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Structural studies of water-soluble polysaccharides of an edible mushroom, *Termitomyces eurhizus*. A reinvestigation

Soumitra Mondal, Indranil Chakraborty, Malay Pramanik, Dilip Rout and Syed S. Islam*

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

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Abstract—The structure of two polysaccharides isolated from the hot aqueous extract of fruiting bodies of the mushroom, *Termitomyces eurhizus*, have been reinvestigated. These consist of two homogeneous fractions PS-I and PS-II. PS-I contains only D-glucose as the monosaccharide constituent. From methylation analysis and periodate oxidation studies, followed by GLC–MS analysis the linkages, the sugar units in PS-I were identified as $(1\rightarrow3)$-D-Glc*p* and $(1\rightarrow6)$-D-Glc*p*. PS-II contains D-glucose, and the mode of linkage of D-glucose was identified as $(1\rightarrow6)$-D-Glc*p*. Finally, the following possible structures of the polysaccharides were assigned using ^{1}H, 2D-COSY, TOCSY, NOESY and ^{13}C NMR spectral analysis:

$$\left[\rightarrow6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\right]_m\rightarrow\left[3)\text{-}\alpha\text{-D-Glc}p\text{-}(1\right]_n\rightarrow \quad m{:}n \;=\; 2.5{:}1$$

PS-I (α-Glucan)

$$\left[\rightarrow6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow\right]_n$$

PS-II (α-Glucan)

Keywords: Mushroom polysaccharide; *Termitomyces eurhizus*; Structure; NMR analysis

1. Introduction

Mushroom polysaccharides have drawn the attention of chemists and immunobiologists in recent years on account of their immunomodulation and antitumour properties.[1–3] Wild mushrooms of the genus *Termitomyces*, namely, *Termitomyces eurhizus*, *T. clypeatus*, *T. striatus*, *T. robustus* and *T. microcarpus* have been identified as edible mushrooms with high nutritive value. Several enzymes of the species *T. microcarpus*[4] and *T. clypeatus*[5–9] have been reported as having high therapeutic value. *T. robustus* and *T. clypeatus* contained[10] 31% protein, 32% carbohydrate and 10–14% ascorbic acid. The nutrient content of *T. robustus*[11,12] has also been analyzed. The mycelium of *T. eurhizus*,[13] prepared in liquid culture medium, was found to contain protein (14–27%) and 10 amino acids. *T. eurhizus* grows abundantly in guts of termites found in the laterite forest soil in Southwest Bengal, India during the month of September–October every year. Local people consume them as delicious food material.

Water-soluble polysaccharides were isolated from a hot aqueous extract of *T. eurhizus*. A heteropolysaccharide consisting of D-glucose, D-mannose, D-galactose and D-galacturonic acid from the aqueous extract of *T. eurhizus*, was reported earlier[14] where the results were shown only on the basis of GLC analysis. Moreover, in some fractions of the crude extract, an antitumour glucan has been located. For this reason a thorough investigation was carried out with the whole aqueous extract of the same mushroom. The

* Corresponding author. Tel.: +91-3222-276558x437 (O), +91-3222-268387 (R); fax: +91-3222-275329; e-mail: sirajul_1999@yahoo.com

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water-soluble extract was repeatedly purified by precipitating in alcohol, dialyzing through a DEAE cellulose bag, and gel filtration. Finally fractionation of the water-soluble polysaccharides through a Sephadex G-50 column yielded two fractions, PS-I and PS-II. Detailed structural studies of these materials were carried out using GLC–MS analysis, followed by NMR (^{1}H, ^{13}C, 2D-COSY, TOCSY and NOESY) spectroscopic studies that are reported herein.

2. Results and discussion

Fruit bodies of the mushroom, *T. eurhizus*, 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 precipitated with ethanol (1:5, v/v). 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 molecules. The solution was then precipitated with ethanol (1:5, v/v). The precipitated material was collected through centrifugation and freeze dried. The water-soluble material was purified through a Sephadex G-50 column, and two fractions, PS-I and PS-II, were obtained. Both fractions (PS-I and PS-II) were hydrolyzed using 2 M trifluoroacetic acid, and analysis by PC and GLC showed the presence of only glucose in both the fractions. PS-I and PS-II showed specific rotations of $[\alpha]_D^{25}$ +34.8 (*c* 0.72, water) and $[\alpha]_D^{25}$ +110 (*c* 0.43, water), respectively. The molecular weights of PS-I and PS-II were determined using a calibration curve of standard dextrans[15] and found to be ~9000 and ~6000, respectively. The total sugar content of PS-I and PS-II was estimated as 96% and 100%, respectively, using the phenol–sulfuric acid method.[16] Protein was estimated as 4% in PS-I by Lowry's method.[17] The absolute configurations of the monosaccharides were determined as the D-gluco configuration for both fractions by GLC analysis of their acetylated (+)-2-octyl glycosides according to a modified method.[18] GLC analysis of the alditol acetates of the acid-hydrolyzed product of PS-I showed the presence of only D-glucose. PS-I and PS-II were methylated twice by the Ciucanu and Kerek method,[19] followed by the Purdie and Irvine method,[20] and then hydrolyzed. The alditol acetates of the methylated material from PS-I were analyzed by GLC using column A and column B (see Section 3). Methylated sugars were also identified by GLC–MS analysis using an HP-5 capillary column. PS-I showed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol in a molar ratio of 1.00:2.50, and PS-II showed only 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol (Table 1). From these results the linkages of constituent sugars were identified as (1→3)-linked D-Glc*p* and (1→6)-linked D-Glc*p* in PS-I and (1→6)-linked D-Glc*p* in PS-II. These linkages were further confirmed by periodate oxidation experiments. The methylated products of periodate oxidized–reduced polysaccharides were hydrolyzed and acetylated. GLC analysis of periodate-oxidized, methylated PS-I showed only one peak for (1→3)-linked D-Glc*p*, but no peak corresponding to a (1→6)-linked D-Glc*p* was found. For PS-II, no peak was found corresponding to a (1→6)-linked D-Glc*p*.

The 500-MHz ^{1}H NMR spectrum of PS-I (Fig. 1) at 37 °C contained two H-1 signals at δ 5.33 ($^3J_{H\text{-}1,H\text{-}2}$ 3.0 Hz) and 5.08 ($^3J_{H\text{-}1,H\text{-}2}$ 3.0 Hz) ppm corresponding to (1→3)-D-Glc*p* (residue B) and (1→6)-D-Glc*p* (residue

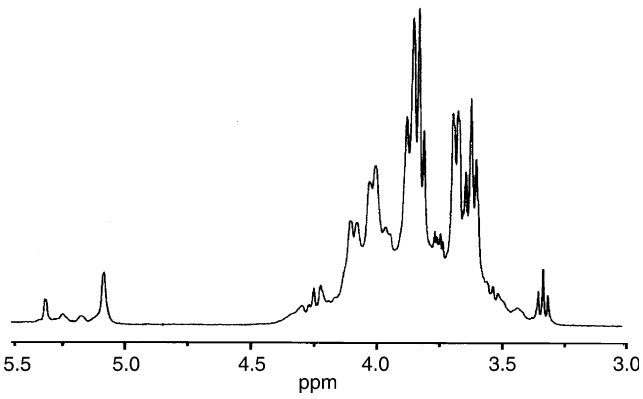

Figure 1. ^{1}H NMR (500 MHz) spectrum of PS-I recorded at 37 °C using D_2O as solvent.

Table 1. GLC and GLC–MS data for the alditol acetates derived from the methylated PS-I and PS-II

Methylated sugar (as alditol acetate)	T^a	T^b	Main mass fragments (*m*/*z*)	Molar ratio	Mode of linkage
PS-I					
2,4,6-Me$_3$-Glc*p*	1.95	1.82	43, 58,71, 87, 101, 117, 129, 161, 201, 233	3.49	→3)-Glc*p*-(1→
2,3,4-Me$_3$-Glc*p*	2.49	2.22	43, 58, 71, 87, 99, 101, 117, 129, 161, 189, 233	3.50	→6)-Glc*p*-(1→
PS-II					
2,3,4-Me$_3$-Glc*p*	2.49	2.22	43, 58, 71, 87, 99, 101, 117, 129, 161, 189, 233	1.00	→6)-Glc*p*-(1→

[a] Relative retention time with respect to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on 3% ECNSS-M on Gas Chrome Q at 170 °C.
[b] Relative retention time with respect to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on 1% OV-225 on Gas Chrome Q at 170 °C.

Table 2. ^{1}H NMR chemical shifts of polysaccharides recorded in D_2O at 37 °C for PS-I and PS-II[a]

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6
PS-I						
(1→3)-α-D-Glc*p*	5.33	3.60	3.81	3.34	3.62	3.83[b] 4.22[c]
(1→6)-α-D-Glc*p*	5.08	3.68	3.85	3.64	3.70	4.01[b] 4.08[c]
PS-II						
(1→6)-α-D-Glc*p*	4.92	3.52	3.68	3.50	3.56	3.81[b] 3.95[c]

[a]Values of chemical shifts were taken with respect to the HOD signal fixed at δ 4.67 ppm.
[b,c]Interchangeable.

A), respectively, in a molar ratio of 1.00:2.49. All proton signals of PS-I (Table 2) were assigned from correlation of 2D-COSY and TOCSY NMR experiments (Fig. 2, right panel). The 400-MHz ^{1}H NMR spectrum of PS-II (Fig. 3) at 37 °C showed one anomeric proton (H-1) signal at δ 4.92 ppm (unresolved) for the (1→6)-linked D-glucopyranosyl moiety. All proton signals of PS-II (Table 2) were assigned from correlation of 2D-COSY NMR experiments.

The 100-MHz ^{13}C NMR spectrum of PS-I at 27 °C (Table 4, Fig. 4) showed two anomeric carbon signals at δ 97.01 and 99.18 ppm corresponding to (1→3)-D-Glc*p* and (1→6)-D-Glc*p*, respectively. The coupling constant values of anomeric protons in the ^{1}H NMR spectrum ($J_{1,2}$ = 3.0 Hz) and appearance of anomeric carbon signals in ^{13}C NMR spectrum indicated that all the sugar moieties in PS-I were present in the α-anomeric configuration. The C-6 signals at δ 61.78 and 67.06 ppm were observed for (1→3)-linked and (1→6)-linked glucose moieties, respectively. The C-6 peak showed a downfield shift at 67.06 ppm due to glycosylation at the C-6

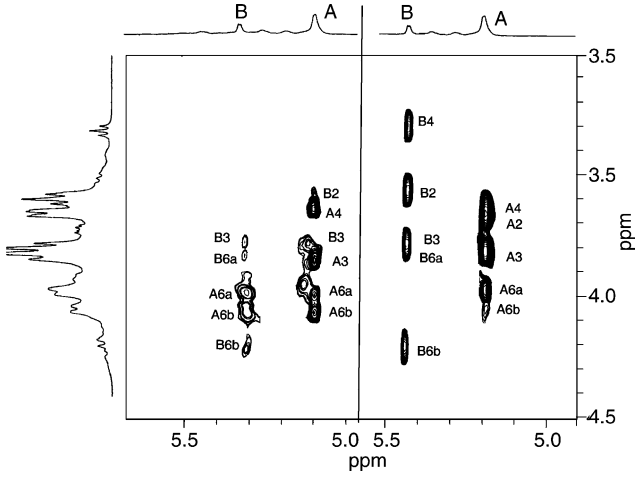

Figure 2. The TOCSY (right panel) and NOESY (left panel) spectra of PS-I isolated from *T. eurhizus*. The mixing time for the TOCSY spectrum shown was 150 ms. Complete assignment required several TOCSY experiments requiring several mixing times ranging from 60 to 300 ms.

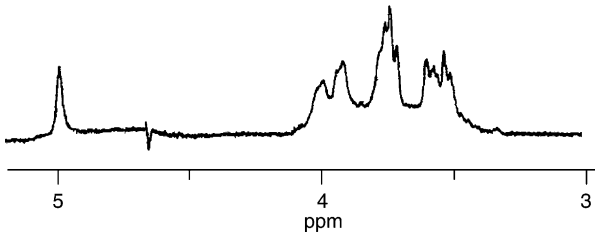

Figure 3. ^{1}H NMR (400 MHz, D_2O) spectrum of PS-II recorded at 37 °C.

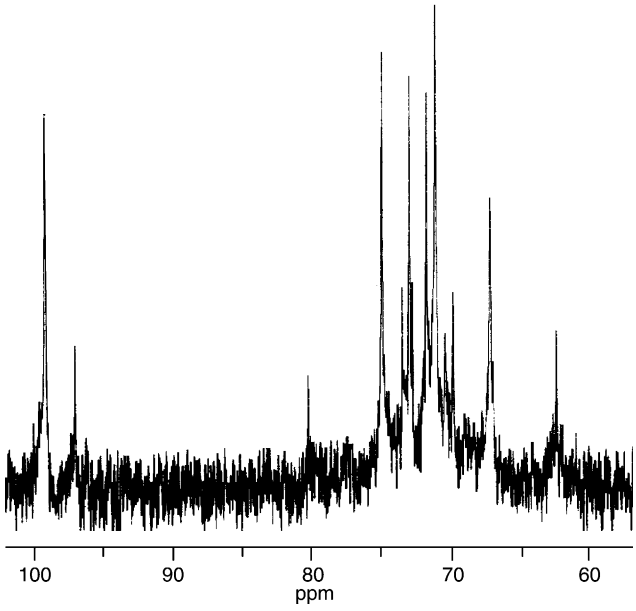

Figure 4. ^{13}C NMR (100 MHz, D_2O) spectrum of PS-I recorded at 27 °C.

position. The C-3 signal of (1→3)-linked glucose showed a downfield shift due to the glycosylation effect and appeared at 80.06 ppm. The C-2, C-3, C-4 and C-5 signals of these sugars were assigned by comparing their spectra with those of standard methyl glycosides.[21] The rest of the signals of (1→3)-linked glucose were C-2 (70.33), C-4 (69.75) and C-5 (73.41). The (1→6)-linked

Table 3. NOE data for the PS-I fraction isolated from *T. eurhizus*

Anomeric proton		NOE contact to proton		
Glycosyl residue	δ	δ	Intensity[a]	Residue, atom
A → 6)-α-D-Glc*p*-(1→	5.08	3.64	s	A H-4
		3.85	m	A H-3
		4.01	s	A H-6a
		4.08	s	A H-6b
		3.81	s	B H-3
		3.60	w	B H-2
B → 3)-D-Glc*p*-(1→	5.33	3.81	m	B H-3
		3.83	m	B H-6a
		4.22	m	B H-6b
		4.01	s	A H-6a
		4.08	s	A H-6b

[a]The intensities are estimated from visual inspection of the NOESY spectrum shown in Figure 2 and are given as: s = strong, m = medium and w = weak.

glucose showed carbon signals at 72.87, 74.87, 71.02 and 71.65 ppm for C-2, C-3, C-4 and C-5, respectively.

The sequence of glycosyl residues of PS-I was determined from a NOESY experiment (Fig. 2, left panel; Table 3). Residue A has a NOE contact from H-1 to H-3 of residue B, in addition to intraresidue NOE contacts to H-4, H-3, H-6a and H-6b and a weak interresidue contact to H-2 of residue B. Since residue A is linked at the 3-position of residue B, the following sequence was established:

$$\underset{\text{A}}{\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow} \underset{\text{B}}{3)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow}$$

Residue B has strong NOE contacts from H-1 to H-6a and H-6b of residue A, in addition to intraresidue contacts to H-3 and H-6, indicating that residue B is linked at the 6-position of residue A. Thus, the following sequence was established:

$$\underset{\text{B}}{\rightarrow 3)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow} \underset{\text{A}}{6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow}$$

Since PS-I contains (1 → 6)-linked α-D-Glc*p* in a larger proportion than (1 → 3)-linked α-D-Glc*p*, the structure of PS-I is assigned as,

$$[\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1]_m \rightarrow [3)\text{-}\alpha\text{-D-Glc}p\text{-}(1]_n \rightarrow$$
$$m : n = 2.5 : 1$$

The 100-MHz ^{13}C NMR spectrum of PS-II at 27 °C (Table 4, Fig. 5) showed a signal in the anomeric region at δ 98.76 ppm for the (1 → 6)-linked D-glucopyranosyl residues. ^{1}H as well as ^{13}C NMR spectra indicated that the sugar residue is of the α-anomeric configuration. The C-6 signal at δ 66.74 ppm indicated the presence of a 6-linked glycoside residue.[22] The signals other than C-1 of (1 → 6)-linked glucose were identified as δ 71.25, 74.60, 70.79 and 72.47 ppm for C-2, C-3, C-4 and C-5,

Table 4. ^{13}C NMR chemical shifts of polysaccharides (PS-I and PS-II) recorded in D_2O at 27 °C[a]

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
PS-I						
(1 → 3)-α-D-Glc*p*	97.01	70.33	80.06	69.75	73.41	61.78
(1 → 6)-α-D-Glc*p*	99.18	72.87	74.87	71.02	71.65	67.06
PS-II						
(1 → 6)-α-D-Glc*p*	98.76	71.25	74.60	70.79	72.47	66.74

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

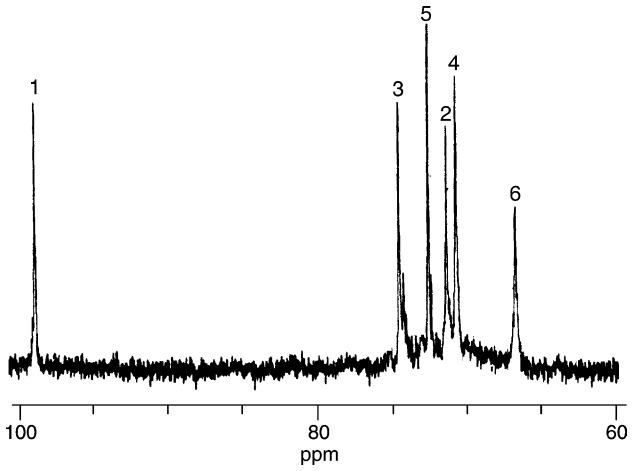

Figure 5. ^{13}C NMR (100 MHz, D_2O) spectrum of PS-II recorded at 27 °C.

respectively. Therefore, based on the above evidences, the structure of PS-II is assigned as,

$$\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1 \rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1 \rightarrow$$

3. Experimental

3.1. General methods

All evaporations were carried out at 40 °C. Paper chromatography was performed on Whatmann nos. 1 and 3 mm papers. Solvent systems (v/v) used were as follows: (A) BuOH–HOAC–H_2O (4:1:5, upper layer) and (B) EtoAC–pyridine–H_2O (8:2:1). The spray reagent used was alkaline $AgNO_3$ solution.[23] All solvents (except D_2O) were distilled before use. Optical rotations were measured with a Perkin–Elmer model 241 MC spectropolarimeter. Gel filtrations were performed on columns of Sephadex G-50 using a Redifrac fraction collector. Colorimetric estimations were conducted with a Shimadzu UV–vis spectrophotometer, model 1601. Gas–liquid chromatogrphy (GLC) was performed using a Hewlett–Packard Model 5730A gas chromatograph having a flame-ionization detector. Glass columns

(1.8 m × 6 mm) packed with A, 3% ECNSS-M on Gas Chrom Q (100–120 mesh); (B) 1% OV-225 on Gas Chrom Q (100–120 mesh) were used for GLC. GLC–MS experiments were performed on a Hewlett–Packard 5970 MSD instrument fitted with an HP-5 fused silica capillary column using a temperature programme from 150 °C (2 min) to 200 °C (5 min) at 2 °C min^{-1}. ^{13}C NMR and ^{1}H NMR experiments were performed with Bruker Avance DPX-500 and Bruker AMX-400 instruments at 300 K.

3.2. Isolation and purification of the polysaccharide

The fruit body of the mushroom, *T. eurhizus* (1.5 kg) was collected from the local forest and washed with water. It was swollen in 250 mL of distilled water and boiled for 4 h. The whole mixture was kept overnight at 4 °C and then filtered through linen cloth. The aqueous extract was collected and lyophilized. The lyophilized material was dissolved in 20 mL of distilled water and dialyzed through a DEAE cellulose bag against distilled water for 4 h to remove low molecular weight materials. The aqueous solution was then collected from the dialysis bag and precipitated in ethanol (1:5, v/v). It was kept overnight at 4 °C and then centrifuged at 10,000 rpm at 4 °C for 1 h. The precipitated material (polysaccharide) was washed with ethanol four times and then dried. This process was carried out thrice for better purification of the polysaccharide. The dried polysaccharide was then dissolved in a minimum volume of distilled water and lyophilized to give 1.2 g of crude polysaccharide.

The crude polysaccharide (40 mg) was purified through a Sephadex G-50 gel-permeation column (90 × 2.1 cm) using water as eluant with a flow rate of 0.4 mL min^{-1}. A total of 110 test tubes (2 mL each) were collected and monitored spectrophotometrically at 490 nm using the phenol–sulfuric acid method.[16] Two homogeneous fractions, PS-I (test tubes 36–58) and PS-II (test tubes 62–85) were collected and freeze dried, yielding 16 mg of PS-I and 20 mg of PS-II. This purification process was carried out in five lots, and each individual fraction was again purified and collected. PS-I, yield 65 mg, and PS-II, yield 70 mg.

3.3. Determination of molecular weight

The molecular weights of PS-I and PS-II were determined by a gel-chromatographic technique. Standard dextrans T-40 and T-10 were passed through a Sephadex G-50 column, and then the elution volumes were plotted against the logarithm of their respective molecular weights. The elution volumes of PS-I and PS-II were plotted in the same graph, and the molecular weights were determined.

3.4. Monosaccharide analysis

The polysaccharides PS-I and PS-II (1.5 mg each) were hydrolyzed separately with 2 M CF_3COOH (1 mL each) for 18 h at 100 °C. The excess acid was completely removed by co-distillation with water, and then the hydrolyzed products were divided into two parts. One part of both of the polysaccharides was examined by PC in solvent systems A and B. Another part of both of them was reduced with $NaBH_4$ (9 mg), followed by acidification with dilute CH_3COOH, and then co-distilled with pure CH_3OH. The reduced sugars (alditols) were acetylated with 1:1 pyridine–Ac_2O in a boiling water bath for 1 h, and the resulting alditol acetates were analyzed by GLC (columns A and B). Absolute configurations of monosaccharides of both PS-I and PS-II were determined according to the method reported by Gerwig et al.[18]

3.5. Methylation analysis

PS-I and PS-II (4.0 mg each) were methylated separately using the method of Ciucanu and Kerek.[19] The methylated products were isolated by partition between $CHCl_3$ and H_2O (5:1, v/v). The product was methylated again by the Purdie method.[20] The product showed no band in the region 3600–3300 cm^{-1} in the IR spectrum. The product was then hydrolyzed with 90% HCOOH (1 mL) for 1 h, and excess HCOOH was evaporated by co-distillation with distilled water. The hydrolyzed product was then reduced with $NaBH_4$ (24 mg) and acetylated with pyridine and Ac_2O. The alditol acetates of the methylated sugars were analyzed by GLC (using columns A and B) and GLC–MS (using HP-5 fused silica capillary column).

3.6. Periodate oxidation study

PS-I and PS-II (5 mg) were added separately to 1.5 mL 0.1 M sodium metaperiodate solution in a round-bottom flask, and the mixture was kept for 48 h in the dark at 4 °C. The excess periodate was destroyed by adding ethylene glycol (1.0 mL), and the solution was dialyzed against distilled water for 1 h. The product was then reduced with $NaBH_4$ (30 mg) followed by acidification with CH_3COOH and dried by co-distillation with CH_3OH. The periodate-reduced material was divided into two portions. One portion was hydrolyzed by 2 M CF_3COOH (1 mL) for 16 h, and the alditol acetates were prepared as usual. Another portion was methylated by the method of Ciucanu and Kerek,[19] and the alditol acetates of this methylated product were prepared. The alditol acetates were analyzed by GLC using columns A and B.

3.7. NMR studies

PS-I and PS-II were dried over P_2O_5 in vacuum for several days and then deuterium exchanged[24] four times, followed by lyophilization with D_2O. The ^1H NMR spectrum was recorded by suppressing HOD signal (fixed at δ 4.67 ppm) using the WEFT pulse sequence.[25] The TOCSY experiment was recorded at mixing time 60–300 ms, and the NOESY mixing delay was 200 ms. The ^{13}C NMR experiment of PS-I and PS-II was carried out taking acetone as the internal standard, fixing the methyl carbon signal at δ 31.05 ppm using D_2O as solvent.

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