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Carbohydrate Research 340 (2005) 629–636
Carbohydrate RESEARCH

Structural investigation of a polysaccharide (Fr. II) isolated from the aqueous extract of an edible mushroom, *Pleurotus sajor-caju*

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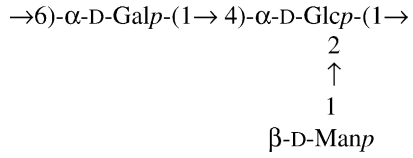
Received 5 March 2004; accepted 31 December 2004

Abstract—A water-soluble polysaccharide, (Fr. II) isolated from aqueous extract of an edible mushroom *Pleurotus sajor-caju*, was found to consist of D-glucose, D-galactose, and D-mannose in a molar proportion of 1:1:1. Compositional analysis, methylation analysis, periodate oxidation study, partial hydrolysis, and NMR experiments (^{1}H, ^{13}C, 2D-COSY, TOCSY, NOESY, HSQC, and HMBC) revealed the presence of the following repeating unit in the polysaccharide:



Keywords: *Pleurotus sajor-caju*; Mushroom polysaccharide; Structure; NMR experiments

1. Introduction

Among different types of mushrooms of the genus *Pleurotus*, namely *Pleurotus sajor-caju*,[1,2] *Pleurotus ostreatus*,[3,4] *Pleurotus citrinopileatus*,[5] and *Pleurotus florida*[6–8] are reported as commonly available edible mushrooms having antitumor materials. These are also known as oyster mushrooms or abalone mushrooms. *Pleurotus sajor-caju*[2] is a delicious edible fungus, which was first found by an Indian, Yan Dai ke, at the foot of the Himalayas and then distributed to many other countries throughout the world. Currently, it is cultivated all over the world. This mushroom contains[2] very little lipid or starch but some protein. The aqueous extract contains vitamin B_1, B_2, and C, and it can reduce the cholesterol level in blood. Thus, it is considered as an important health tonic. Extracellular polysaccharides obtained from liquid culture medium of the mycelium of *P. sajor-caju* have been reported.[9] *P. sajor-caju* was found effective in the degradation of the pesticides, *p*,*p*′-DDT and lindane.[10] Some polysaccharides have been isolated from the fruit bodies of this mushroom using different solvents, and an antitumor[2] polysaccharide has been reported but no detailed structural studies were carried out with any of the polysaccharides isolated from aqueous as well as other extracts. We are reporting herein the detailed structural studies of Fr. II polysaccharide from an aqueous extract of *Pleurotus sajor-caju*.

2. Results and discussion

Fresh mushrooms (1 kg) were washed with distilled water and then extracted with hot water. The whole extract was cooled and centrifuged. The filtrate was collected and freeze dried. The dried material was

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doi:10.1016/j.carres.2004.12.032

dissolved in water and precipitated in alcohol (1:5, v/v). The precipitate was collected through centrifugation and dried. It was again dissolved in water, kept overnight, and centrifuged. The filtrate (water-soluble part) was collected, freeze dried, and the residue (water-insoluble part) was washed repeatedly with water, and dried. The water-soluble material was dialyzed overnight to remove low-molecular weight carbohydrate materials and freeze dried. This material on fractionation by Sepharose 6B gel-permeation chromatography gave two fractions, Fr. I and Fr. II. Both the fractions were collected and freeze dried. Fr. I was identified as soluble glucan, and Fr. II as a hetero polysaccharide composed of D-mannose, D-glucose, and D-galactose. The molecular weights of both Fr. I and Fr. II were estimated from a calibration curve prepared with standard dextrans,[11] and they were nearly 2.4×10^5 and 3.5×10^4, respectively. We are reporting herein the structural characterization of Fr. II, only.

The total carbohydrate of Fr. II was estimated 97.4% using the phenol–sulfuric acid method.[12] Protein was estimated by Lowry's method.[13] On hydrolysis by 2 M TFA, the presence of D-glucose, D-mannose, and D-galactose was detected by PC as well as GLC analysis. These sugars were found to be present in a molar ratio of 1:1:1 as revealed by GLC analysis. The absolute configuration of the monosaccharides were determined by GLC examination of acetylated (+)-2-octyl glycosides and showed that all have D configurations.[14]

GLC analysis of methylated[15,16] alditol acetates of Fr. II using column A, 3% ECNSS-M on Gaschrom-Q (100–120 mesh), and B, 1% OV-225 on Gaschrom-Q (100–120 mesh), followed by GLC–MS examination, revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol, **A** (*m*/*z*: 28, 43, 71, 87, 101, 117, 129, 145, 161, 205); 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-D-glucitol, **C** (*m*/*z*: 28, 43, 71, 87, 99, 113, 129, 173, 189, 233) and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol, **B** (*m*/*z*: 28, 43, 71, 87, 99, 101, 117, 129, 161, 189, 233) in a molar ratio of almost 1:1:1 (Fig. 1, Table 1). Further GLC analysis of the alditol acetates of methylated, periodate-oxidized, reduced polysaccharide showed the presence of 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-D-glucitol. Periodate-oxidized, reduced material of Fr. II, upon hydrolysis with TFA followed by GLC analysis, showed the presence of D-glucose, only. This indicated that the other sugars, D-mannose and D-galactose, are consumed during periodate oxidation. Now, considering the results of methylation and periodate oxidation studies, it may be

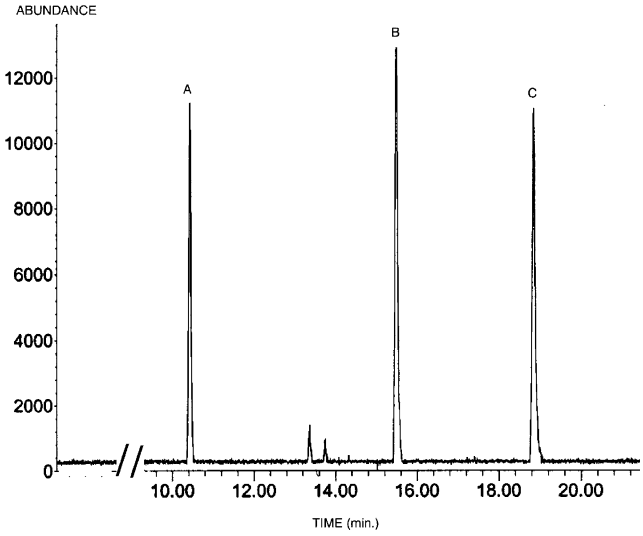


Figure 1. GLC of the alditol acetates derived from polysaccharide, Fr. II isolated from *P. sajor-caju* using an HP-5 fused silica capillary column.

Table 1. GLC and GLC–MS data for methylated alditol acetates of the polysaccharide (Fr. II) isolated from *P. sajor-caju* and oligomers (O-1a and O-1b)

Methylated sugar	t_R[a]	t_R[b]	Mass fragmentation (*m*/*z*)[c]	Molar ratio	Mode of linkage
Fr. II					
2,3,4,6-Me$_4$-Man*p* **A**	1.00	1.00	28, 43, 71, 87, 101, 117, 129, 145, 161, 205	1	Man*p*-(1→
2,3,4-Me$_3$-Gal*p* **B**	3.41	2.89	28, 43, 71, 87, 99, 101, 117, 129, 161, 189, 233	1	→6)-Gal*p*-(1→
3,6-Me$_2$-Glc*p* **C**	4.40	3.73	28, 43, 71, 87, 99, 113, 129, 173, 189, 233	1	→2,4)-Glc*p*-(1→
O-1a					
2,3,4,6-Me$_4$-Gal*p*	1.25	1.19	28, 43, 71, 87, 101, 117, 129, 145, 161, 205	1	Gal*p*-(1→
2,3,6-Me$_3$-Glc*p*	2.50	2.32	28, 43, 71, 87, 99, 101, 117, 129, 161, 173, 233	1	→4)-Glc*p*
O-1b					
2,3,4,6-Me$_4$-Glc*p*	1.00	1.00	28, 43, 71, 87, 101, 117, 129, 145, 161, 205	1	Glc*p*-(1→
2,3,4-Me$_3$-Gal*p*	3.41	2.89	28, 43, 71, 87, 101, 117, 129, 161, 189, 233	1	→6)-Gal*p*

[a] Retention time in GLC with reference to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol of 3% ECNSSM column on Gaschrom-Q at 170 °C.
[b] Retention time in GLC with reference to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol of 1% OV-225 column on Gaschrom-Q at 170 °C.
[c] Equipped with a HP-5 fused-silica capillary column using a temperature program from 150 °C (2 min) to 200 °C (5 min) at 2 °C min^{-1}.

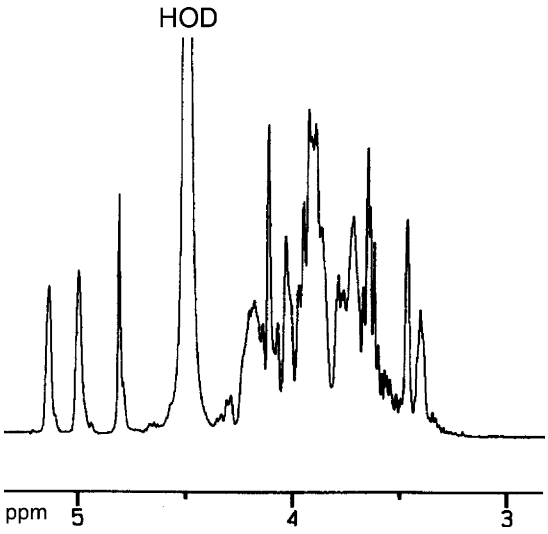


Figure 2. ^{1}H NMR (500 MHz, D_2O, 50 °C) spectrum of polysaccharide, Fr. II isolated from *P. sajor-caju*.

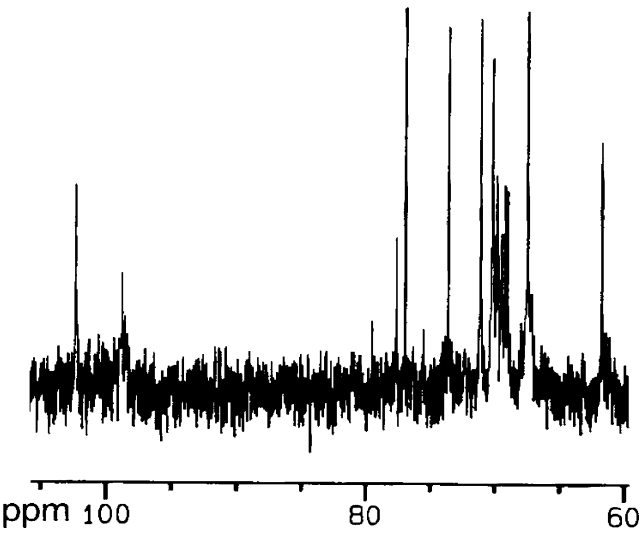


Figure 3. ^{13}C NMR (125 MHz, D_2O, 50 °C) spectrum of polysaccharide, Fr. II isolated from *P. sajor-caju*.

concluded that D-mannose is present as a nonreducing terminal, D-galactose as 1,6-linked residue in the main chain and D-glucose as 1,2,4-linked branched-chain moiety in the polymer. A Smith degradation experiment[17] was also carried out to further prove these linkages. IO_4^- oxidized, reduced Fr. II was treated with 0.5 M TFA, and the released oligomers were identified as α-D-Glc*p*-(1→3)-Gro. The analysis of the alditol acetates of methylated, reduced Smith-degraded product showed the appearance of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and 1-*O*-acetyl-2,3-di-*O*-methyl-D-glycerol, which indicates that 1,2,4-linked D-glucose is present in the backbone of the polysaccharide, Fr. II.

The ^{1}H (500 MHz, Fig. 2, Table 2), ^{13}C (125 MHz, Fig. 3, Table 3) and proton-coupled ^{13}C NMR experiments were carried out at 50 °C. The 500 MHz ^{1}H NMR spectrum showed three signals in the anomeric region at δ 5.14, 5.00, and 4.81 ppm, with a small value of coupling constants in a ratio of nearly 1:1:1. Since a small $J_{1,2}$ value for the D-mannosyl residue does not provide information about the anomeric configuration, a one-bond C-1–H-1 heteronuclear NMR experiment

Table 2. The ^{1}H NMR data at 50 °C for the polysaccharide (Fr. II) isolated from *P. sajor-caju*[a]

Glycosyl residue	H-1	H-2	H-3	H-4	H-5	H-6a[b]	H-6b[b]
β-D-Man*p*-(1→ **A**	4.81	4.12	3.65	3.79	3.40	3.92	4.18
→6)-α-D-Gal*p*-(1→ **B**	5.00	3.87	3.55	4.11	4.04	3.89	3.93
→2,4)-α-D-Glc*p*-(1→ **C**	5.14	3.95	4.02	3.72	3.97	4.07	4.14

[a] Values of chemical shifts were recorded with respect to the HOD signal fixed at δ 4.51 ppm at 50 °C.
[b] a and b are interchangeable.

Table 3. The ^{13}C NMR data at 50 °C for the polysaccharide (Fr. II) isolated from *P. sajor-caju*[a]

Glycosyl residue	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Man*p*-(1→ **A**	102.41 (162 Hz)[b]	71.13	73.63	67.52	76.95	61.77
→ 6)-α-D-Gal*p*-(1→ **B**	99.30 (171 Hz)[b]	69.89	71.13	70.21	69.0	69.5
→ 2,4)-α-D-Glc*p*-(1→ **C**	102.41 (170 Hz)[b]	77.67	73.63	76.95	70.21	61.77

[a] Values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm at 50 °C.
[b] J_{C-H} coupling constant value.

was carried out. All the ^{1}H and ^{13}C signals were assigned using 2D-COSY (spectrum not shown), TOCSY (Fig. 4, top panel), HSQC (not shown), and HMBC (Fig. 5) NMR experiments. The three glycosyl moieties were designated as **A**, **B**, and **C** according to their increasing anomeric shifts.

The proton chemical shifts of **A** were assigned from H-1 to H-6 by 2D-COSY and TOCSY (Fig. 4, top panel) spectra. The large coupling constants $J_{H-3,H-4}$ (~7.5 Hz) and $J_{H-4,H-5}$ (~10 Hz) for moiety **A** indicate that it has a *manno* configuration. The carbon chemical shifts from the C-1 to C-6 for **A** were assigned from the HSQC spectrum, and these correspond nearly to the standard values of methyl glycosides[17,18] of D-mannose. The anomeric signals for moiety **A** at δ 4.81 ($J_{H-1,H-2} \sim 0$) ppm and $J_{H-1,C-1}$ 162 Hz indicate that D-mannose residue is β linked. The C-1 signal of **A** at 102.4 ppm was confirmed by the appearance of cross-peak **A** C-1, **C** H-2 in HMBC experiment (Table 5; Fig. 5, right panel). Thus, considering the results of methylation analysis and NMR experiments, it may be concluded that **A** is a β-glycosidically linked, nonreducing end D-mannose moiety.

All the proton chemical shifts of **B** (H-1 to H-6, Table 2) were identified from COSY as well as TOCSY (Fig. 4, top panel) spectra. A large $J_{H-2,H-3}$ (>5 Hz) and relatively small $J_{H-3,H-4}$ (<5 Hz) indicated that **B** has *galacto* configuration. The chemical shifts of the C-1 to C-6 of moiety **B** were assigned from the HSQC spectrum. The anomeric chemical shift for moiety **B** at δ 5.00 ppm ($J_{H-1,H-2} \sim 0$) and $J_{H-1,C-1}$ 171 Hz indicated that the D-galactose is α-linked. The anomeric carbon signal of **B** at δ 99.3 ppm was confirmed by the presence of a cross-peak **B** C-1, **C** H-4 in the HMBC experiment (Table 5; Fig. 5, right panel). The downfield shift of C-6 (δ 67.5) indicates that **B** is a 6-linked D-galactose moiety.

Residue **C** has an anomeric proton signal at δ 5.14 ppm, and the $J_{H-1,H-2}$ coupling constant is very small, but $J_{H-1,C-1}$ 170 Hz indicates that it is an α-linked moiety. The proton signals (Table 2) of **C** from H-1 to H-6 were assigned using the 2D-COSY and TOCSY (Fig. 4, top panel) experiments. Large coupling constants $J_{H-2,H-3}$ (~10 Hz) and $J_{H-3,H-4}$ (~10 Hz) were observed for **C**, supporting that it is D-glucosyl moiety. The carbon signals (Table 3) from C-1 to C-6 for residue **C** were identified from the HSQC spectrum. The downfield shifts of C-2 (δ 77.67) and C-4 (δ 76.95) carbon signals with respect to standard values[18,19] indicate that the moiety **C** is linked at these positions. These observations also support the GC–MS data for this linkage. Hence 1,2,4-linked D-glucose is present in the Fr. II polysaccharide. The ^{13}C signal for the anomeric carbon of the D-glucosyl moiety was observed at δ 102.4 ppm, and also confirmed by HMBC experiment, where couplings corresponding to **C** C-1, **B** H-6a and also **C** H-1, **B** C-6 appeared (Fig. 5). Thus, the peak at δ 102.4 ppm corresponds to the anomeric carbons of both the β-D-mannosyl and α-D-glucosyl moieties, for which it is almost double the proportion of D-galactosyl moiety appearing at δ 99.3 ppm.

The sequence of glycosyl moieties was determined from NOESY studies (Table 4; Fig. 4, bottom panel), followed by confirmation with HMBC (Table 5, Fig. 5) experiments. Moiety **A** has an NOE contact from

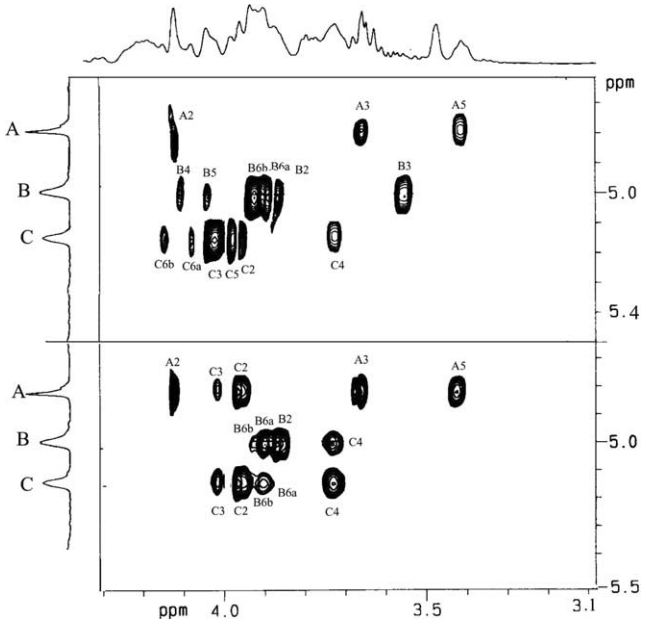


Figure 4. TOCSY (top panel) and NOESY (bottom panel) spectra of polysaccharide, Fr. II isolated from *P. sajor-caju*. The mixing time for the TOCSY spectrum shown was 150 ms. Complete assignment required several TOCSY experiments having mixing times ranging from 60–300 ms. NOESY mixing delay was 300 ms.

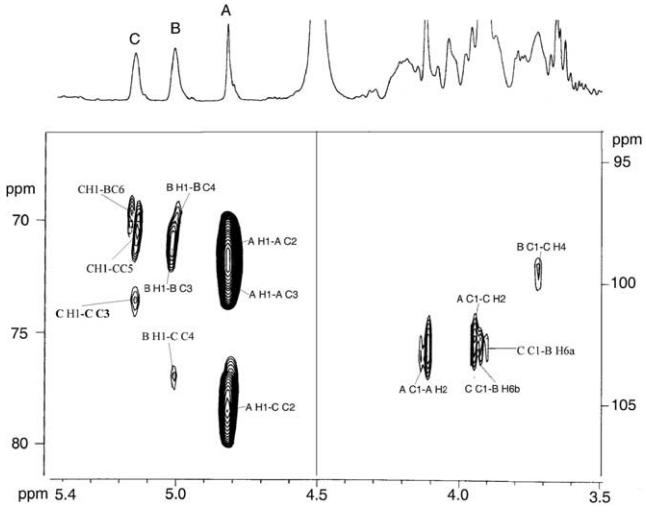


Figure 5. HMBC spectrum (anomeric proton region in left panel and anomeric carbon region in right panel) of polysaccharide, Fr. II isolated from *P. sajor-caju*. The delay time in the HMBC experiment was 80 ms.

Table 4. NOE data for the polysaccharide (Fr. II) isolated from *P. sajor-caju*

Anomeric proton	NOE contact to proton			
Glycosyl residue	δ	δ	Intensity[a]	Residue, atom
β-D-Man*p*-(1→ **A**	4.81	3.40	s	**A** H-5
		3.65	s	**A** H-3
		4.12	s	**A** H-2
		3.95	s	**C** H-2
		4.01	w	**C** H-3
→6)-α-D-Gal*p*-(1→ **B**	5.00	3.87	s	**B** H-2
		3.89	s	**B** H-6a
		3.93	w	**B** H-6b
		3.72	s	**C** H-4
→2,4)-α-D-Glc*p*-(1→ **C**	5.14	3.72	s	**C** H-4
		3.95	s	**C** H-2
		4.02	m	**C** H-3
		3.89	s	**B** H6a
		3.93	w	**B** H6b

[a] The intensities are estimated from visual inspection of the NOESY spectrum shown in Figure 4, and are given as: s = strong, m = medium and w = weak.

H-1 to H-2 of moiety **C** in addition to intraresidue strong NOE contacts to H-2, H-3, H-5 and a weak interresidue contact to H-3 of **C**. Since moiety **A** is linked at the C-2 position of the **C** moiety, the following sequence is established as,

β-D-Man*p*-(1→2)-α-D-Glc*p*-(1→

A **C**

Moiety **C** has a strong interresidue contact from H-1 to H-6a, and a weak contact to H-6b of moiety **B** in addition to strong intraresidue contacts to H-2, H-4, and also a medium contact to H-3, indicating that moiety **C** is linked to the 6-position of moiety **B**. Further, moiety **B** has a strong NOE contact from H-1 to H-4 of **C**, along with strong intraresidue contacts to H-2, H-6a and a weak contact to H-6b, indicating that **B** is also linked to the 4-position of **C**. Thus, **C** is a 1,2,4-linked moiety, and the trisaccharide repeating unit is assigned as shown below,

B **C**

→6)-α-D-Gal*p*-(1→ 4)-α-D-Glc*p*-(1→

2

↑

1

β-D-Man*p*

A

Long-range ^{13}C–^{1}H correlations obtained from an HMBC spectrum (Table 5, Fig. 5) corroborated the assigned disaccharide as well as trisaccharide elements deduced from the NOESY experiment. 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 5, Fig. 5). Cross-peaks were found between H-1 of D-mannose (δ 4.81) and C-2 of D-glucose (**A** H-1, **C** C-2); C-1 of D-mannose (δ 102.4) and H-2 of D-glucose (**A** C-1, **C** H-2), with other intraresidual couplings between H-1 of D-mannose with its own C-2 and C-3 atoms. Similarly, cross-peaks between C-1 (δ 99.30) of D-galactose and H-4 of D-glucose (**B** C-1, **C** H-4); H-1 of D-galactose (δ 5.00) and C-4 (δ 76.95) of D-glucose (**B** H-1, **C** C-4) were observed with other intraresidue couplings between H-1 of D-galactose with its C-3 and C-4. The cross-peaks between C-1 of D-glucose (δ 102.41) and H-6a and H-6b of D-galactose (**C** C-1, **B** H-6a and **C** C-1, **B** H-6b) and H-1 of D-glucose (δ 5.14) with C-6 of D-galactose (δ 69.50), **C** H-1 **B** C-6 were observed along with other intraresidual couplings between H-1 of D-glucose with its C-3 and C-5 positions.

Table 5. The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide (Fr. II) of *P. sajor-caju*

Residue	Sugar linkage	H-1/C-1	Observed connectivities		
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	β-D-Man*p*-(1→	4.81	71.13	**A**	C-2
			73.63	**A**	C-3
			77.67	**C**	C-2
		102.41	3.95	**C**	H-2
B	→6)-α-D-Gal*p*-(1→	5.00	71.13	**B**	C-3
			70.21	**B**	C-4
			76.95	**C**	C-4
		99.30	3.72	**C**	H-4
C	→2,4)-α-D-Glc*p*-(1→	5.14	70.21	**C**	C-5
			73.63	**C**	C-3
			69.50	**B**	C-6
		102.41	3.89, 3.93	**B**	H-6a, H-6b

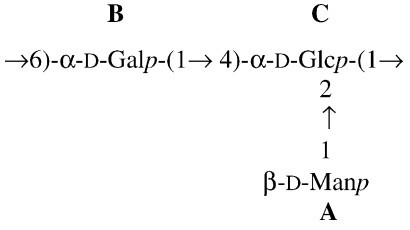
Thus, the appearance of these cross-peaks clearly supports the presence of a trisaccharide repeating unit in the polysaccharide, Fr. II of *Pleurotus sajor-caju*.

Partial acid hydrolysis was also conducted to isolate the oligomers released from the polysaccharide. Fr. II (30 mg) on hydrolysis with 0.5 M CF_3COOH for 1 h at 100 °C yielded an oligomer (10.2 mg), R_f value ~ 1.0 with reference to maltose. This is possibly a dimer, which on hydrolysis showed the presence of D-glucose and D-galactose in the molar ratio of 1:1. No D-mannose was detected, and its methylated sugar showed the presence of four peaks that were identified as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol, and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol. This indicates that the released oligomer is a mixture of two disaccharides, which was further separated into **1a** and **1b** through preparative paper chromatography, and identified by GC–MS (Table 1) as,

D-Gal*p*-(1→4)-D-Glc*p* (**1a**) and
D-Glc*p*-(1→6)-D-Gal*p* (**1b**)

The formation of these oligomers indicates that non-reducing end D-mannose is easily cleaved during partial acid hydrolysis, and the C-2 position of D-glucose becomes free. Therefore, the presence of linkages in the polysaccharide, Fr. II as deduced from NOESY and HMBC experiments, is further confirmed from the isolation and characterization of these disaccharides.

Based on all these evidences the structure of the repeating unit present in polysaccharide, Fr. II is assigned as:



3. Experimental

3.1. Isolation and purification of the polysaccharide

The fresh fruiting bodies of *Pleurotus sajor-caju* (1 kg), collected from a local firm, were washed with water, crushed, and allowed to boil in distilled water (400 mL) for 6 h. The whole extract was cooled and filtered. The filtrate was centrifuged at 10,000 rpm (using a Heraeus Biofuge stratos centrifuge) for 45 min at 6 °C. The supernatant was collected (200 mL) and precipitated by the addition in 1:10 EtOH at room temperature. After overnight precipitation at 4 °C, the sample was centrifuged as above. The centrifugate was collected and freeze dried to give 2.5 g. The material was redissolved in distilled water (100 mL), recovered through precipitation by addition of 1:5 EtOH, and collected as above. The resulting material was again dissolved in distilled water (100 mL), and the low-molecular weight carbohydrate materials were removed by exhaustive dialysis through a DEAE cellulose bag against distilled water for 20 h at room temperature. The contents of the dialysis bag were freeze dried to get crude polysaccharide (1.73 g). The purity of the polysaccharide was determined by gel-permeation chromatography on a Sepharose-6B column (65 × 2 cm) loading 30–35 mg crude polysaccharide for each run. The column was eluted with distilled water with a flow rate of 0.24 mL min^{-1}. Test tubes (120 containing 2 mL eluant each) were collected using a Redifrac fraction collector and monitored by the phenol–H_2SO_4 procedure[12] at 490 nm. Fractions (test tube nos. 38–63 and 84–111) were collected, and freeze dried; yield, Fr. I (~11 mg), Fr. II (~15 mg).

3.2. Molecular weight determination

The average molecular weight of the polysaccharide was determined by a gel-chromatographic technique performed on a Sepharose-6B column (65 × 2 cm) eluting with distilled water at a flow rate of 0.24 mL min^{-1}. The elution volume of Fr. II was plotted in a standard calibration curve prepared by plotting the elution volume of standard dextrans (T-10, T-40, T-200) against the logarithm of their respective molecular weights.

3.3. Sugar composition

Fr. II was hydrolyzed by treatment with 2 M CF_3COOH (100 °C, 18 h). Excess acid was removed by co-distillation with water. The released sugars were reduced by $NaBH_4$, followed by acidification with acetic acid. The solvent was then co-distilled with MeOH to remove excess boric acid, and the residue was dried over P_2O_5. Thereafter, the whole mass was acetylated with pyridine and Ac_2O to give the alditol acetates, which were analyzed by GLC performed with a Hewlett–Packard 5810 gas chromatograph equipped with a flame-ionization detector. The instrument was fitted with a glass column (1.8 m × 6 mm) packed with A (3% ECNSS-M) on Gaschrom-Q (100–120 mesh) and B (1% OV-225) on Gaschrom-Q (100–120 mesh).

3.4. Linkage analysis

The polysaccharide was methylated twice using the procedure described by Ciucanu and Kerek,[15] followed by the Purdie method.[16] The methylated polysaccharide was hydrolyzed by treatment with 90% HCOOH (100 °C, 1 h), and the monosaccharides were converted to their corresponding methylated alditol acetates as

usual. The sugar linkages of the constituent methylated alditol acetates were analyzed by GLC using columns A and B as above and also by GLC–MS analysis, performed on a Hewlett–Packard 5988A automatic GLC–MS system with an HP-5 fused silica capillary column using a temperature program from 150 °C (2 min) to 200 °C (5 min) at 2 °C min^{-1}.

3.5. Periodate oxidation study

A solution of Fr. II (4.0 mg) in 0.1 M $NaIO_4$ was kept in the dark for 48 h at room temperature. Excess $NaIO_4$ was destroyed by addition of ethylene glycol, and the mixture were dialyzed against distilled water. The product was reduced overnight with $NaBH_4$, neutralized with CH_3COOH and dried by addition of CH_3OH. This IO_4^--reduced polysaccharide was then hydrolyzed by 2 M CF_3COOH for 16 h, and the alditol acetates were prepared and analyzed by GLC using columns A and B.

3.6. Smith degradation

A sample (25 mg) was oxidized with 0.05 M sodium metaperiodate (10 mL) at 25 °C in the dark during 48 h. The oxidation 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 at 25 °C, neutralized with 50% acetic acid, and again dialyzed against distilled water and freeze dried. The product was subjected to mild hydrolysis with 0.05 M trifluroacetic acid for 15 h at 25 °C to eliminate residues of oxidized sugars attached to the polysaccharide chain (Smith degradation). Acid was removed after repeated addition and evaporation of water. The Smith-degraded material was collected by descending paper chromatography using the solvent system *n*-butanol–glacial acetic acid–water (4:1:5, upper phase) to give 5 mg of product. A part of the material (2 mg) was reduced by $NaBH_4$ and methylated by the usual procedure, and the alditol acetates were analyzed by GLC.

3.7. Partial acid hydrolysis

Oligosaccharides were produced by partial acid hydrolysis of the polysaccharide. Fr. II (30 mg) was hydrolyzed by 0.5 M CF_3COOH (2 mL) for 1 h at 100 °C. The acid was removed by co-distillation with water. The hydrolyzate was dissolved in distilled water (0.5 mL), and subjected to 3-mm preparative paper chromatography for separation in a descending system of *n*-butanol–glacial acetic acid–water (4:1:5, upper phase). The zone containing the oligosaccharides was located with alkaline $AgNO_3$ reagent[20] and washed with a 5% $Na_2S_2O_3$ solution. Finally, this was extracted with distilled water, and the extract was freeze dried. The oligomers were separated by repeated chromatography with the same solvent for 96 h for each run. The resulting oligosaccharides were methylated, the alditol acetates were prepared and analyzed by GC and GLC–MS as stated earlier.

3.8. NMR spectroscopy

The ^1H and ^{13}C NMR experiments were recorded at 500 MHz and 125 MHz on a Bruker Avance DPX-500 spectrometer, respectively, using a 5-mm broad-band probe. For NMR measurements Fr. II was dried in a vacuum over P_2O_5 for several days, and then exchanged with deuterium[21] by lyophilizing with D_2O for several times. The deuterium-exchanged polysaccharide (5 mg) was dissolved in 0.7 mL D_2O (99.96% atom ^2H, Aldrich). The ^1H and ^{13}C (both ^1H coupled and decoupled) NMR spectra were recorded at 50 °C. Acetone was used as an internal standard (δ 31.05 ppm) for the ^{13}C spectrum. The ^1H NMR spectrum was recorded fixing the HOD signal at δ 4.51 ppm at 50 °C. The 2D-COSY NMR experiment was performed using standard Bruker software. The mixing times in the TOCSY and NOESY experiments were 150 and 300 ms, respectively. Complete assignment was done using several TOCSY experiments having mixing times ranging from 60–300 ms. The delay time in the HMBC experiment was 80 ms.

Acknowledgements

The authors are thankful to Dr. S. Lahiri, IACS, Dr. Asish Sen (Jr.), IICB, Dr. P. K. Roychowdhuri, Chembiotek Research International, and Dr. S. Roy, Bose Institute, Kolkata for helpful suggestions and instrumental facilities. Mr. Barun Majumder, Bose Institute, Kolkata, is gratefully acknowledged for preparing NMR spectra.

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