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NMR and MALDI-TOFMS analysis of a heteroglycan isolated from hot water extract of edible mushroom, *Volvariella bombycina*

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Abstract—A water-soluble polysaccharide, isolated from the hot aqueous extract of an edible mushroom, *Volvariella bombycina*, consists of D-glucose, D-mannose, and D-galactose in a molar ratio 2:1:1. On the basis of total hydrolysis, methylation analysis, periodate oxidation, and NMR studies (^{1}H, ^{13}C, TOCSY, DQF-COSY, NOESY, ROESY, HMQC, and HMBC), and MALDI-TOFMS analysis, the repeating unit of the polysaccharide is established as

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

Keywords: *Volvariella bombycina*; Polysaccharide; Structure; NMR spectroscopy; MALDI-TOFMS analysis

1. Introduction

Mushrooms are 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. Their polysaccharides[2–6] are considered to be one of the most useful antitumor agents for clinical uses. A few glucans[7–13] and some heteroglycans[14–18] have been isolated from some edible mushrooms in our laboratory and reported in this journal. Mushrooms of the genus *Volvariella*, namely *V. bombycina, V. volvacea,* and *V. diplasia*, are commonly available edible mushrooms and grow abundantly in South East Asia. These belong to well known *Pluteaceae*[19] family and are known as straw or paddy straw mushroom. It has been reported that the cultured mycelia of *V. bombycina* exhibit antioxidant[20] activity, and the protein-polysaccharide complexes extracted from cultured mycelia of this polysaccharide show antitumor[21] activity against sarcoma 180 in ICR mice. No report relating to the structure of polysaccharide isolated from the fruit bodies of *V. bombycina* are in the literature. A water-soluble polysaccharide was isolated from hot aqueous extract of fruit bodies of *V. bombycina* with a view to study the structural as well as immunological properties of this macromolecule. We are reporting herein the detailed structural studies of this polysaccharide.

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2. Results and discussion

Fruit bodies of the mushroom *V. bombycina* were successively washed, boiled with water, cooled and the dialyzed material on precipitation in alcohol yielded crude polysaccharide (1.5 g). This water-soluble polysaccharide (30 mg) on fractionation through a Sepharose 6B column yielded only one homogeneous fraction (22 mg). The molecular weight[22] of the polysaccharide (PS) was estimated from a calibration curve prepared with standard dextrans as $\sim 1.6 \times 10^5$ Da. The polysaccharide showed a specific rotation of $[\alpha]_D^{25}$ +47.67 (*c* 0.074, water).

On acid hydrolysis with 2 M CF_3COOH, followed by alditol acetate conversion and analysis through GLC, the PS was found to contain D-glucose, D-galactose, and D-mannose in a molar ratio of 2:1:1. The absolute configuration of the monosaccharides was determined by the method of Gerwig et al.[23] which showed that each monosaccharide belongs to the D-series. The PS was then methylated using the Ciucanu and Kerek method[24] followed by the Purdie and Irvine method[25] followed by hydrolysis and alditol acetate preparation.[26] Alditol acetates were then analyzed by GLC as well as GLC–MS, which showed the presence of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol, and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-mannitol in a molar ratio of 2:1:1. These results indicate that (1→6)-linked D-glucopyranosyl, (1→4,6)-linked D-mannopyranosyl and terminal D-galactopyranosyl moieties are present in the PS in a molar ratio of 2:1:1. The GLC analysis of the alditol acetates of the periodate-oxidized,[27,28] reduced PS and IO_4-oxidized, reduced, methylated PS showed that all these sugars were destroyed during oxidation. These results confirm the mode of linkage of the monosaccharide components present in the PS.

The proton NMR spectrum (500 MHz, Fig. 1) of this PS at 27 °C contains signals at 5.12, 4.98, and 4.51 ppm for anomeric protons in a molar ratio of almost 1:2:1. The response of the signal at δ 4.98 is almost double

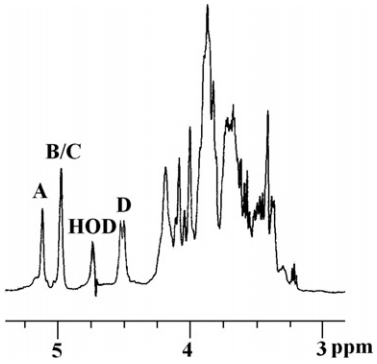

Figure 1. ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) of the polysaccharide isolated from *Volvariella bombycina*.

of the ratio of the other peaks, which indicates two sugar residues. They were designated as **A**, **B**, **C**, and **D** according to their decreasing anomeric proton chemical shifts (Table 1). In the ^{13}C NMR spectrum (125 MHz, Fig. 2) at 27 °C four anomeric signals appeared at δ 103.2, 102.1, and 98.2 ppm (Table 1) in a ratio of nearly 2:1:1. On the basis of the anomeric proton assignments, the anomeric carbon signals were readily assigned from the HMQC spectrum. Signal at δ 98.2 ppm was assigned to anomeric carbons of **A** and **B** residues. Signals at δ 102.1 and 103.2 ppm were assigned to anomeric carbons of **C** and **D** residues, respectively. All the ^{1}H and ^{13}C signals (Table 1) were assigned using DQF-COSY, TOCSY, HMQC, and HMBC experiments. The proton coupling constants were measured from DQF-COSY experiments.

In the case of residue **A**, the anomeric proton chemical shift at δ 5.12 ppm and $J_{H\text{-}1,H\text{-}2} \sim 3.4$ Hz, $J_{H\text{-}1,C\text{-}1} \sim 170$ Hz indicate that it is an α-anomer. Large coupling constants $J_{H\text{-}2,H\text{-}3}$ (∼10 Hz) and $J_{H\text{-}3,\ H\text{-}4}$ (∼10 Hz) for residue **A** indicate that it is a glucosyl moiety. The downfield shift of C-6 (δ 66.8 ppm) signal with respect to the standard value of methyl glycosides[29,30] indicates that residue **A** is (1→6)-linked α-D-glucopyranose.

The anomeric proton chemical shift for moiety **B** at δ 4.98 ppm ($J_{H\text{-}1,H\text{-}2} \sim 3.2$ Hz and $J_{H\text{-}1,C\text{-}1} \sim 171$ Hz) indicates that it is α-linked. A large $J_{H\text{-}2,H\text{-}3}$ (>5 Hz) and small $J_{H\text{-}3,H\text{-}4}$ (<5 Hz), respectively, indicates that it is an α-linked D-galactosyl unit. The carbon chemical shifts of **B** from C-1 to C-6 correspond nearly to the standard values for a methyl glycoside of α-D-galactose. Thus considering the results of methylation analysis and NMR experiments, it is concluded that it is an α-linked terminal D-galactose.

Residue **C** has an anomeric signal at δ 4.98 ppm, $J_{C\text{-}1,H\text{-}1} \sim 170$ Hz and relative small $J_{H\text{-}1,H\text{-}2}$ and $J_{H\text{-}2,H\text{-}3}$ coupling constants and large $J_{H\text{-}3,H\text{-}4}$ (∼7.5 Hz) and $J_{H\text{-}4,H\text{-}5}$ (∼10 Hz) coupling constants, indicating that residue **C** is an α-D-mannopyranose. The downfield shifts of the C-4 (δ 74.1 ppm) and C-6 (δ 69.2 ppm) signals with respect to the standard values for methyl glycosides indicate that residue **C** is a (1→4,6)-linked-α-D-mannopyranosyl residue.

Residue **D** was assigned to D-Glc*p* as it showed large coupling constants $J_{H\text{-}2,H\text{-}3}$ (∼9.3 Hz) and $J_{H\text{-}3,H\text{-}4}$ (∼9.5 Hz). The anomeric proton chemical shift at δ 4.51 ppm ($J_{H\text{-}1,H\text{-}2} \sim 7.5$ Hz) and anomeric carbon chemical shift at δ 103.2 ppm ($J_{H\text{-}1,C\text{-}1} \sim 160$ Hz) indicate that D-glucose is β-linked. The downfield shift of C-6 (δ 67.5 ppm) indicates that it is a β-(1→6)-linked-D-glucopyranosyl residue.

The sequence of glycosyl residues of the PS was determined from NOESY as well as ROESY experiments followed by confirmation with an HMBC experiment. From inter-residue NOE contacts (Fig. 3, Table 2), the following sequences were established:

A **D** **B** **C**
α-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→ ; α-D-Gal*p*-(1→ 4)-α-D-Man*p*-(1→

C **A** **D** **C**
→ 4)-α-D-Man*p*-(1→6)-α-D-Glc*p*-(1→ ; →6)-β-D-Glc*p*-(1→6)-α-D-Man*p*-(1→
4
↑

Table 1. The ^{1}H and ^{13}C NMR chemical shifts for the polysaccharide isolated from *Volvariella bombycina*[a,b] in D_2O at 27 °C

Glycosyl residue	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5	C-6/H-6a,H-6b
A	98.2	72.1	74.6	69.9	70.8	66.8
→6)-α-D-Glc*p*-(1→	5.12	3.95	4.0	3.85	3.93	4.05, 4.19
B	98.2	68.9	70.1	69.8	71.0	61.4
α-D-Gal*p*-(1→	4.98	3.84	3.7	4.09	3.87	3.68, 3.73
C	102.1	71.9	70.4	74.1	73.3	69.2
→4,6)-α-D-Man*p*-(1→	4.98	3.87	3.54	3.91	3.65	3.74, 3.88
D	103.2	74.0	76.6	70.8	75.9	67.5
→6)-β-D-Glc*p*-(1→	4.51	3.33	3.49	3.41	3.62	3.7, 3.77

[a] Values of the ^{13}C chemical shifts were recorded with reference to using acetone as internal standard and fixed at 31.05 ppm at 27 °C.
[b] Values of the ^{1}H chemical shifts were recorded and assigned with respect to the HOD signal fixed at 4.73 ppm at 27 °C.

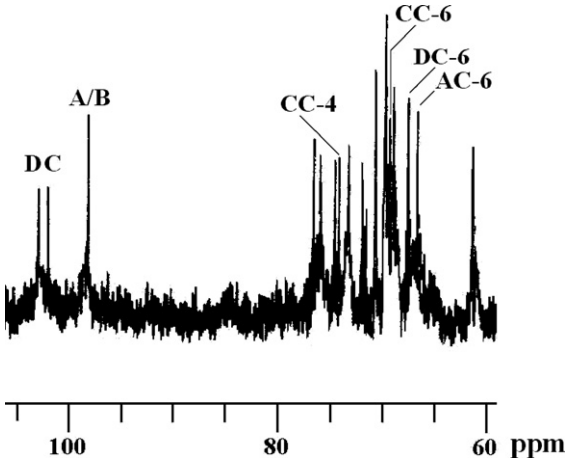

Figure 2. ^{13}C NMR spectrum (125 MHz, D_2O, 27 °C) of the polysaccharide isolated from *Volvariella bombycina*.

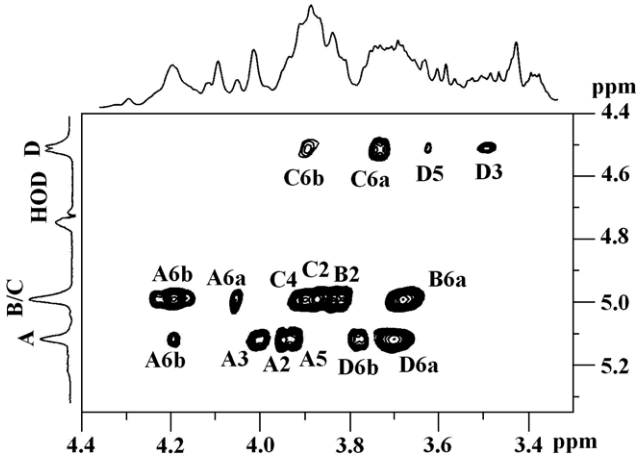

Figure 3. The NOESY spectrum of the polysaccharide isolated from *Volvariella bombycina* recorded with a mixing time of 300 ms.

Long-range ^{13}C–^{1}H correlations obtained from the HMBC spectrum (Fig. 4, Table 3) corroborated the assigned sequences deduced from both ROESY and NOESY experiments. The cross peaks of both anomeric protons and carbons of each of the sugar moieties were examined, and both inter- and intraresidual connectivities were observed from the HMBC experiment. Cross peaks were found between H-1 (5.12 ppm) of residue **A** and C-6 (67.5 ppm) of residue **D** (**A** H-1, **D** C-6); C-1 (98.2 ppm) of residue **A** and H-6a (3.7 ppm) and H-6b (3.77 ppm) of residue **D** (**A** C-1, **D** H-6a and **A** C-1, **D** H-6b). Similarly cross peaks between H-1 (4.98 ppm) of residue **B** and C-4 (74.1 ppm) of residue **C** (**B** H-1, **C** C-4); C-1 (98.2 ppm) of residue **B** and H-4 (3.91 ppm) of residue **C** (**B** C-1, **C** H-4) were observed. Cross peaks between H-1 (4.98 ppm) of residue **C** and C-6 (66.8 ppm) of residue **A** (**C** H-1, **A** C-6); C-1 (102.1 ppm) of residue **C** and H-6a (4.05 ppm) and H-6b (4.19 ppm) of residue **A** (**C** C-1, **A** H-6a and **C** C-1, **A** H-6b) were observed. The cross peaks between H-1 (4.51 ppm) of residue **D** and C-6 (69.2 ppm) of residue **C** (**D** H-1, **C** C-6); C-1 (103.2 ppm) of residue **D** and H-6a (3.74 ppm) and H-6b (3.88 ppm) of residue **C** (**D** C-1, **C** H-6a and **D** C-1, **C** H-6b) were observed.

Table 2. NOE data for the polysaccharide isolated from *Volvariella bombycina*

Anomeric proton		NOE contact protons	
Glycosyl residue	δ	δ	Residue, atom
→6)-α-D-Glc*p*-(1→	5.12	3.70	**D** H-6a
A		3.77	**D** H-6b
		3.95	**A** H-2
		4.00	**A** H-3
		3.93	**A** H-5
		4.19	**A** H-6b
α-D-Gal*p*-(1→	4.98	3.91	**C** H-4
B		3.84	**B** H-2
		3.68	**B** H-6a
→4,6)-α-D-Man*p*-(1→	4.98	4.05	**A** H-6a
C		4.19	**A** H-6b
		3.87	**C** H-2
→6)-β-D-Glc*p*-(1→	4.51	3.74	**C** H-6a
D		3.88	**C** H-6b
		3.49	**D** H-3
		3.62	**D** H-5

Thus these cross peaks and NOESY connectivities clearly support a tetrasaccharide repeating unit in the polysaccharide isolated from *Volvariella bombycina* as

D **C** **A**
→6)-β-D-Glc*p*-(1→6)-α-D-Man*p*-(1→6)-α-D-Glc*p*-(1→
4
↑
1
α-D-Gal*p*
B

The MALDI-TOF analysis of this PS was carried out to support the proposed structure. The nomenclature of the different fragments (Fig. 5) and their peaks follows that used by Harvey et al.[31] for high mannose N-linked glycan from ribonuclease B recorded from 2,5-dihydroxybenzoic acid (DHB). The molecule showed distinct mass peaks from *m*/*z* 500 to 3500 (Table 4) with reference to myoglobulin (M_W 30 000) as standard for external calibration in reflector mode and are shown in Figure 6a and b. The ion peaks of the repeating oligosaccharide at *m*/*z* 655 and 671.3 were observed due to breaking of the glycosidic linkages with or without oxygen, respectively, at both sides of the repeating unit by double cleavage. Peaks at *m*/*z* 1141.1, 1303.0, 1789.0, 1951.0, 2113.0, 2437.0, 2615.2, and 3425.0 were probably the result of double cleavage phenomenon at glycosidic linkages of more than one oligosaccharide repeating units. The peaks at *m*/*z* 582.0, 625.8, 714.6, 774.0, 876.0, 1375.8, 1892.1, and 2672.4 were observed due to the breaking of the glycosidic linkages in one side and of ring of different sugar residues in linear chain in another side through double cleavage. The other fragments at *m*/*z* 611.0, 994.7, 1023.0, 1080.23, 1743.0, 1832.7, and 2290.4 appeared due to either breaking of the glycosidic linkages only or breaking along with the ring cleavage of different sugar residues through triple cleavage phenomenon. Higher fragments above 3500 showed poor response in reflector mode and this may explain why the higher ranges of mass values were not-observed. Thus, double or triple cleavage of the

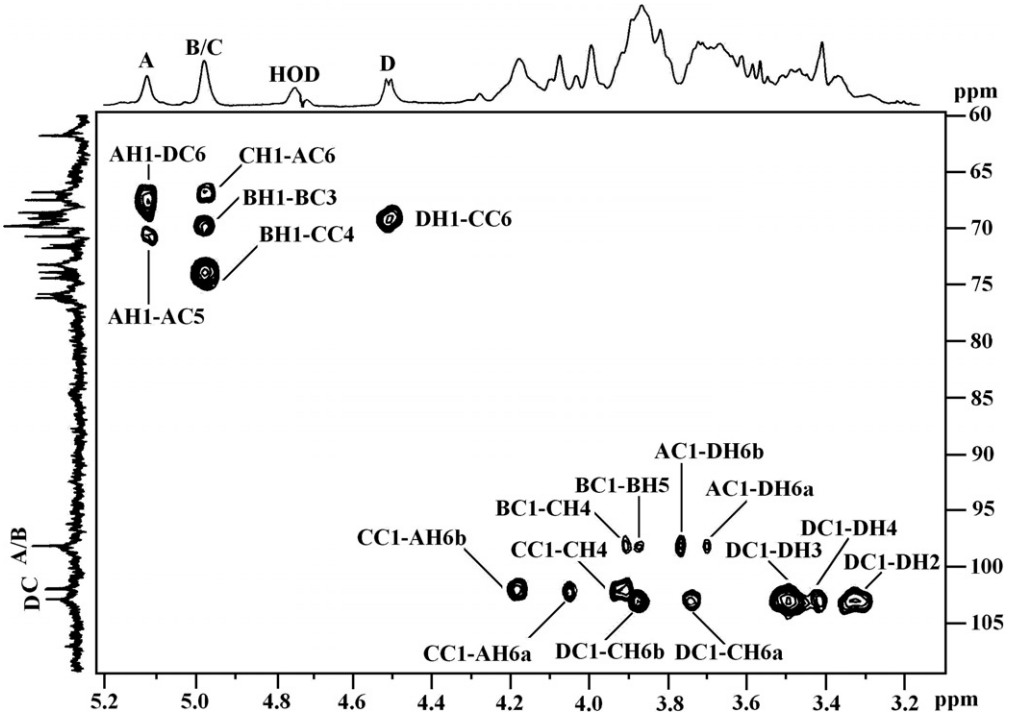

Figure 4. HMBC spectrum of the polysaccharide isolated from *Volvariella bombycina* recorded with a delay time of 80 ms.

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 *Volvariella bombycina*

Residues	Sugar linkage	H-1/C-1	Observed connectivities		
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	→6)-α-D-Glc*p*-(1→	5.12	67.5	**D**	C-6
			70.8	**A**	C-5
		98.2	3.7	**D**	H-6a
			3.77	**D**	H-6b
B	α-D-Gal*p*-(1→	4.98	74.1	**C**	C-4
			70.1	**B**	C-3
		98.2	3.91	**C**	H-4
			3.87	**B**	H-5
C	→4,6)-α-D-Man*p*-(1→	4.98	66.8	**A**	C-6
		102.1	4.05	**A**	H-6a
			4.19	**A**	H-6b
D	→6)-β-D-Glc*p*-(1→	4.51	69.2	**C**	C-6
		103.2	3.74	**C**	H-6a
			3.88	**C**	H-6b
			3.49	**D**	H-3

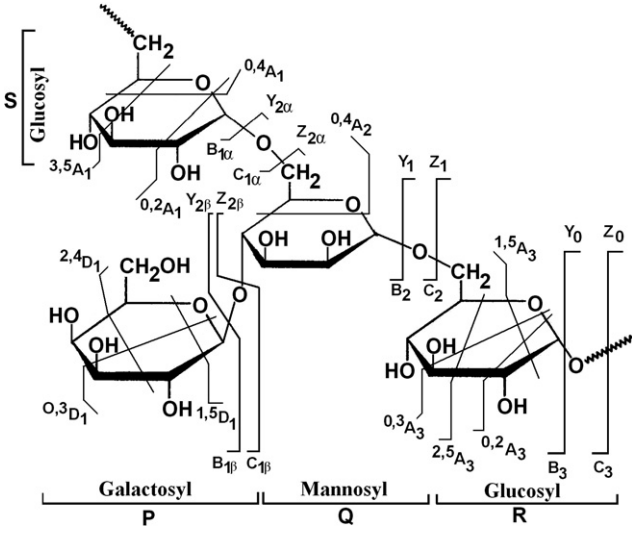

Figure 5. Fragmentation scheme explaining the ions obtained by MALDI-TOF analysis of the polysaccharide isolated from *Volvariella bombycina*.

polymeric chain showed different mass fragments from where the cross linking and branching information[32,33] of the molecule was established.

3. Experimental

3.1. Isolation and purification of the polysaccharide

The fresh fruiting bodies of the mushroom, *Volvariella bombycina* (1 kg), were collected from the local firm and washed with water. It was swollen in 250 mL of distilled water and boiled with distilled water for 4 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 with 1:5 (v/v) EtOH. The precipitated material was collected through centrifugation followed by dialysis against distilled water and then centrifuged at 10 000 rpm at 6 °C for 45 min. The residue was rejected, and the filtrate (water soluble part) was freeze-dried, yielding 1.5 g of

Table 4. Different fragmentations of different sugar residues of the repeating unit of the polysaccharide in MALDI-TOFMS analysis

Double cleavage				Triple cleavage			
Glycosidic cleavage	*m/z*	Ring breaking with glycosidic cleavage	*m/z*	Glycosidic cleavage	*m/z*	Ring breaking with glycosidic cleavage	*m/z*
B_3	655.0	$0,3A_3$	582.0	$C_5/Y_{2\beta}$	994.7	$C_3/2,4D_1$	611.0
C_3	671.3	$1,5A_3$	625.8	$C_{11}/Y_{2\beta}$	2290.4	$C_5/1,5D_1$	1023.0
B_5	1141.1	$0,4A_4$	714.6			$B_5/2,4D_1$	1080.23
B_6	1303.0	$0,2A_4$	774.0			$1,5A_9/Z_{2\beta}$	1743.0
B_8	1789.0	$0,4A_5$	876.0			$C_9/1,5D_1$	1832.7
B_9	1951.0	$3,5A_7$	1375.8				
$B_{10\alpha}$	2113.0	$2,5A_9$	1892.1				
B_{11}	2437.0	$3,5A_{13}$	2672.4				
C_{12}	2615.2						
$C_{16\alpha}$	3425.0						

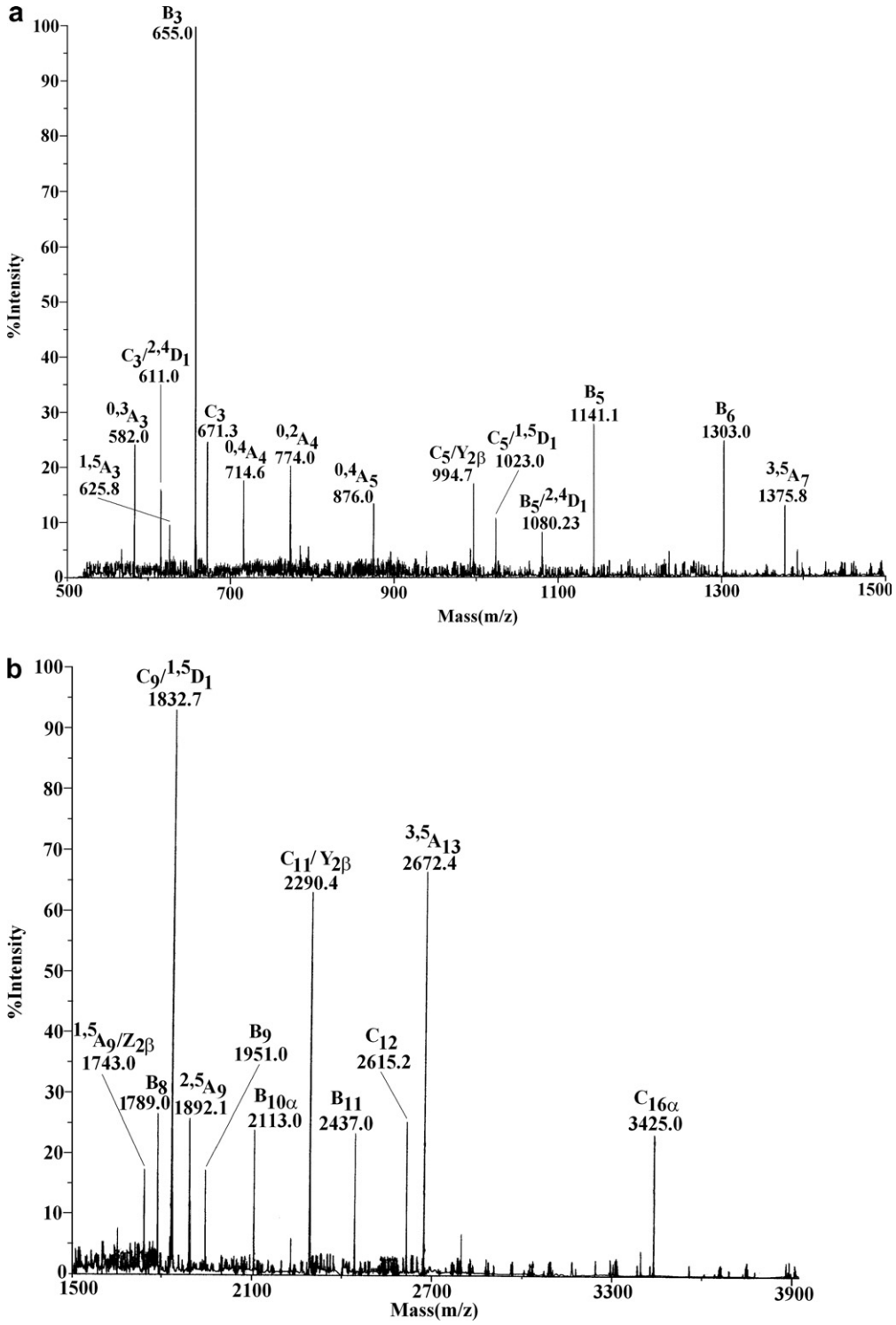

Figure 6. (a) and (b) MALDI-TOF mass spectrum of the polysaccharide isolated from *Volvariella bombycina*. Sample was prepared using DHB (10 mg/mL) matrix dissolved in acetonitrile–water (2:1) containing 1% sodium salt of TFA.

crude polysaccharide. 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 each) were collected, and monitored spectrophotometrically at 490 nm with phenol–sulfuric acid reagent[34] using Shimadzu UV–vis spectrophotometer, model-1601. One homogeneous fraction (test tubes 22–48) was collected and freeze-dried, yielding 22 mg of material. The purification process was carried out in seven lots, and the collected polysaccharides overall recovery was 154 mg.

3.2. Monosaccharide analysis

The polysaccharide sample (3.0 mg) was hydrolyzed with 2 M CF_3COOH (2 mL) in a round-bottomed 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 (alditol) were acetylated with 1:1 pyridine–Ac_2O in a boiling water bath for 1 h to give the alditol acetates,[32] which were analyzed by GLC and GLC–MS. Quantitation was carried out from the peak area, using response factors from standard monosaccharides.

3.3. Methylation analysis

The polysaccharide was methylated using the procedure described by Ciucanu and Kerek,[24] followed by the Purdie and Irvine method.[25] The methylated product was isolated by making partition between 5:2 $CHCl_3$–water. The organic layer-containing product was washed with water several times and dried. The methylated polysaccharide was hydrolyzed with 90% HCOOH (1 mL) at 100 °C for 1 h, and excess HCOOH was evaporated by co-distillation with distilled water. The hydrolyzed product was then reduced with $NaBH_4$ and acetylated with pyridine and Ac_2O. The alditol acetates of the methylated sugars were analyzed by GLC and GLC–MS.

3.4. Periodate oxidation

The PS (5 mg) was oxidized by 2 mL of 0.1 M $NaIO_4$ in the dark for 48 h at room temperature. Excess of $NaIO_4$ was destroyed by the addition of ethylene glycol, and dialyzed against distilled water for 3–4 h. The dialyzed product was reduced with $NaBH_4$ overnight and neutralized with AcOH. The resulting material was obtained by co-distillation with CH_3OH. The residue was subjected to both hydrolysis and methylation by the same process described earlier and the product was analyzed by GLC.

3.5. Absolute configuration of monosaccharides

The method used was based on Gerwig et al.[23] 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 TMS-derivatives were prepared with 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*-TMS-(+)-2-butylglycosides obtained were identified by comparison with those prepared from the D and L enantiomers of different monosaccharides.

3.6. Optical rotation

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

3.7. Determination of molecular weight

The molecular weight of polysaccharide was determined by gel–chromatographic technique. Standard dextrans[22] 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.

3.8. NMR studies

The polysaccharide was kept over P_2O_5 in vacuum for several days, and then exchanged with deuterium[35] by lyophilizing with D_2O (99.96% atom ^2H, Aldrich) four times. ^1H, TOCSY, DQF-COSY, NOESY, and HMBC NMR spectra were recorded in D_2O at 27 °C with a Bruker Avance DPX-500 spectrometer. The ^1H and ^{13}C (both ^1H coupled and decoupled) NMR spectra were recorded at 27 °C. The ^1H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.73) using the WEFT pulse sequence.[36] The 2D-DQF-COSY experiment was carried out using standard BRUKER software at 27 °C. The TOCSY experiment was recorded at a mixing time of 150 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 the polysaccharide dissolved in D_2O was recorded at 27 °C using acetone as internal standard, fixing the methyl carbon signal at δ 31.05. The delay time in the HMBC experiment was 80 ms.

3.9. GLC–MS experiments

All the GLC–MS experiments were carried out in a Hewlett–Packard 5970 MSD instrument using HP-5 fused silica capillary column. 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.

3.10. MALDI-TOF mass spectrometry[37,38]

2,5-Dihydroxybenzoic acid (DHB, 2 mg) was dissolved in 200 μL of the matrix solvent (2:1 acetonitrile–water, v/v) and 1% sodium trifluoroacetate was added to it. Sodium trifluoroacetate and acetonitrile percentages were

optimized using different concentrations of 0.1–3.5% and 20–80%, respectively, using the same instrumental settings. The polysaccharide (1.0 mg) was dissolved in 200 μL Milli Q deionized water. Two microliter of the sample solution was taken in a vial where DHB matrix (2 μL) was added and centrifuged. One microliter of the solution mixture was transferred to the MALDI sample plate for analysis. MALDI-TOFMS was performed on a Voyager-DE PRO (Applied Biosystems) mass spectrometer, equipped with a nitrogen laser operating at 337 nm (laser power 30–35 J and accelerating voltage 25 kV). The instrument was calibrated with myoglobin (sigma) prior to analysis and the spectra were recorded in the reflector mode and in positive ion detection using DHB (10 mg/mL) as matrix.

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