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Note

Structural analysis of a water-soluble glucan (Fr.I) of an edible mushroom, *Pleurotus sajor-caju*

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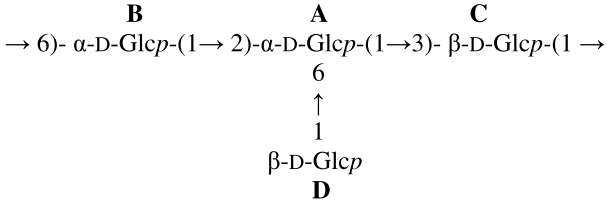
Abstract—A water-soluble glucan was obtained from the fruit bodies of an edible mushroom, *Pleurotus sajor-caju*, by hot water extraction, ethanol precipitation, dialysis, and Sepharose 6B gel filtration. On the basis of total hydrolysis, methylation analysis, periodate oxidation, and NMR studies (^{1}H, ^{13}C, DQF-COSY, TOCSY, NOESY, and HSQC), the structure of the repeating unit of the glucan is determined as



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

Pleurous mushrooms, namely *Pleurotus sajor-caju*,[1–5] *Pleurotus ostreatus*,[6,7] *Pleurotus citrinopileatus*,[8] and *Pleurotus florida*,[9–13] are reported as commonly available edible mushrooms, and are considered to be one of the most efficient producers of food protein, producing 30% of its dry weight. They contain vitamins (B1, B2, B12, C, D folates, and niacin), minerals, lipids, and carbohydrates. *P. sajor-caju* was originally found in India, growing naturally on a succulent plant (*Euphorbia royleans*) in the foothills of the Himalayas. Now, this mushroom is cultivated in the period of February to mid-April and September–November in our area Midnapore, West Bengal, India.

Mushroom polysaccharides, such as (1→3), (1→6)-β-glucans,[14] (1→3)-α-glucans,[15] heteroglycan,[16] α-manno-β-glucan,[17] act as immunomodulating and anti-tumor materials. Two water-soluble polysaccharides (Fr.I and Fr.II) have been isolated from the fruit bodies of *P. sajor-caju*. Fr.II was characterized and reported[3] by our group in *Carbohydrate Research*. Now, a detailed structural investigation on Fr.I was carried out, and reported herein.

The molecular weight of this polysaccharide fraction was estimated from a calibration curve prepared with standard dextrans[18] and found to be $\sim 2.4 \times 10^5$ Da. The total sugar content of Fr.I was estimated as 97.8% using the phenol–sulfuric acid method.[19] Protein was estimated by Lowry's method.[20] Fr.I was hydrolyzed with 2 M trifluoro acetic acid (TFA), and the alditol acetate on analysis through GLC using columns A (3% ECNSS-M) and B (1% OV-225) indicated the presence of only glucose. One part of the hydrolyzate on paper chromatographic (PC) analysis showed only the spot of glucose. The absolute configuration of the monosaccharide was determined as D configuration by GLC

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analysis of acetylated (+)-2-octyl glycosides according to a modified method.[21] Fr.I was then methylated using the method of Ciucanu and Kerek,[22] and also by the method of Purdie and Irvine,[23] followed by hydrolysis and conversion into alditol acetates. The alditol acetates were analyzed by GLC using columns A and B, and also by GLC–MS using an HP-5 fused silica capillary column. Fr.I on GLC and GLC–MS analyses showed the presence of 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-D-glucitol; 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol; 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, in a molar ratio of nearly 1:1:1:1 (Table 1). These results indicate that (1→2,6), (1→6), (1→3) and nonreducing end D-glucosyl moieties are present in the polysaccharide. GLC analysis of the alditol acetates of methylated, periodate-oxidized reduced polysaccharide showed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol (residue **C**) only. This indicates that only the (1→3)-linked D-glucosyl moiety is retained while the other residues are consumed during oxidation. Thus, periodate oxidation study supports the mode of linkage of the D-glucosyl moieties present in Fr.I.

The ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR experiments were carried out with Fr.I at 27 °C and 50 °C, respectively. The 500 MHz ^{1}H NMR spectrum (Fig. 1) showed four signals in the anomeric region at δ 5.08, 4.94, 4.47, and 4.46 ppm in a molar ratio of nearly 1:1:1:1, and these were assigned to residues **A**, **B**, **C**, and **D**, respectively, according to their decreasing ^{1}H chemical shift values. The ^{13}C NMR spectrum (Fig. 2) showed four anomeric carbon signals at δ 102.72, 102.42, 98.79, and 98.43 ppm, in a molar ratio of 1:1:1:1. DEPT-135 NMR experiment was carried out to find the C-6 signals in the polysaccharide. The free C-6 carbon signals appearing at δ 60.60 and the linking C-6 carbons at 68.56 ppm were assigned from their downward signals in DEPT spectrum (Fig. 3). All the ^{1}H (Table 2) and ^{13}C (Table 3) signals were assigned from DQF-COSY, TOCSY, and HSQC NMR experiments.

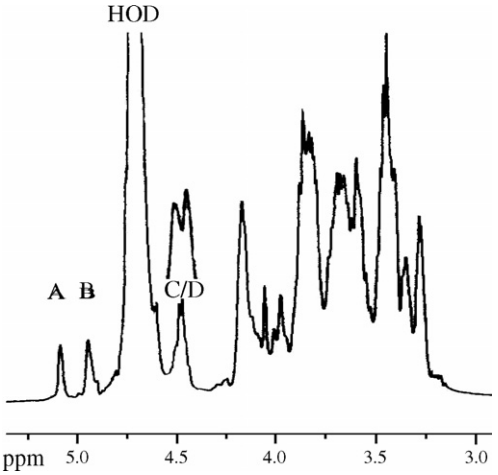

Figure 1. ^{1}H NMR (500 MHz, D_2O, 27 °C) spectrum of a polysaccharide Fr.I isolated from *Pleurotus sajor-caju*.

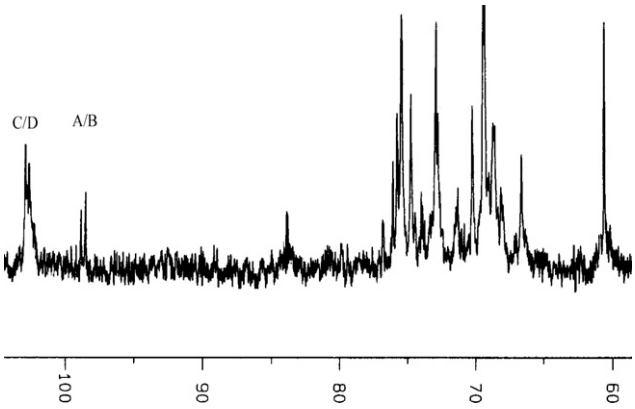

Figure 2. ^{13}C NMR (125 MHz, D_2O, 50 °C) of a polysaccharide Fr.I isolated from *Pleurotus sajor-caju*.

Residue **A** has an anomeric proton signal at δ 5.08 ppm, and the small coupling constant value (unresolved) indicates that it is α-linked. The ^{13}C signal for the anomeric carbon of **A** was observed at δ 98.79 ppm. The 4.56 ppm downfield shifts of C-2 (δ 76.96 ppm) and 6.67 ppm for C-6 (68.56 ppm) with

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

Methylated sugar	t_R[a]	t_R[b]	Characteristic fragments[c] (*m*/*z*)	Molar ratio	linkage type
3, 4-Me_2-Glc*p* **A**	4.40	3.73	87, 99, 129, 189	1	→2,6)-Glc*p*-(1→
2,3,4-Me_3-Glc*p* **B**	2.49	2.22	71, 87, 99, 101, 117, 129	1	→6)-Glc*p*-(1→
2,4,6-Me_3-Glc*p* **C**	1.95	1.82	71, 87, 101, 117, 129, 143, 161, 173	1	→3)-Glc*p*-(1→
2,3,4,6-Me_4-Glc*p* **D**	1.00	1.00	87, 101, 117, 129, 161	1	Glc*p*-(1→

[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 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}.

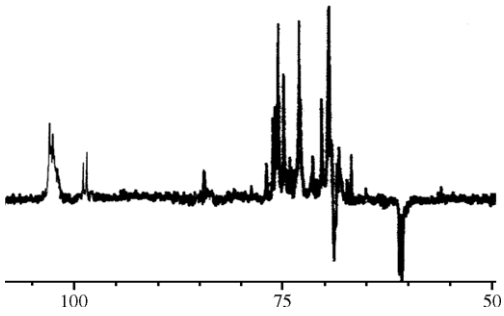

Figure 3. DEPT-135 NMR (D_2O, 50 °C) spectrum of a polysaccharide Fr.I isolated from *Pleurotus sajor-caju.*

respect to standard values[24,25] indicates that moiety **A** is linked at C-2 and C-6. The branching of residue **A** at C-6 is also evident from DEPT-135 spectrum (Fig. 3). These observations indicate that **A** is 1,2,6-linked-α-D-glucopyranosyl moiety.

The appearance of anomeric proton signal for moiety **B** at δ 4.94 ppm and a small coupling constant value (unresolved) indicate that it has an α-anomeric configuration. The carbon signal at 98.43 ppm was assigned to C-1 of residue **B**. The 6.76 ppm downfield shift of C-6 (68.56 ppm) with respect to standard values of methyl glycosides[25] indicate that moiety **B** is linked at C-6, which is further supported by DEPT-135 NMR (Fig. 3). Hence, **B** is a 1,6-linked-α-D-glucopyranosyl moiety.

Residue **C** has an anomeric proton signal at δ 4.47 ppm, and a large coupling constant value, $^3J_{1-2} \sim 8.5$ Hz, indicates that it is a β-linked moiety. Its anomeric carbon signal appears at δ 102.72 ppm. The 7.09 ppm downfield shift of C-3 compared to the resonance of standard methyl glycoside is due to the α-effect of glycosylation.[25] **C** is thus a 1,3-linked-β-D-glucopyranosyl moiety.

The anomeric proton and carbon signals for Residue **D** were assigned at δ 4.46 and 102.42 ppm, respectively. The large coupling constant value, $^3J_{1-2} \sim 9$ Hz, indicates that residue **D** is β-linked. The signals from C-1 to C-6 for residue **D** correspond nearly to the standard values of methyl glycosides[25,26] of D-glucose. Thus, it may be concluded that **D** is a nonreducing end β-D-glucopyranosyl moiety.

The sequence of glycosyl moieties was determined from NOESY experiment (Table 4, Fig. 4, bottom panel). Residue **D** has interresidue NOE contacts from

Table 2. The ^{1}H NMR data at 27 °C of the polysaccharide (Fr.I) isolated from *P. sajor-caju*[c]

Glycosyl residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
→2,6)-α-D-Glc*p*-(1→ **A**	5.08	3.86	3.43	4.08	4.00	3.89	3.96
→6)- α-D-Glc*p*-(1→ **B**	4.94	3.80	3.52	3.67	3.94	3.84	4.15
→3)-β-D-Glc*p*-(1→ **C**	4.47	3.44	3.81	3.36	3.55	3.62	4.08
β-D-Glc*p*-(1→ **D**	4.46	3.27	3.41	3.28	3.33	3.58	3.87

[a,b] Interchangeable.
[c] Values of chemical shifts were recorded with respect to the HOD signal fixed at δ 4.69 ppm at 27 °C.

Table 3. The ^{13}C NMR data at 50 °C of the polysaccharide (Fr.I) isolated from *P. sajor-caju*

Glycosyl residue	C-1	C-2	C-3	C-4	C-5	C-6
→2,6)-α-D-Glc*p*-(1→ **A**	98.79	76.96	73.33	70.30	69.69	68.56
→6)-α-D-Glc*p*-(1→ **B**	98.43	69.39	66.66	70.30	69.69	68.56
→3)-β-D-Glc*p*-(1→ **C**	102.72	73.03	83.79	76.06	73.33	60.60
β-D-Glc*p*-(1→ **D**	102.42	73.33	74.84	76.06	76.06	60.60

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

Anomeric proton Glycosyl residue	δ	δ	NOE contact to proton Residue, atom
→ 2,6)-α-D-Glc*p*-(1→	5.08	3.86	**A** H-2
		3.89	**A** H-6a
A		3.96	**A** H-6b
		3.81	**C** H-3
→ 6)-α-D-Glc*p*-(1→	4.94	3.67	**B** H-4
		3.80	**B** H-2
B		3.84	**B** H-6a
		4.15	**B** H-6b
		3.86	**A** H-2
→3)-β-D-Glc*p*-(1→	4.47	3.44	**C** H-2
		3.55	**C** H-5
C		3.62	**C** H-6a
		3.81	**C** H-3
		3.84	**B** H-6a
		4.15	**B** H-6b
β-D-Glc*p*-(1→	4.46	3.27	**D** H-2
		3.28	**D** H-4
D		3.41	**D** H-3
		3.58	**D**H-6a
		3.89	**A** H-6a
		3.96	**A** H-6b

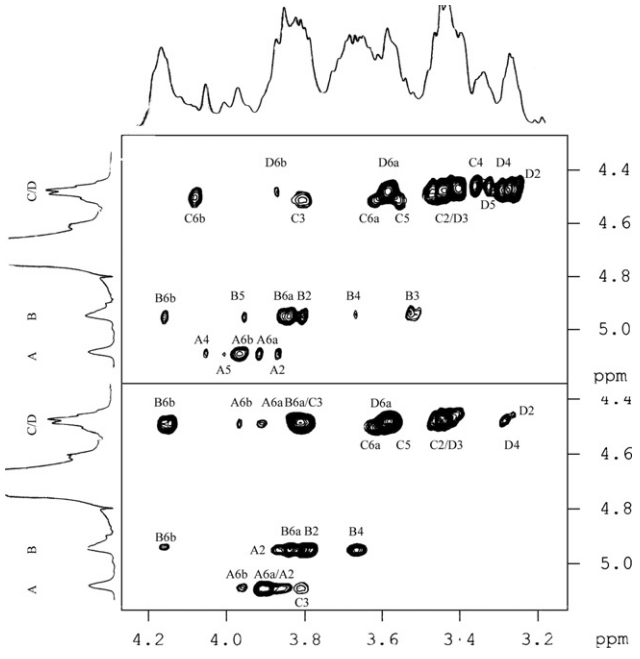

Figure 4. TOCSY (top panel) and NOESY (bottom panel) spectra of a polysaccharide Fr.I isolated from *Pleurotus sajor-caju.* The mixing time for the TOCSY spectrum shown was 150 ms. Complete assignment required several TOCSY experiments having mixing times ranging from 60 to 300 ms. The NOESY mixing delay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

H-1 to H-6a, and H-6b of residue **A**. Hence, residue **D** is linked at C-6 of residue **A**, indicating the following sequence;

D **A**
β-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→
↑
2

Residue **B** has an interresidue NOE contact from H-1 to H-2 of residue **A**. Hence, residue **B** is linked at C-2 of residue **A**. Thus, the following sequence is established;

B **A**
→ 6)-α-D-Glc*p*-(1→ 2)-α-D-Glc*p*-(1→
6
↑
1
β-D-Glc*p*
D

Residue **A** has an interresidue NOE contact from H-1 to H-3 of residue **C**. Hence, residue **A** is linked at C-3 of residue **C**. This indicates the following sequence;

A **C**
→2)-α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→
6
↑
1
β-D-Glc*p*
D

Again, residue **C** has interresidue NOE contacts from H-1 to H-6a, and H-6b of residue **B**. Hence, residue **C** is linked at C-6 of residue **B**, indicating the following sequence;

C **B**
→3)-β-D- Glc*p*(1 → 6)-α-D- Glc*p*-(1→

Hence, from the above chemical and spectroscopic evidences the following tetrasaccharide repeating unit was assigned for Fr.I;

B **A** **C**
→ 6)-α-D-Glc*p*-(1→ 2)-α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1 →
6
↑
1
β-D-Glc*p*
D

1. Experimental

1.1. Isolation and purification of the polysaccharide

The polysaccharide, Fr.I, was extracted from the fruit body of *P. sajor-caju* as described earlier.[3] From 1 kg of fruits, 584.5 mg of Fr.I was obtained.

1.2. Determination of molecular weight

The molecular weight of Fr.I was determined by a gel-chromatographic technique. Standard dextrans T-200,

T-70, and T-40 were passed through a Sepharose 6B column, and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution volume of Fr.I was then plotted in the same graph, and the molecular weight of Fr.I was determined.

1.3. Monosaccharide analysis

The polysaccharide Fr.I (2 mg) was hydrolyzed in 2 M CF_3COOH (1.5 mL) at 100 °C for 18 h in a boiling water bath. The hydrolyzate was then converted into its respective alditol acetates and analyzed by gas liquid chromatography (GLC) using a Hewlett–Packard 5810 instrument equipped with a flame ionization detector. The alditol acetates were resolved on a glass column (1.8 m × 6 mm) containing 3% ECNSS-M on Gaschrom-Q (100–120 mesh) and 1% OV-225 on Gaschrom-Q (100–120 mesh). Gas liquid chromatography–mass spectrometric (GLC–MS) analysis was also performed on a Hewlett–Packard 5970 A automatic GLC–MS system, using an HP-5 silica 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). Quantization was carried out from the peak area, using response factors from standard monosaccharide.

1.4. Paper chromatographic studies

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

1.5. Methylation analysis

Fr.I was methylated using the method of Ciucanu and Kerek,[22] and the product was isolated by partitioning between $CHCl_3$ and H_2O. It was methylated again by the Purdie method.[23] The methylated polysaccharide was hydrolyzed by treatment with 90% HCO_2H, 1 h. Excess HCO_2H was evaporated off by co-distillation with distilled water. The hydrolyzate was then reduced with $NaBH_4$, and the alditol acetate was prepared as usual. The alditol acetate of the methylated sugar was analyzed by GLC (using columns A and B) and also by GLC–MS using an HP-5 fused silica capillary column.

1.6. Periodate oxidation

The polysaccharide (8 mg) was oxidized with 0.1 M sodium metaperiodate (2 mL) at 4 °C in the dark for 48 h. The oxidation process was stopped by the 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 material was obtained by co-distillation with MeOH. It was subjected to both hydrolysis and methylation by as usual procedures described above and the alditol acetates were analyzed by GLC–MS.

1.7. NMR spectroscopy

The freeze-dried polysaccharide was kept over P_2O_5 in vacuum for several days, and then deuterium exchanged three times followed by lyophilization with D_2O. The ^1H, TOCSY, DQF-COSY, NOESY, and HSQC NMR spectra were recorded with a Bruker Avance DPX-500 spectrometer in D_2O at 27 °C. The ^{13}C and DEPT-135 NMR experiment was carried out at 50 °C. ^1H Chemical shifts were referred to the residual signal of HOD at δ 4.69 ppm. 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 to 300 ms.

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