experiment. Thus considering the results of methylation analysis and the NMR experiment, it may be concluded that residue III is a β-glycosidically linked, nonreducing-end D-mannose moiety.

All the proton chemical shifts of IV (H-1–H-6) were identified from the DQF-COSY as well as the TOCSY spectra (Fig. 3, top panel). A large $^{3}J_{2,3}$ (~9 Hz) and relatively small $^{3}J_{3,4}$ (~3 Hz) indicated that residue IV has the galacto configuration. The chemical shifts of C-1–C-6 of residue IV were assigned from the HSQC spectrum. The anomeric proton chemical shift for residue IV at δ 4.51 ppm ($^{3}J_{1,2} \sim 7.7$ Hz) and carbon chemical shift of

Table 2. NOE data for the polysaccharide (Fraction-II) isolated from *P. florida*

Anomeric proton		NOE contact protons	
Glycosyl residue	δ	δ	Residue, atom
→2,4,6)-α-D-Gal*p*-(1→	5.12	3.93	I H-2
		3.99	I H-3
I		4.20	I H-4
		3.69	I H-6a
		3.71	I H-6b
		4.80	III H-1
		4.09	III H-2
		3.84	IV H-6a
		3.91	IV H-6b
α-D-Glc*p*-(1→	4.98	3.85	II H-2
II		4.01	II H-3
		3.60	II H-4
		3.88	II H-5
		4.20	I H-4
β-D-Man*p*-(1→	4.80	4.09	III H-2
III		3.58	III H-3
		3.38	III H-5
		5.12	I H-1
		3.93	I H-2
		3.99	I H-3
→6)-β-D-Gal*p*-(1→	4.51	3.32	IV H-2
IV		3.49	IV H-3
		3.63	IV H-5
		3.84	IV H-6a
		3.91	IV H-6b
		3.69	I H-6a
		3.71	I H-6b

δ 103.4 ppm ($J_{C,H} \sim 160$ Hz) indicated that the D-galactose is β-linked. The anomeric signal of residue IV at δ 103.4 ppm was confirmed by the presence of a cross-peak IVC-1, IH-6a and IVC-1, IH-6b in the HMBC experiment. In conclusion, it is important to note that the peak at δ 102.1 ppm corresponds to the anomeric carbon of both the β-D-mannosyl residue III and α-D-glucosyl residue II for which it is almost double the proportion of D-galactosyl moieties appearing at δ 103.4 ppm and δ 98.3 ppm.

The sequence of glycosyl residues of Fraction-II was determined from the NOESY experiment (Fig. 3, bottom panel), followed by confirmation with HMBC experiments. Residue I has an NOE contact from H-1 to both H-6a and H-6b of residue IV, in addition to intraresidue NOE contacts to H-2, H-3, H-4, H-6a, and H-6b (Table 2). Hence, this indicates that residue I is linked at the 6-position of residue IV. Similarly residue IV has interresidue contacts from H-1 to H-6a and H-6b of residue I, in addition to the intraresidue NOE contacts to H-2 (very weak), H-3, H-5, H-6a, and H-6b, indicating that residue IV is linked to the 6-position of residue I. Thus the above NOE results suggest the following sequence between two residues of I and IV.

→6)-α-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→
I IV

Residue II has an NOE contact from H-1 to H-4 of residue I in addition to intraresidue contacts to H-2, H-3, H-4, and H-5, indicating that residue II is linked at the 4-position of residue I. On the other hand, residue III has interresidue NOE contacts from H-1 to H-1, H-2, and H-3 of residue I in addition to intraresidue contacts to H-2, H-3, and H-5. The interresidual NOE contacts from H-1 of residue I to H-1, and H-2 of residue III are also observed in the NOE spectrum. Thus, it indicates that residue III is also linked to residue I at the 2-position. Hence, residue I is a 1,2,4,6-linked moiety, and the following tetrasaccharide repeating unit is established.

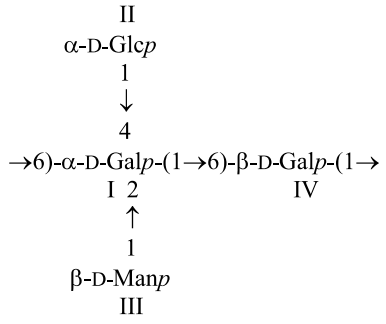

Table 3. The ^{13}C NMR chemical shifts for the polysaccharide (Fraction-II) isolated from *P. florida*[a] in D_2O at 27 °C

Glycosyl residue	C-1	C-2	C-3	C-4	C-5	C-6
→2,4,6)-α-D-Gal*p*-(1→	98.3	76.1	67.2	76.6	67.2	69.3
I						
α-D-Glc*p*-(1→	102.1	72.2	74.1	69.9	71.9	61.2
II						
β-D-Man*p*-(1→	102.1	70.9	74.8	68.4	77.4	61.5
III						
→6)-β-D-Gal*p*-(1→	103.4	70.9	73.5	69.5	73.3	68.9
IV						

[a] Values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm at 27 °C.

Long-range ^{13}C–^{1}H correlations obtained from the HMBC spectrum (Fig. 4) corroborated the assigned tetrasaccharide repeating unit deduced from the NOESY experiments. The cross-peaks of both anomeric protons and carbons of each of the sugar moieties were examined, and intra- and inter-residual connectivities were observed from the HMBC experiment (Table 4). Cross-peaks were found between H-1 of α-D-galactose (δ 5.12) and C-6 of β-D-galactose (IH-1, IVC-6); C-1 of α-D-galactose (δ 98.3) and both H-6a and H-6b of β-D-galactose (IC-1, IVH-6a, and IVH-6b), with other intraresidual coupling between H-1 of α-D-galactose with its own C-2 and C-3 atoms. Similarly, cross-peaks between C-1 (δ 102.1) of α-D-glucose and H-4 of α-D-galactose (IIC-1, IH-4); H-1 of α-D-glucose and C-4 of α-D-galactose (IIH-1, IC-4) were observed. Intraresidual coupling between H-1 of α-D-glucose with its own C-2 and C-3 atoms were also observed. The cross-peaks between C-1 of D-mannose (δ 102.1) and H-2 of α-D-galactose (IIIC-1, IH-2); H-1 of D-mannose (δ 4.80) and C-2 of α-D-galactose (IIIH-1, IC-2) were observed with other intraresidual coupling between H-1 of D-mannose with its C-2 and C-3 positions. The cross-peaks between H-1 of β-D-galactose (δ 103.4) with C-6 of α-D-galactose (IVH-1, IC-6) and C-1 of β-D-galactose with H-6a and H-6b of α-D-galactose (IVC-1, IH-6a and IVC-1, IH-6b) were observed along with other intraresidual coupling between H-1 of β-D-galactose with its C-5. Thus, the appearance of these cross-peaks clearly supports the presence of a tetrasaccharide repeating unit in the polysaccharide, Fraction-II of *P. florida.*

3. Experimental

3.1. Isolation and purification of the polysaccharide

Two kilograms of fresh mushroom fruit bodies was collected from a local firm where they were grown on rice straw supplemented with cottonseed powder at a temperature of 28 °C. After washing with distilled H_2O and EtOH, the mushroom bodies were pulverized for extraction of the polysaccharide by boiling with water for 4 h as applied in our earlier works.[17–19] The aqueous extract was filtered with linen cloth, and the filtrate was collected and kept overnight at 4 °C. The filtrate was centrifuged at 8000 rpm at 6 °C for 45 min to obtain a clear solution, and then the polysaccharide was precipitated with EtOH (80%, final concentration). After keeping the precipitate at 4 °C overnight, it was collected by centrifugation at 6 °C for 1 h. Then, it was dissolved in a minimum volume of water and then dialyzed against distilled water for 12 h to remove low-molecular-weight carbohydrate materials. The aqueous extract was then reprecipitated with 1:5 v/v EtOH. It was again centrifuged, and the precipitate was collected and freeze dried (yield, 2 g). When this polysaccharide was dissolved in 1% NaCl solution, one portion became soluble while another portion remained insoluble. These two parts were separated by centrifugation. The soluble part was purified by gel-permeation chromatography. The polysaccharide (30 mg) was dissolved in 1% NaCl solution (3 mL). The soluble portion was separated and applied all at once onto a Sephadex G-75 column

Table 4. The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide (Fraction-II) of *P. florida*

Residue	Sugar linkage	H-1/C-1 δ_H/δ_C	Observed connectivities		
			δ_H/δ_C	Residue	Atom
I	→2,4,6)-α-D-Gal*p*-(1→	5.12	67.2	I	C-3
			76.1	I	C-2
			68.9	IV	C-6
		98.3	3.84	IV	H-6a
			3.91	IV	H-6b
II	α-D-Glc*p*-(1→	4.98	72.2	II	C-2
			74.1	II	C-3
			3.85	II	H-2
		102.1	76.6	I	C-4
			4.20	I	H-4
III	β-D-Man*p*-(1→	4.80	70.9	III	C-2
			74.8	III	C-3
			76.1	I	C-2
		102.1	3.93	I	H-2
IV	→6)-β-D-Gal*p*-(1→	4.51	73.3	IV	C-5
			69.3	I	C-6
			3.69	I	H-6a
		103.4	3.71	I	H-6b

(100 cm × 1 cm) saturated with 1% NaCl solution and fractionated (0.2 mL/min) using a Redifrac fraction collector. Fractions were collected and analyzed with the phenol–sulfuric acid reagent[19] at 490 nm using a Shimadzu UV–vis spectrophotometer, model 1601. Fractions (test tubes 40–52 and 56–76) corresponding to two peaks were pooled together separately and freeze dried. After reducing the volume by freeze drying, the fractions were dialyzed against distilled water to remove NaCl and then again freeze dried (yield, Fraction-I, 7 mg and Fraction-II, 12 mg). This same procedure was followed several times. The apparent molecular weight of Fraction-II was estimated using standard dextrans (T_{100}, T_{40}, and T_{10} from Pharmacia). Total carbohydrate of the polysaccharide (Fraction-II) was determined using the phenol–sulfuric acid reagent.

3.2. Sugar composition

The polysaccharide sample (3 mg) was hydrolyzed in 2 M trifluoroacetic acid (2 mL) at 100 °C for 16 h in a boiling water bath. The components of the hydrolyzate were then converted into their alditol acetates and analyzed by gas–liquid chromatography (GLC) using a Hewlett–Packard model 5730 instrument equipped with a flame-ionization detector. Peaks were identified and estimated with arabinose as the internal standard. The alditol acetates were resolved on a glass column (1.8 m × 6 mm) containing 3% ECNSS-M on Gas Chrom Q (100–120 mesh) at 170 °C. Gas–liquid chromatography–mass spectrometric (GLC–MS) analysis was also performed on a Hewlett–Packard 5970A automatic GLC–MS system, using an HP-5 capillary column (25 m × 25 mm) and a temperature program starting at 150 °C (2 min), followed by an increase of 2 °C/min to 200 °C (5 min). Quantitation was carried out from the peak areas using response factors from standard monosaccharides.

3.3. Paper chromatographic studies

Paper partition chromatographic studies were performed on Whatmann nos 1 and 3 mm sheets. Solvent systems used were: (X) BuOH–HOAc–H_2O (v/v/v, 4:1:5, upper phase) and (Y) EtOAc–pyridine–H_2O (v/v/v, 8:2:1). The spray reagent used was alkaline silver nitrate solution.[20]

3.4. Absolute configuration of monosaccharides

The method used was based on that of Gerwig et al.[11] After trifluoroacetic acid (TFA) hydrolysis of 1 mg of polysaccharide, the acid was removed by co-distillation with water. A solution of 250 μL of 0.625 M HCl in (+)-2-butanol was added to the residue, and the mixture was heated at 80 °C for 16 h. The reactants were then evaporated, and per-*O*-TMS-derivatives were prepared with *N*,*O*-bis (trimethylsilyl)trifluoroacetamide (BSTFA). The products were analyzed by GLC using a capillary column (SPB-1, 30 m × 0.26 mm) with a temperature program (3 °C/min) from 150 to 210 °C. The (+)-2-butyl 2,3,4,6-tetra-*O*-TMS-glycosides obtained were identified by comparison with those prepared from the D and L enantiomers of the monosaccharides.

3.5. Linkage analysis

The polysaccharide (4 mg) was methylated according to the methods of Ciucanu and Kerek,[12] and the products were isolated by partition between 5:2 $CHCl_3$–H_2O. The organic layer containing products was washed with 3 mL water three times and dried. The methylated products were then hydrolyzed with 90% HCO_2H (1 mL) at 100 °C for 1 h, reduced with $NaBH_4$, acetylated with 1:1 Ac_2O–pyridine and analyzed by GLC and GLC–MS using an HP-5 capillary column and the same temperature program indicated above. Quantitations were carried out from peak areas.

3.6. Periodate oxidation study

The polysaccharide (5 mg) was oxidized with 0.1 M sodium metaperiodate (2 mL) at 27 °C in the dark during 48 h. The oxidation process was stopped by addition of 1,2-ethanediol, and the solution was dialyzed against distilled water. The dialyzed material was reduced with $NaBH_4$ for 15 h and neutralized with HOAc. The resulting residual material was obtained by repeated addition of MeOH, followed by distillation. The residue was subjected to both hydrolysis and methylation by the usual procedures described above, and the products were analyzed by GLC–MS.

3.7. Smith degradation

The polysaccharide (Fraction-II) (15 mg) was oxidized with 0.1 M sodium metaperiodate (4 mL) at 27 °C in the dark during 48 h. The oxidation was stopped by addition of 1,2-ethanediol, and the solution was dialyzed against distilled H_2O. The dialyzed material was reduced with $NaBH_4$ for 15 h at 27 °C, neutralized with 50% HOAc and again dialyzed against distilled water and freeze dried. This portion was subjected to mild hydrolysis with 0.5 M TFA for 15 h at 25 °C to eliminate the residues of oxidized sugars attached to the polysaccharide chain (Smith degradation).[13,14] The acid was removed after repeated addition and evaporation of water at 37 °C. This material (2 mg) was reduced by

$NaBH_4$ and methylated by the usual procedure and analyzed by GLC–MS.

3.8. NMR studies

The freeze-dried polysaccharide was kept over P_2O_5 in vacuum for several days and then deuterium exchanged three times, followed by lyophilization[21] with D_2O. The ^1H, TOCSY, DQF-COSY, NOESY, and HMBC NMR spectra were recorded with a Bruker Avance DPX-500 spectrometer in D_2O at 35 °C. Chemical shifts were referenced to the residual signal of HOD at δ 4.66 ppm. The TOCSY experiment was recorded at mixing time of 150 ms, and complete assignment required several TOCSY experiments requiring several mixing times ranging from 60 to 300 ms. The NOESY mixing delay was 200 ms. The DQF-COSY spectrum was obtained by using standard pulse sequences. For the ^{13}C NMR spectrum of the native polysaccharide, a solution in D_2O was recorded with a Bruker Avance DPX-500 instrument at 27 °C. Acetone was used (δ 31.05 ppm) as the internal standard for the ^{13}C NMR spectrum. The delay time in the HMBC experiment was 80 ms.

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