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Note

NMR and MALDI-TOF analysis of a water-soluble glucan from an edible mushroom, *Volvariella diplasia*

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ABSTRACT

A polysaccharide (Fr. II) has been isolated from the hot aqueous extract of the fruit bodies of an edible mushroom, *Volvariella diplasia*. The polysaccharide contains glucose only. On the basis of total acid hydrolysis, methylation analysis, NMR studies (^{1}H, ^{13}C, 2D-COSY, TOCSY, NOESY, ROESY, HMQC, and HMBC), and MALDI-TOFMS analysis, the structure of the repeating unit of the polysaccharide was established as

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

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Mushroom polysaccharides have immense utility, but the most important aspect is their immunomodulatory and anti-cancer activity.[1,2] Several (1→3),(1→6)-β-glucans,[3] (1→3)-β-glucans,[4,5] (1→3)-α-glucans,[6] a heteroglycan[7] and an α-*manno*-β-glucan[8] act as immunomodulating and anti-tumor materials. Some mushroom polysaccharides have been developed, which are used as biological response modifiers (BRMs) or immunopotentiators.[9]

Among the different types of straw mushrooms of the genus *Volvariella*, *Volvariella volvacea*, *Volvariella bombycina*, and *Volvariella diplasia* are commonly available edible mushrooms with high nutritive value that have been cultivated in India. These mushroom polysaccharides showed anti-oxidant activity.[10] Mycelial extracellular polysaccharides isolated from two liquid cultures of *V. volvacea* exhibited hypocholesterolemic activity[11] in rats. *V. diplasia* produced cellulolytic enzymes[12] when grown in shake culture at $_{P}$H 5.4, 28 °C with 0.5% cellulose powder as carbon source. Two water-soluble polysaccharides (Fr. I and Fr. II) have been isolated from the fruit bodies of *V. diplasia*. Fr. I has been characterized and reported[13] by our group in this journal. Now, a detailed structural investigation on Fr. II is carried out and reported herein.

The fruit body of the mushroom, *V. diplasia* (1.0 kg), was washed with distilled water and then extracted with hot water. After centrifugation, the supernatant was precipitated in ethanol (1:5, v/v). The precipitated material was lyophilized and treated with 1% aq acetic acid. The solution was centrifuged, dialyzed, and freeze-dried, yielding 2.5 g of crude product. Then, the pure polysaccharide Fr. II (10 mg) was isolated from each lot (30 mg) adopting the procedure as described earlier.[13] Fr. II showed $[\alpha]_{D}^{25}$ +4.8 (*c* 0.68, water). The molecular weight of this polysaccharide fraction was estimated from a calibration curve prepared with standard dextrans[14] and found to be ~70,000 Da. The total sugar content of Fr. II was estimated as 97.2% using the phenol–sulfuric acid method.[15] Protein was estimated as 2.5% by the Lowry's et al. method.[16] Fr. II was hydrolyzed with 2 M TFA, and the alditol acetate on analysis through GLC indicated the presence of glucose only. Another part of the hydrolyzate on paper chromatographic analysis[17] showed only the spot of glucose. The absolute configuration of the monosaccharide was determined as D by the method of Gerwig et al.[18] Fr. II was methylated by the Ciucanu and Kerek method[19] and then hydrolyzed. The alditol acetates of the methylated product were analyzed by GLC–MS analysis. Fr. II showed the presence of 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol, and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol in a ratio of nearly 1:1:1:1. These results

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indicated the presence of (1→4,6), (1→6), (1→4), and terminal D-glucosyl moieties in the polysaccharide.

The 500 MHz ^{1}H NMR spectrum of the polysaccharide Fr. II (Fig. 1) showed four anomeric proton signals at δ 5.05, δ 4.91, δ 4.42, and δ 4.40 ppm in a ratio of nearly 1:1:1:1. The sugar residues were assigned as **A**, **B**, **C**, and **D** according to their decreasing anomeric chemical shifts (Table 1). In the ^{13}C NMR (125 MHz) spectrum (Fig. 2, Table 1), four anomeric carbon signals appeared at δ 103.4, δ 99.0, and δ 98.5 ppm in a ratio of nearly 2:1:1. All the ^{1}H and ^{13}C signals were assigned using DQF-COSY, TOCSY, HMQC, and HMBC (Fig. 3) NMR experiments.

The anomeric signals of residue **A** at δ 5.05 (unresolved) and $J_{H\text{-}1,C\text{-}1} \sim 171$ Hz indicate that it is an α-linked residue. Large coupling constants $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ (~10 Hz) were observed for **A**, supporting that it is a D-glucosyl moiety.[20,21] The anomeric carbon chemical shift of **A** at 99.0 ppm was confirmed by the presence of cross peaks **A**C-1, **C**H-6a and **A**C-1, **C**H-6b in the HMBC experiment (Fig. 3, Table 3). The downfield shift of C-4 (76.6 ppm) with respect to standard value[22] indicates that residue **A** is a 4-linked α-D-Glc*p* moiety. The carbon values of residue **A** (Table 1) indicate that it is (1→4)-linked α-D-glucopyranose.

Residue **B** has an anomeric proton chemical shift at 4.91 (unresolved) and $J_{H\text{-}1,C\text{-}1} \sim 170$ Hz, indicating that it is an α-linked residue. The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ values (9–10 Hz) for **B** support that it is D-glucosyl moiety. The ^{13}C signal for the anomeric carbon of the D-glucosyl moiety was observed at 98.5 ppm and also confirmed by an HMBC experiment, where coupling corresponding to **B**C-1, **A**H-4 (Fig. 3, Table 3) is found. The downfield shift of C-4 (77.4 ppm) and C-6 (68.7 ppm) signals with respect to the standard value of methyl glycosides[22] is due to the effect of glycosylation. The carbon values of residue **B** (Table 1) indicate that it is a (1,4,6)-linked D-glucose.

Residue **C** has an anomeric proton signal at δ 4.42, and the $J_{H\text{-}1,H\text{-}2} \sim 7.86$ Hz, and $J_{H\text{-}1,C\text{-}1} \sim 160$ Hz indicate that it is a β-linked moiety. Large coupling constants $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ (~10 Hz) were observed from the DQF-COSY spectrum for residue **C**, supporting that it is a D-glucosyl moiety.[21] The C-1 signal of residue **C** at 103.4 ppm was confirmed by the appearance of cross peak **C**C-1, **B**H-4 in an HMBC experiment (Fig. 3, Table 3). The downfield shift of C-6 (69.2 ppm) indicates that residue **C** is a 6-linked β-D-Glc*p* moiety. Hence, the carbon values of residue **C** (Table 1) indicate that it is (1→6)-linked β-D-glucopyranose.

The anomeric proton chemical shift for residue **D** is δ 4.40. A large coupling constant $J_{H\text{-}1,H\text{-}2}$ value (~7.86 Hz) and $J_{H\text{-}1,C\text{-}1}$ value (161 Hz) indicate that it is a β-linked residue. The $J_{H\text{-}2,H\text{-}3}$ value (~9.7 Hz) and the $J_{H\text{-}3,H\text{-}4}$ value (~10 Hz) for residue **D** indicate that it is a β-D-glucosyl residue. The anomeric carbon chemical shift of moiety **E** at 103.4 ppm was confirmed by the presence of two cross-peaks **D**C-1, **B**H-6a and **D**C-1, **B**H-6b in the HMBC experiment (Fig. 3, Table 3). From the results of methylation analysis and NMR experiments, it may be concluded that **D** is terminal β-D-glucose.

The sequence of glycosyl residues of the polysaccharide (Fr. II) was determined from NOESY (Fig. 4, Table 2) as well as ROESY experiments, followed by confirmation with a HMBC experiment. From inter-residue NOE contacts (Fig. 4, Table 2), the following sequences were established as

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

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

The above sequences of glycosyl residues of the polysaccharide were confirmed from cross peaks observed from HMBC experiment (Fig. 3, Table 3). The cross peaks of both anomeric proton and carbon of each of the sugar moieties were examined, and intra- and inter-residual connectivities were observed. Cross peaks were observed between H-1 (δ 5.05) of residue **A** and C-6 (δ 69.2) of residue **C** (**A**H-1, **C**C-6); C-1 of residue **A** (δ 99.0) and H-6a and H-6b of residue **C** (**A**C-1, **C**H-6a and **A**C-1, **C**H-6b), with other intra-residual coupling between H-1 (δ 5.05) of residue **A** with its own C-3 atom (**A**H-1, **A**C-3). The cross peaks between H-1 (δ 4.42) of residue **C** and C-4 (δ 77.4) of residue **B** (**C**H-1, **B**C-4), C-1 (δ 103.4) of residue **C** and H-4 (δ 3.55) of residue **B** (**C**C-1, **B**H-4) were observed. Similarly, the cross peaks between H-1 (δ 4.91) of residue **B** and C-4 (δ 76.6) of residue **A** (**B**H-1, **A**C-4), C-1 (δ 98.5) of residue **B** and H-4 (δ

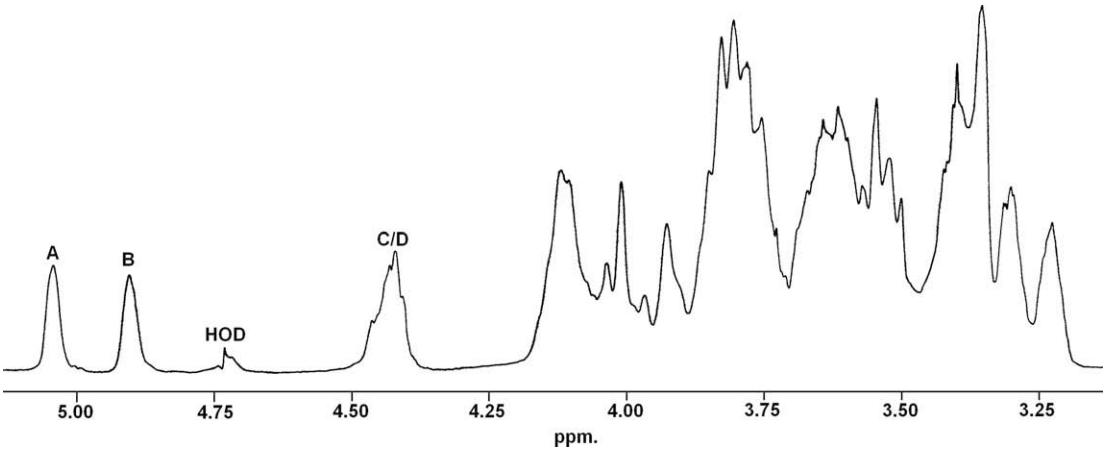


Figure 1. ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) of polysaccharide Fr. II, isolated from *Volvariella diplasia*.

Table 1
^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts of the polysaccharide (Fr. II) isolated from *Volvariella diplasia* recorded in D_2O at 27 °C

Sugar 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
→4)-α-D-Glc*p*-(1→ **A**	5.05 99.0	3.85 72.4	3.93 73.1	3.61 76.6	4.04 70.8	3.97, 3.83 61.4
→4,6)-α-D-Glc*p*-(1→ **B**	4.91 98.5	3.76 72.4	4.01 73.1	3.55 77.4	3.73 69.9	4.12, 3.78 68.7
→6)-β-D-Glc*p*-(1→ **C**	4.42 103.4	3.43 73.8	3.64 76.0	3.53 70.8	3.50 75.1	4.12, 4.04 69.2
β-D-Glc*p*-(1→ **D**	4.40 103.4	3.23 73.4	3.40 76.6	3.31 70.8	3.36 76.0	3.81, 3.67 61.2

[a] The values of chemical shifts were recorded keeping HOD signal fixed at δ 4.73 ppm.
[b] Values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm at 27 °C.

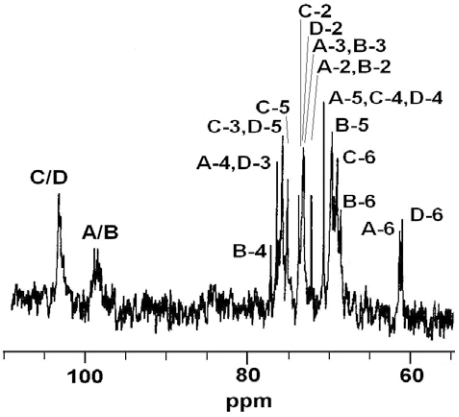
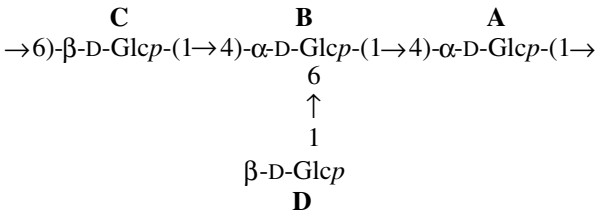


Figure 2. ^{13}C NMR (125 MHz, D_2O, 27 °C) spectrum of polysaccharide Fr. II, isolated from *Volvariella diplasia.*

3.61) of residue **A** (**B**C-1, **A**H-4) were observed. The cross peaks between H-1 (δ 4.40) of residue **D** with C-6 (δ 68.7) of residue **B** (**D**H-1, **B**C-6) and C-1 (δ 103.4) of residue **D** with H-6a and H-6b of residue **B** (**D**C-1, **B**H-6a and **D**C-1, **B**H-6b) were also observed.



Thus, based on all these chemical and spectroscopic evidences the structure of the polysaccharide (Fr. II) is established as:

MALDI-TOFMS analysis of this polysaccharide was carried out to support the proposed structure. The nomenclature of fragments (Fig. 5) and their peaks are presented following Harvey et al.[23] for a high mannose N-linked glycan using B2,5-dihydroxybenzoic acid (DHB) as solid matrix. The molecule showed distinct mass peaks from *m*/*z* 500 to 4559 (Table 4, Fig. 6a and b) in reflector mode. The ion peaks of the repeating oligosaccharide at *m*/*z* 655.0 and 671.1 were observed due to breaking of the glycosidic linkages without or with oxygen at both sides of the repeating unit by double cleavage. The peaks at *m*/*z* 508.4, 817.0, 833.0, 1140.4, 1156.0, 1303.0, 1319.0, 1465.0, 1481.0, 1789.0, 1805.0, 1967.0, 2129.0, 2437.0, 2453.0, 2615.0, 2777.1, 3263.0, 3425.0,

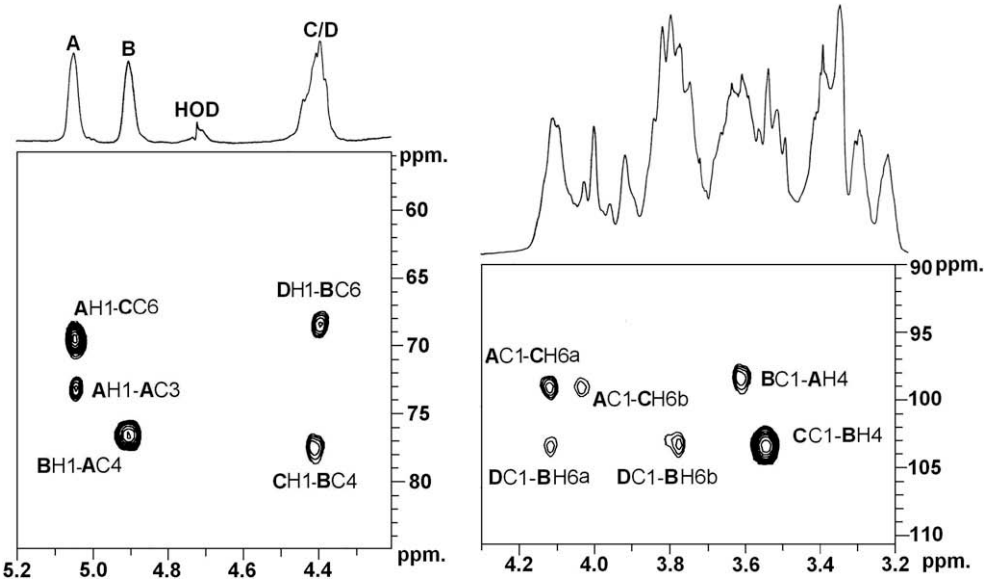


Figure 3. HMBC spectrum (anomeric proton region in the left panel and anomeric carbon region in the right panel) of polysaccharide Fr. II, isolated from *Volvariella diplasia.* The delay time in the HMBC experiment was 80 ms.

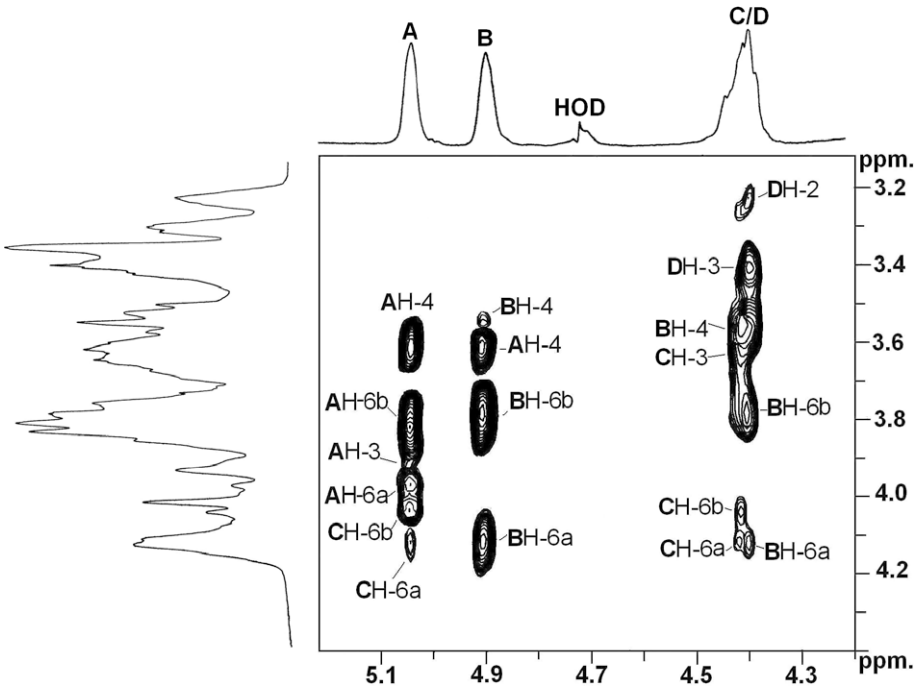


Figure 4. NOESY spectrum of polysaccharide Fr. II, isolated from *Volvariella diplasia*. The NOESY mixing time was 300 ms.

Table 2
NOE data for the polysaccharide (Fr. II) isolated from *Volvariella diplasia*

Glycosyl residue	Anomeric proton	NOE contact to proton	
	δ_H	δ_H	Residue, atom
→4)-α-D-Glc*p*-(1→	5.05	4.12	**C**H-6a
		4.04	**C**H-6b
A		3.93	**A**H-3
		3.61	**A**H-4
		3.97	**A**H-6a
		3.83	**A**H-6b
→4,6)-α-D-Glc*p*-(1→	4.91	3.61	**A**H-4
		3.55	**B**H-4
B		4.12	**B**H-6a
		3.78	**B**H-6b
→6)-β-D-Glc*p*-(1→	4.42	3.55	**B**H-4
		3.64	**C**H-3
C		4.12	**C**H-6a
		4.04	**C**H-6b
β-D-Glc*p*-(1→	4.40	4.12	**B**H-6a
		3.78	**B**H-6b
D		3.23	**D**H-2
		3.40	**D**H-3

Table 3
The significant $^3J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide (Fr. II), isolated from. *Volvariella diplasia.*

Residue	Sugar linkage	H-1/C-1 δ_H/δ_C	Observed connectivities		
			δ_H/δ_C	Residue	Atom
A	→4)-α-D-Glc*p*-(1→	5.05	69.5	**C**	C-6
			73.1	**A**	C-3
		99.0	4.12	**C**	H-6a
			4.04	**C**	H-6b
B	→4,6)-α-D-Glc*p*-(1→	4.91	76.6	**A**	C-4
		98.5	3.61	**A**	H-4
C	→6)-β-D-Glcp-(1→	4.42	77.4	**B**	C-4
		103.4	3.55	**B**	H-4
D	β-D-Glcp-(1→	4.40	68.7	**B**	C-6
		103.4	4.12	**B**	H-6a
			3.78	**B**	H-6b

3911.0, 4397.0, and 4559.0 were solely the results of a double cleavage at glycosidic linkages of more than one oligosaccharide repeating unit. Peaks at *m*/*z* 566.0, 595.7, 611.5, 727.7, 758.0, 774.4, 1214.0, 1243.6, and 1260.0 were observed due to the breaking of the glycosidic linkages in one side and of the ring of different sugar residues in linear chain of another side through double cleavage. The other fragments at *m*/*z* 978.1 and 935.0 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. The higher fragments above *m*/*z* 4559 were of poor intensity in reflector mode. This might explain why higher fragmentations resulting from this kind of cleavage were not observed. Thus double or triple cleavage of the polymeric chain showed different mass fragments from where the cross-linking and branching information[24,25] of the molecule was established.

1. Experimental

1.1. Isolation and purification of the polysaccharide

The fresh edible mushrooms, *V.diplasia* (1.0 kg), were collected from the local market and the crude polysaccharide (2.5 g) was isolated as described in an earlier report.[13] The purity of the polysaccharide was determined by gel-permeation chromatography on a Sepharose-6B column (65 × 2 cm), loading 30 mg crude polysaccharide for each run. Test tubes (95 containing 2 mL eluant each) were collected using a Redifrac fraction collector and monitored by the phenol–sulfuric acid procedure[15] at 490 nm using a Shimadzu UV–Vis spectrophotometer, model-1601. Fractions (test tube nos. 20–45 and 68–89) were collected and freeze-dried; yield, Fr. I (12 mg), and Fr. II (10 mg).

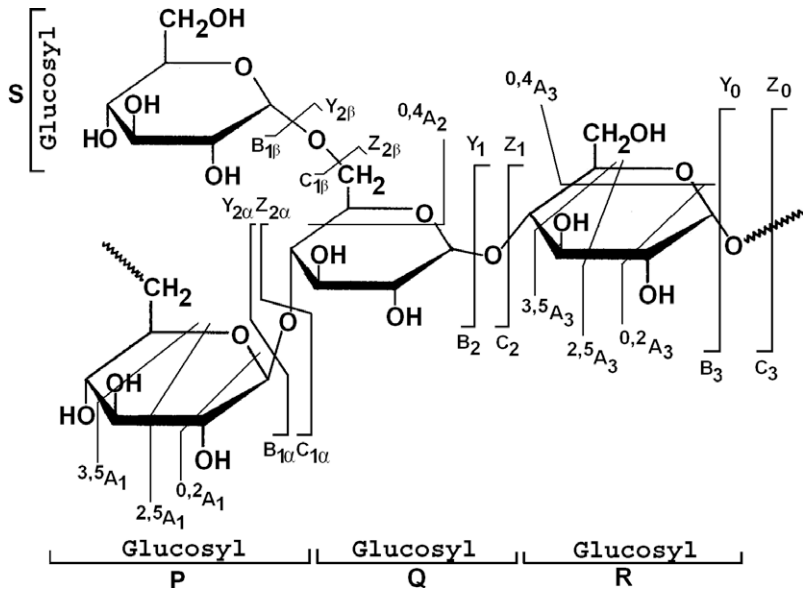


Figure 5. Fragmentation patterns (MALDI-TOFMS) of different mass fragments of polysaccharide Fr. II, isolated from *Volvariella diplasia.*

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

Double cleavage				Triple cleavage			
Glycosidic cleavage	*m/z*	Ring breaking with glycosidic cleavage	*m/z*	Glysidic cleavage	*m/z*	Ring breaking with glycosidic cleavage	*m/z*
C_2	508.4	$3,5A_3$	566.0	$C_3/Y_{20\beta}$	508.4	$C_5/0,4A_5$	935.0
B_3	655.0	$2,5A_3$	595.7	$C_5/Z_{17\beta}$	978.1		
C_3	671.1	$0,4A_3$	611.5	$B_6/Y_{17\beta}$	1140.4		
$B_{4\alpha}$	817.0	$0,2A_3$	611.5	$C_6/Y_{17\beta}$	1156.0		
$C_{4\alpha}$	833.0	$3,5A_4$	727.7				
B_5	1140.4	$2,5A_4$	758.0				
C_5	1156.0	$0,2A_4$	774.4				
B_6	1303.0	$3,5A_6$	1214.0				
C_6	1319.0	$2,5A_6$	1243.6				
$B_{7\alpha}$	1465.0	$0,2A_6$	1260.0				
$C_{7\alpha}$	1481.0						
B_8	1789.0						
C_8	1805.0						
C_9	1967.0						
$C_{10\alpha}$	2129.0						
B_{11}	2437.0						
C_{11}	2453.0						
C_{12}	2615.0						
$C_{13\alpha}$	2777.1						
C_{15}	3263.0						
$C_{16\alpha}$	3425.0						
C_{18}	3911.0						
C_{20}	4397.0						
C_{21}	4559.0						

1.2. Molecular weight determination

The molecular weight of polysaccharide Fr. II was determined by a gel-chromatographic technique. Standard dextrans[14] T-200, T-70, and T-40 were passed through a Sepharose 6B column, and then the elution vol was plotted against the logarithms of their respective molecular weights. The elution vol of Fr. II was then plotted in the same graph and the molecular weight of Fr. II was determined.

1.3. Optical rotation

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

1.4. Paper chromatographic studies

Paper partition chromatographic studies were performed on Whatmann nos. 1 and 3 mm sheets. Solvent systems used were (X) BuOH–AcOH–water (v/v/v, 4:1:5, upper phase) and (Y) EtOAc–pyridine–water (v/v/v, 8:2:1). The spray reagent used was alkaline silver nitrate solution.[17]

1.5. Monosaccharide analysis

Polysaccharide Fr. II (3.0 mg) was hydrolyzed with 2 M CF_3COOH (2 mL) 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 divided into two parts. One part was examined by paper chromatography in solvent systems X and Y. Another part was reduced with $NaBH_4$ (9 mg), followed by acidification with dilute AcOH, and then co-distilled with pure MeOH to remove excess boric acid. The reduced sugars (alditol) 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 using column (A) 3% ECNSS-M on Gas Chrom Q (100–120 mesh) and column (B) 1% OV-225 on Gas Chrom Q (100–120 mesh) at 170 °C. Gas-liquid chromatography–mass spectrometric (GLC–MS) analysis was also performed on Hewlett–Packard 5970A automatic GLC–MS system, using an HP-5 capillary column (25 m × 25 mm). 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.

1.6. Methylation analysis

Polysaccharide Fr. II (4.0 mg) was methylated using the method of Ciucanu and Kerek.[19] The methylated products were isolated by

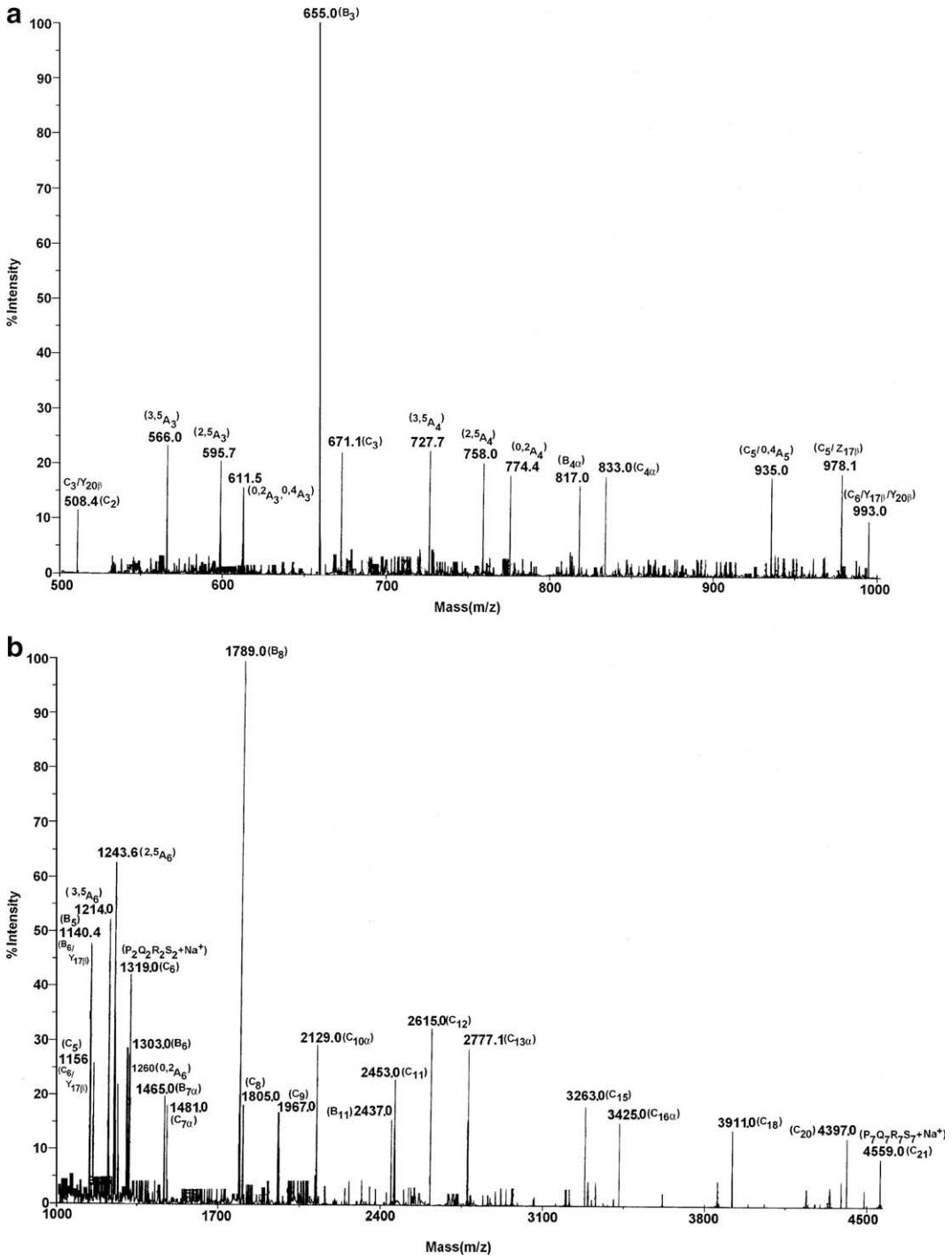


Figure 6. (a and b) MALDI-TOF mass spectrum of polysaccharide Fr. II, isolated from *Volvariella diplasia.*

partition between $CHCl_3$ and water (5:2, v/v). The organic layer containing products was washed with 3 mL water for three times and dried. The methylated products were then hydrolyzed with 90% HCOOH (1 mL) at 100 °C for 1 h. Excess HCOOH was evaporated off by co-distillation with distilled water. The hydrolyzate was then reduced with $NaBH_4$, and acetylated with 1:1 acetic anhydride–pyridine. The alditol acetates of the methylated sugar were analyzed by GLC (using columns A and B) and GLC–MS (using an HP-5 fused silica capillary column and the same temperature program as indicated in Section 1.5).

1.7. Absolute configuration of monosaccharides

The method used was based on that of Gerwig et al.[18] After TFA hydrolysis of 1.0 mg of polysaccharide, the acid was removed by co-distillation with water. A soln 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)trifluoroacetamide (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*-TMSi-(+)-2-butylglycosides obtained were identified by comparison with those prepared from the D and L enantiomers of different monosaccharides.

1.8. NMR studies

The freeze-dried polysaccharide was kept over P_2O_5 in vacuum for several days and then deuterium exchanged[26] four times followed by lyophilization with D_2O (99.96% atom ^2H, Aldrich). With a Bruker Avance DPX-500 spectrometer, ^1H, TOCSY, DQF-COSY, NOESY, ROESY, HSQC, 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 4.73 ppm) using the WEFT pulse sequence.[27] The 2D-DQF-COSY experiment was carried out using standard Bruker software at 27 °C. The TOCSY experiment was recorded at 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 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.

1.9. MALDI-TOF mass spectrometry

1.9.1. Preparation of the DHB matrix

Two milligrams of 2,5-dihydroxybenzoic acid (DHB) were dissolved in the matrix solvent 2:1 MeCN-water (200 μL) and 1% CF3COONa was added. The sodium trifluoroacetate/MeCN proportions were optimized (0.5–3.5% and 20–80% respectively) using the same instrumental settings.

1.9.2. MALDI-TOFMS analysis[28,29]

The polysaccharide (1.0 mg) was dissolved in 200 μL MilliQ (deionized water). Two microliters of the sample soln was taken in a vial where DHB matrix (2 μL) was added and centrifuged. One microliter of the solution mixture was taken in a MALDI sample plate for analysis. MALDI-TOF mass spectrometry was performed on a Voyager-DE PRO (Applied Biosystem) 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. Mass spectra were recorded in reflector mode and in positive ion detection using DHB (10 mg/mL) as matrix.

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