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Note

Structural analysis of a polysaccharide isolated from the aqueous extract of an edible mushroom, *Pleurotus sajor-caju*, cultivar Black Japan

Sadhan K. Roy, Debabrata Maiti, Subhas Mondal, Debsankar Das and Syed S. Islam*

Department of Chemistry and Chemical Technology, Vidyasagar University, Midnapore 721102, West Bengal, India

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Abstract—A water-soluble polysaccharide, isolated from the aqueous extract of an edible mushroom *Pleurotus sajor-caju*, cultivar Black Japan was found to consist of D-glucose and D-galactose in a molar ratio of 3:1. On the basis of total acid hydrolysis, methylation analysis, and NMR experiments (^{1}H, ^{13}C, DQF-COSY, TOCSY, NOESY, ROESY, HMQC, and HMBC) the structure of the polysaccharide was established as

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

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

Among different types of mushrooms of the genus *Pleurotus*, namely *Pleurotus sajor-caju*,[1–4] *Pleurotus ostreatus*,[5,6] *Pleurotus citrinopileatus*,[7] and *Pleurotus florida*[8–14] are reported as commonly available edible mushrooms having components with immunostimulating and antitumor properties. These are also known as oyster mushrooms or abalone mushrooms. A cultivar of *P. sajor-caju* called Black Japan is commercially available in large scale near Midnapore city during the period of December–January. The main objective of this work is to investigate any difference that arises in the constituent of polysaccharide of this variety from the original mushroom *P. sajor-caju*. The aqueous extract of the edible mushroom *P. sajor-caju* was found to consist of two fractions and identified as glucan[15] (Fr. I) and heteroglycan[16] (Fr. II) consisting of D-mannose, D-glucose, and D-galactose in equimolar ratio and reported in *Carbohydrate Research*. The aqueous extract of this variety was found to consist of D-glucose and D-galactose in a molar ratio of 3:1. With a view to study the structural as well as immunological properties of this molecule we are reporting herein the detailed structural characterization of this polysaccharide, isolated from *P. sajor-caju* (cv Black Japan).

The molecular weight of this polysaccharide was estimated from a calibration curve prepared with standard dextrans[17] as $\sim 2.8 \times 10^4$ Da. The polysaccharide showed specific rotation of $[\alpha]_D^{25}$ +24.0 (*c* 0.80, water). We report herein the structural characterization of this polysaccharide fraction. The polysaccharide on acid hydrolysis by 2 M trifluoroacetic acid showed the presence of D-glucose and D-galactose that were detected by GLC analysis and found to be present in a molar ratio of 3:1, respectively. The total carbohydrate of this

* Corresponding author. Tel.: +91 03222 276558x437; fax: +91 03222 275329; +91 9932629971 (M); e-mail: sirajul_1999@yahoo.com

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fraction was estimated to be 97.6% using the phenol–sulfuric acid method.[18] The absolute configuration[19] of each monosaccharide was determined by GLC examination of 2,3,4,6-tetra-*O*-trimethylsilyl-(+)-2-butyl 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,[20] followed by formolysis and alditol acetate preparation. The alditol acetates were then analyzed through GLC as well as by GLC–MS using an HP-5 fused silica capillary column, and the presence of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol, 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol, and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol in a molar ratio of 2:1:1 was detected. These results indicate that (1→6)-linked D-glucopyranosyl, (1→4, 6)-linked D-glucopyranosyl moieties and terminal D-galactopyranosyl moieties are present in the polysaccharide.

The ^{1}H NMR (500 MHz) spectrum (Fig. 1) at 27 °C showed three signals in the anomeric region at δ 5.11, 4.97, 4.51 ppm in a ratio of nearly 1:1:2 but expansion of the peak at δ 4.51 ppm showed two overlapped signals at 4.51 and 4.50 ppm. In the ^{13}C NMR spectrum (Fig. 2) at 27 °C, four anomeric signals appeared at δ 103.4, 98.2, and 98.0 ppm in a ratio of 2:1:1, the peak at δ 103.4 ppm with double intensity. All the ^{1}H and ^{13}C signals were assigned using DQF-COSY, TOCSY,

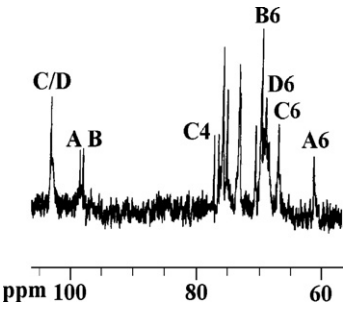


Figure 2. ^{13}C NMR spectrum (125 MHz, D_2O, 27 °C) of the polysaccharide isolated from *P. sajor-caju* (cv Black Japan).

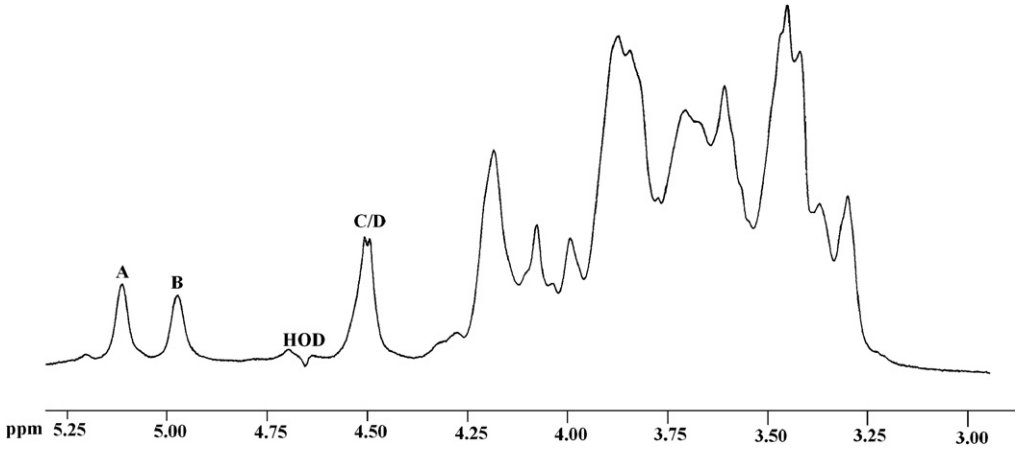


Figure 1. ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) of the polysaccharide isolated from *P. sajor-caju* (cv Black Japan).

Table 1. ^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts for the polysaccharide isolated from *Pleurotus sajor-caju* (cv Black var Japan) in D_2O at 27 °C

Glycosyl residue	H-1/C-1	H-2/ C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a,H-6b/C-6
α-D-Gal*p*-(1→ **A**	5.11 98.2	3.80 69.5	3.89 69.9	4.03 69.9	4.11 70.8	3.70,[c] 3.72[d] 61.4
→6)-α-D-Glc*p*-(1→ **B**	4.97 98.0	3.82 73.3	3.66 75.3	3.76 70.0	3.93 70.6	3.88,[c] 4.19[d] 69.2
→4,6)-β-D-Glc*p*-(1→ **C**	4.51 103.4	3.51 73.4	3.39 76.0	3.84 77.3	3.61 69.9	4.07,[c] 4.18[d] 67.5
→ 6)-β-D-Glc*p*-(1→ **D**	4.50 103.4	3.30 73.4	3.42 76.6	3.46 69.5	3.34 70.0	3.73,[c] 4.17[d] 68.8

[a] Values of the ^{1}H chemical shifts were recorded with respect to the HOD signal fixed at δ 4.67 ppm at 27 °C.
[b] Values of the ^{13}C chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm at 27 °C.
[c,d] Interchangeable.

HMQC, and HMBC NMR experiments. Coupling constants were measured from DQF-COSY spectrum. The four sugar moieties were designated as residues **A**, **B**, **C**, and **D** according to their decreasing chemical shifts (Table 1) in the ^{1}H NMR spectrum.

Residue **A** was assigned to nonreducing-end D-galactosyl unit. The *galacto* configuration was assigned from the large $^{3}J_{2,3}$ coupling constant of ~8 Hz and relatively small $^{3}J_{3,4}$ coupling constant of ~3 Hz. The α configuration of residue **A** (δ 5.11) was assigned from $^{3}J_{1,2}$ coupling constant of ~3 Hz and $^{1}J_{C,H}$ of ~170 Hz. The carbon signal at 98.2 ppm was assigned to C-1 of residue **A**. The carbon signals from C-1 to C-6 of residue **A** correspond nearly to the standard values of methyl glycosides.[21,22] Thus considering the results of methylation analysis and NMR spectroscopy, it is concluded that residue **A** is an α-linked, terminal D-galactopyranosyl moiety.

Residue **B** has an anomeric proton signal at δ 4.97 ppm, and the $^{3}J_{1,2} \sim 3$ Hz, $^{1}J_{C,H}$ of ~171 Hz indicate that it is an α-linked moiety. Large coupling constant $^{3}J_{2,3}$ and $^{3}J_{3,4}$ (~10 Hz) for residue **B** supports that it is a D-glucosyl moiety. The carbon signal at 98.0 ppm was assigned to C-1 of residue **B**. The downfield shift for C-6 (69.2 ppm) with respect to standard values[21,22] indicates that moiety **B** is linked at C-6. The other values for carbons correspond nearly to standard values. **B** is thus a (1→6)-linked-α-D-glucopyranosyl moiety.

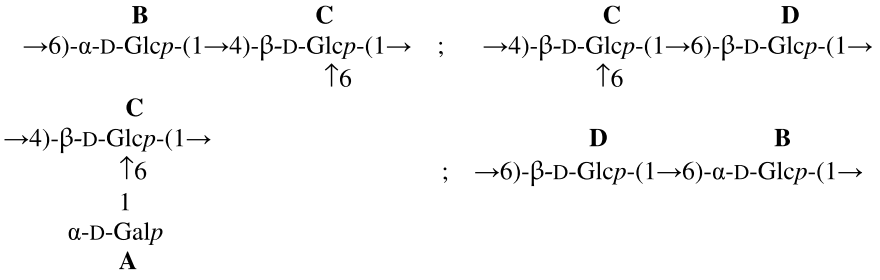
Residue **C** has an anomeric proton signal at δ 4.51 ppm, and the $^{3}J_{1,2} \sim 8$ Hz, $^{1}J_{C,H}$ of ~160 Hz indicate that it is a β-linked moiety. Large coupling constant $^{3}J_{2,3}$ and $^{3}J_{3,4}$ (~10 Hz) for residue **C** supports that it is a D-glucosyl moiety. Its anomeric carbon signal appears at δ 103.4 ppm. The downfield shift of C-4 (δ 77.3 ppm) and for C-6 (67.5 ppm) with respect to standard values[21,22] indicates that moiety **C** is linked at C-4 and C-6. These observations indicate that **C** is 1,4,6-linked β-D-glucopyranosyl moiety.

Residue **D** has an anomeric proton signal at δ 4.50 ppm, and the $^{3}J_{1,2} \sim 8$ Hz, $^{1}J_{C,H}$ of ~160 Hz indicate that it is a β-linked moiety. Large coupling constants $^{3}J_{2,3}$ and $^{3}J_{3,4}$ (~10 Hz) for residue **D** supports that it is a D-glucosyl moiety. Its anomeric carbon signal appears at δ 103.4 ppm. The downfield shift for C-6 (68.8 ppm) with respect to standard values[21,22] indicates that moiety **D** is also linked at C-6. Thus it may be concluded that **D** is (1→6)-linked-β-D-glucopyranosyl moiety.

Table 2. ROESY data for the polysaccharide isolated from *Pleurotus sajor-caju* (cv Black Japan)

Anomeric proton Glycosyl residue	δ	δ	ROE contact protons Residue, atom
α-D-Gal*p*-(1→ **A**	5.11	4.07	**C** H-6a
		4.18	**C** H-6b
		3.80	**A** H-2
		3.89	**A** H-3
→6)-α-D-Glc*p*-(1→ **B**	4.97	3.84	**C** H-4
		3.82	**B** H-2
		3.66	**B** H-3
		3.76	**B** H-4
		3.88	**B** H-6a
		4.19	**B** H-6b
→4,6)-β-D-Glc*p*-(1→ **C**	4.51	3.73	**D** H-6a
		4.17	**D** H-6b
		3.84	**C** H-4
		3.61	**C** H-5
→ 6)-β-D-Glc*p*-(1→ **D**	4.50	3.88	**B** H-6a
		4.19	**B** H-6b
		3.30	**D** H-2
		3.42	**D** H-3
		3.46	**D** H-4

The sequences of glycosyl moieties were determined from ROESY (Table 2, Fig. 3) as well as NOESY experiments and the following connectivities were observed:



The cross peaks of both anomeric protons and carbons from HMBC experiment (Table 3, Fig. 4) of each of the sugar moieties were examined, and both intra- and interresidual connectivities were observed from the HMBC experiment. Cross peaks were found between H-1 of residue **A** (δ 5.11) with C-6 of residue **C** (**A** H-1, **C** C-6); C-1 of residue **A** (δ 98.2) and H-6a, H-6b of residue **C** (**A** C-1, **C** H-6a; **A** C-1, **C** H-6b), with other intraresidual coupling between H-1 of residue **A** with its own C-3 atom. Cross peaks were also observed between H-1 of residue **B** (δ 4.97) with C-4 of residue **C** (**B** H-1, **C** C-4); C-1 of residue **B** (δ 98.0) and H-4 of residue **C** (**B** C-1, **C** H-4) with other intraresidual coupling between H-1 of residue **B** with its own C-5 atom. Cross peaks

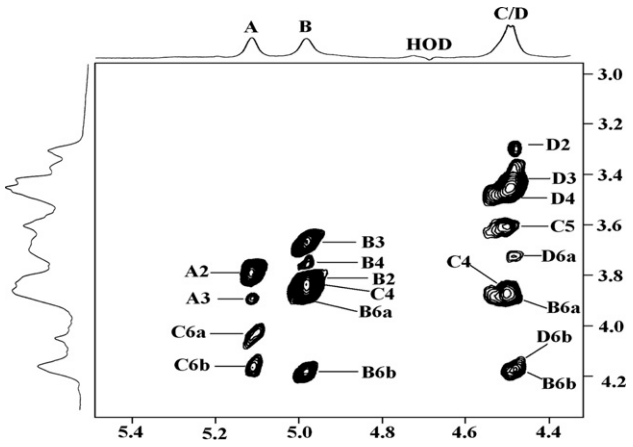
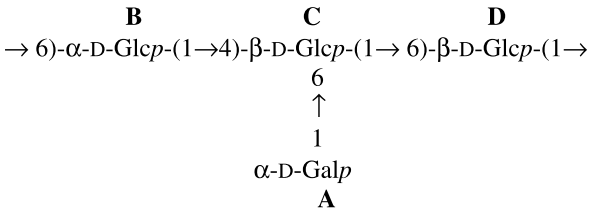


Figure 3. The ROESY spectra of the polysaccharide isolated from *P. sajor-caju* (cv Black Japan). The ROESY mixing time was 300 ms.

between H-1 of residue **C** (δ 4.51) with C-6 of residue **D** (**C** H-1, **D** C-6); C-1 of residue **C** (δ 103.4) and H-6a, H-6b of residue **D** (**C** C-1, **D** H-6a; **C** C-1, **D** H-6b) with other intraresidual coupling between C-1 of residue **C** with its own H-2 atom were observed. Similarly cross peaks were found between H-1 of residue **D** (δ 4.50) with C-6 of residue **B** (**D** H-1, **B** C-6); C-1 of residue **D** (δ 103.4) and H-6a, H-6b of residue **B** (**D** C-1, **B** H-6a; **D** C-1, **B** H-6b) with other intraresidual coupling between C-1 of residue **D** with its own H-2, H-3, and H-5 atoms. The appearance of these cross peaks in the HMBC spectrum firmly supports the presence of a tetrasaccharide repeating unit in this polysaccharide.

Thus, based on all these chemical and spectroscopic evidence, the structure of repeating unit of the polysaccharide is established as



1. Experimental

1.1. Isolation and purification of the polysaccharide

Fruit bodies of the mushroom, *P. sajor-caju*, cultivar Black Japan (1.0 kg) were collected from the local farm and washed with water. It was crushed and boiled in 500 mL of distilled water for 8 h. The whole mixture was kept overnight at 4 °C and then filtered through linen cloth. The filtrate was centrifuged at 8000 rpm (using a Heraeus Biofuge stratos centrifuge) for 45 min at 4 °C. The supernatant was collected and precipitated in ethanol (1:5, v/v). It was kept overnight at 4 °C and again centrifuged as above. The precipitated material (polysaccharide) was washed with ethanol four times and then freeze-dried. The freeze-dried material was dissolved in 30 mL of distilled water and dialyzed through dialysis tubing cellulose membrane (Sigma–Aldrich, retaining >M.W. 12,400) against distilled water for 4 h to remove low molecular weight materials. The aqueous solution was then collected from the dialysis bag and freeze-dried to yield crude polysaccharide (wt. 1.2 g).

The crude polysaccharide (30 mg) was purified by gel permeation chromatography on column (90 × 2.1 cm) of Sepharose 6B in water as eluant (0.4 mL min^{-1}) using Redifrac fraction collector. Ninety-five test tubes (2 mL

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

Residue	Sugar linkage	H-1/C-1	Observed connectivities		
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	α-D-Gal*p*-(1→	5.11	67.5	**C**	C-6
			69.9	**A**	C-3
		98.2	4.07	**C**	H-6a
			4.18	**C**	H-6b
B	→6)-α-D-Glc*p*-(1→	4.97	77.3	**C**	C-4
			70.6	**B**	C-5
		98.0	3.84	**C**	H-4
C	→4,6)-β-D-Glc*p*-(1→	4.51	68.8	**D**	C-6
		103.4	3.73	**D**	H-6a
			4.17	**D**	H-6b
			3.51	**C**	H-2
D	→ 6)-β-D-Glc*p*-(1→	4.50	69.2	**B**	C-6
		103.4	3.88	**B**	H-6a
			4.19	**B**	H-6b
			3.30	**D**	H-2
			3.42	**D**	H-3
			3.34	**D**	H-5

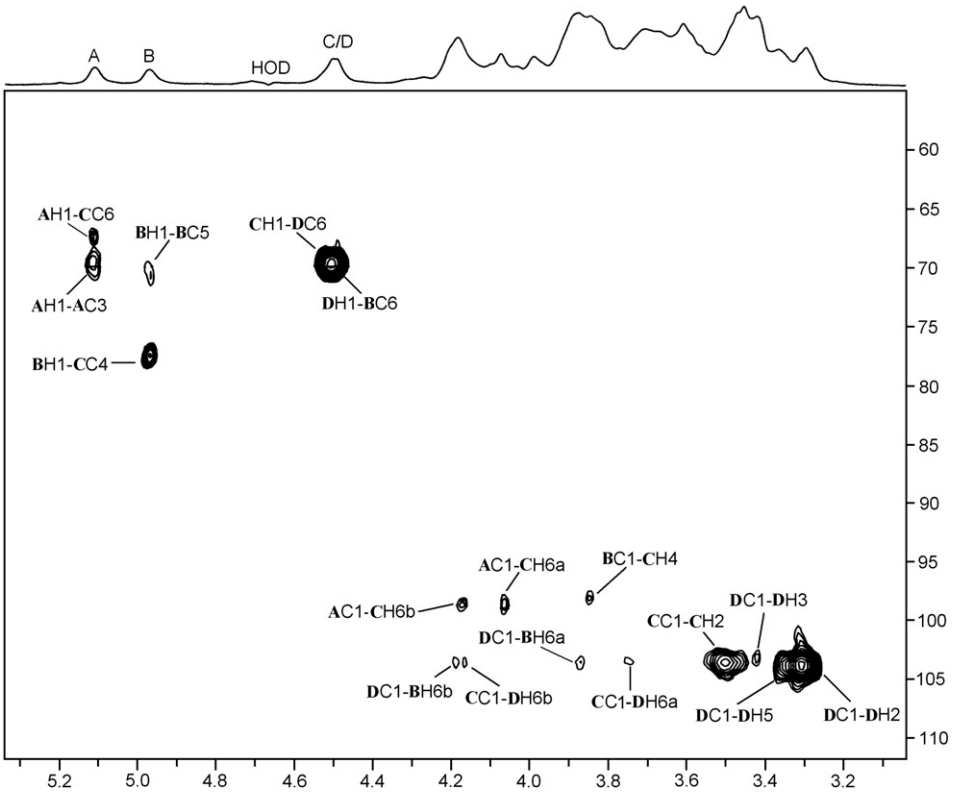


Figure 4. HMBC spectrum of polysaccharide, isolated from *P. sajor-caju* (cv Black Japan). The delay time in the HMBC experiment was 80 ms.

each) were collected and monitored spectrophotometrically at 490 nm with phenol–sulfuric acid reagent[18] using Shimadzu UV–Vis spectrophotometer, model-1601. One homogeneous fraction (test tubes 28–58) was collected and freeze-dried, yielding 22 mg of material. The purification process was carried out in seven lots and the polysaccharide fraction was again purified and collected (yield 150 mg).

1.2. Monosaccharide analysis

The polysaccharide sample (3.0 mg) was hydrolyzed with 2 M CF_3COOH (2 mL) in a round-bottom flask at 100 °C for 18 h in a boiling water bath. The excess acid was completely removed by co-distillation with water. Then the hydrolyzed product was reduced with $NaBH_4$ (9 mg), followed by acidification with dilute CH_3COOH, and then co-distilled with pure CH_3OH to remove excess boric acid. The reduced sugars (alditols) were acetylated with 1:1 pyridine–acetic anhydride in a boiling water bath for 1 h to give the alditol acetates, which were analyzed by GLC and GLC–MS performed on Hewlett–Packard 5970A automatic GLC–MS system, using an HP-5 capillary column (25 m × 25 m). The program was isothermal at 150 °C; hold time 2 min, with a temperature gradient of 4 °C min^{-1} up to a final temperature of 200 °C. Quantitation was carried out from the peak area, using response factors from standard monosaccharides.

1.3. Methylation analysis

The polysaccharide (4.0 mg) was methylated using Ciucanu and Kerek method.[20] The methylated products were isolated by partition in 5:2 $CHCl_3$-water. The organic layer containing products was washed with 3 mL water for three times and dried. The methylated products were then formolyzed with 90% formic acid (1 mL) at 100 °C for 1 h, reduced with sodium borohydride, acetylated with 1:1 acetic anhydride–pyridine and analyzed by GLC–MS (using HP-5 fused silica capillary column) with the same temperature program indicated above.

1.4. Absolute configuration of monosaccharides

The method used was based on Gerwig et al.[19] The polysaccharide (1.0 mg) was hydrolyzed with CF_3COOH, and then the acid was removed. A solution of 250 μL of 0.625 (M) HCl in *R*-(+)-2-butanol was added and heated at 80 °C for 16 h. Then the reactants were evaporated and TMSi-derivatives were prepared with *N*,*O*-bis (trimethylsilyl) trifluroacetamide (BSTFA). The products were analyzed by GLC using a capillary column SPB-1 (30 m × 0.26 mm), a temperature program (3 °C/min)

from 150 to 210 °C. The 2,3,4,6-tetra-*O*-trimethylsilyl-(+)-2-butyl glycosides obtained were identified by comparison with those prepared from the D- and L-enantiomers of different monosaccharides.

1.5. Optical rotation

Optical rotation was measured by a Jasco Polarimeter model P-1020 at 25 °C.

1.6. Determination of molecular weight

The molecular weight of the polysaccharide was determined by gel-chromatographic technique. Standard dextrans[17] 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 polysaccharide was then plotted in the same graph and molecular weight of polysaccharide was determined.

1.7. NMR studies

The polysaccharide was kept over P_2O_5 in vacuum for several days and then exchanged with deuterium[23] by lyophilizing with D_2O (99.96% atom ^2H, Aldrich) for four times. With a Bruker Avance DPX-500 spectrometer, ^1H, TOCSY, DQF-COSY, NOESY, ROESY, HMQC, and HMBC NMR spectra were recorded in D_2O at 27 °C. The ^1H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.67 ppm) using the WEFT pulse sequence.[24] The 2D-DQF-COSY experiment was carried out using standard Bruker software at 27 °C. The TOCSY experiment was recorded at mixing time of 300 ms, and complete assignment required several TOCSY experiments having mixing times ranging from 60 to 300 ms. The NOESY and ROESY mixing delay was 300 ms. The ^{13}C NMR spectrum of polysaccharide solution in D_2O was recorded at 27 °C using acetone as internal standard, fixing the methyl carbon signal at δ 31.05 ppm. The delay time in the HMBC experiment was 80 ms.

Acknowledgments

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