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Note

A water-soluble glucan isolated from an edible mushroom *Termitomyces microcarpus*

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Abstract—A water-soluble glucan was isolated from an edible mushroom, *Termitomyces microcarpus*. On the basis of total acid hydrolysis, methylation analysis, periodate oxidation and NMR studies (^{1}H, ^{13}C, TOCSY, DQF-COSY, NOESY and HSQC), the repeating unit of the polysaccharide is established as

$$\rightarrow 4)\text{-}\alpha\text{-}\text{-Glc}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-}\text{-Glc}p\text{-}(1\rightarrow$$

Keywords: *Termitomyces microcarpus*; Mushroom; Structure; NMR spectroscopy

Mushrooms are a nutritionally functional food and have traditionally been used as folk medicine down through the years. It has been reported that several β-(1→3)-glucans from *Alcaligenes faecalis*,[1] *Poria cocos*,[2] *Auricularia auricula-judae*[3] and *Omphalia lapidescens*,[4] β-(1→3)(1→6)-glucans from *Agaricus blazei*,[5] *Grifola Frondosa* (mitake),[6] *Schizophyllum commune*[7] and *Lentinus edodes*,[8,9] β-(1→6)-glucans from *Gyrophera esculenta*,[10] *Lyophyllum decastes*[11] and *Armillariella tabescens*,[12] both an α-(1→4),β-(1→6)-glucan and an α-(1→4)(1→6)-glucan from *Agaricus blazei*,[13,5] an α-(1→3)(1→4)-glucan from *Gyrophera esculenta*,[10] an α-(1→3),β-(1→6)-glucan from *Agaricus blazei*[5] and a (1→3)-α-glucan from *Armillariella tabescens*,[12] *Amanita muscaria*,[14] and *Agrocybe aegerita*[15] are widely used as antitumour and immunostimulating agents. Thus mushroom polysaccharides have recently drawn the attention of immunobiologists and chemists. The present polysaccharide of *Termitomyces microcarpus* is an α-(1→4),β-(1→3) glucan that is reported herewith for the first time.

Amongst the different types of mushrooms of the genus *Termitomyces*, namely, *Termitomyces eurizus*, *Termitomyces cylpeatus*, *Termitomyces robustus*, *T. microcarpus* and *Termitomyces straitus* are reported as commonly available edible mushrooms. Several value-added materials of the species *T. microcarpus*[16] and *Termitomyces clypeatus*[17–21] have been reported. *T. robustus* and *Termitomyces clypeatus* contained 31% protein, 32% carbohydrate and 10–14% ascorbic acid.[22] The mycelium of *T. eurizus* was found to contain protein (14–27%) and amino acids in a liquid culture medium.[23] Water-soluble and water-insoluble polysaccharides were isolated from the mushroom of the genus *Termitomyces* by our group and are reported in this journal.[24–26]

T. microcarpus generally grows in the termite soil on the laterite forest soil of South Bengal, India, during

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July and August. The fruit body of this mushroom is like a small white jasmine flower. We are reporting herein the structural studies of fraction-I (PS-I) polysaccharide isolated from an aqueous extract of *T. microcarpus*.

The pure polysaccharide (PS-I) has a specific rotation of $[\alpha]_D^{25}$ +6.47 (*c* 0.80, water). The molecular weight of PS-I was estimated as $\sim 1.8 \times 10^5$ Da from a calibration curve prepared with standard dextrans.[27] Total sugar contained in PS-I was estimated by the phenol–sulfuric acid method,[28] and it was found that 85% of the total sugar was contained in this compound. Acid hydrolysis of PS-I was carried out with 2 M trifluoroacetic acid (TFA) for 18 h. Subsequent alditol acetates preparation and GLC analysis of the alditol acetates using column A (3% ECNSS M) and column B (1% OV-225) showed that only glucose was present as the monosaccharide unit. Another part of the hydrolyzed product was set aside for paper chromatographic (PC) study, which showed the presence of only glucose. The absolute configuration of the sugar was determined[29] by GLC analysis of the trimethylsilylated (+)-2-butyl glycoside derivative, and the monosaccharide was found to have the D-configuration. PS-I was methylated by the method of Ciucanu and Kerek[30] and then hydrolyzed by formic acid. The alditol acetates of the methylated product were analyzed by GLC using columns A and B and by GLC–MS using an HP-5 fused-silica capillary column and found to contain 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol (A; *m*/*z*: 87, 99, 101, 113, 117 and 129) and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol (B; *m*/*z*: 87, 99, 101, 117, 129 and 161) in a molar ratio of 1:1 (Table 1). This indicates the presence of (1→4)- and (1→3)-linked D-glucopyranosyl moieties in the glucan (PS-I). A periodate oxidation experiment was carried out with this fraction, and GLC analysis of the methylated periodate oxidized–reduced PS-I showed only one peak corresponding to 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, but no peak corresponding to 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol was obtained. This indicates that (1→3) linked D-glucopyranosyl residue is retained, and the (1→4)-linked D-glucopyranosyl unit is consumed during periodate oxidation.

The 500 MHz ^{1}H NMR spectrum (Fig. 1) of PS-I at 27 °C showed two anomeric proton signals at 5.14 ppm ($J_{H\text{-}1,H\text{-}2} \sim 3.5$ Hz) and 4.55 ppm ($J_{H\text{-}1,H\text{-}2} \sim 6.5$ Hz) in a molar ratio of 1:1. These sugar residues were designated as residue A and residue B according to their decreasing anomeric chemical shift.

The anomeric proton signal at δ 5.14 ppm and the value of the coupling constant ($J_{H\text{-}1,H\text{-}2} \sim 3.5$ Hz) of residue A indicates that it is an α-linked sugar residue. The proton chemical shifts from H-1 to H-6 were assigned from the 2D-DQF-COSY and TOCSY spectra. The anomeric chemical shift at δ 4.55 ppm and the relatively large coupling constant value ($J_{H\text{-}1,H\text{-}2} \sim 6.5$ Hz) of residue B indicates that it is a β-linked sugar residue. The proton chemical shifts from H-1 to H-6 for residue B were assigned from the DQF-COSY and TOCSY spectra (Table 2).

The 500 MHz ^{13}C NMR spectrum (Fig. 2) of PS-I at 27 °C exhibits two anomeric carbon signals at δ 98 ppm

Table 1. GLC and GLC–MS data for the alditol acetates derived from the methylated polysaccharide AQS-I isolated from *Termitomyces microcarpus*

Methylated sugars	t_R [a]	t_R [b]	Characteristic fragments (*m*/*z*)	Molar ratio	Mode of linkage
2,3,6-Me_3-Glc*p*	2.50	2.32	87, 99, 101, 113, 117, 129	1.0	→4)-Glc*p*-(1→
2,4,6-Me_3-Glc*p*	1.95	1.82	87, 99, 101, 117, 129, 161	1.0	→3)-Glc*p*-(1→

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

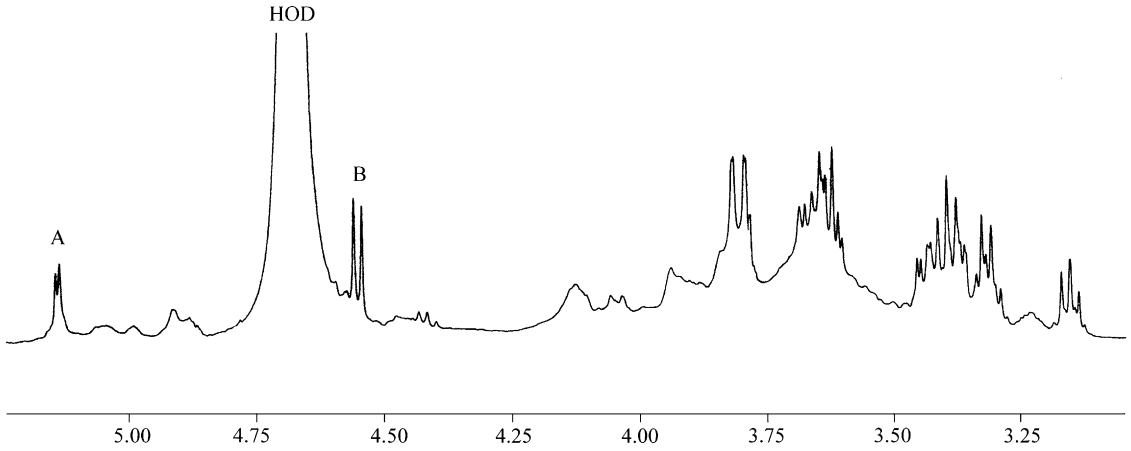

Figure 1. ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) of the polysaccharide (PS-I) isolated from *Termitomyces microcarpus*.

Table 2. The ^{1}H and ^{13}C NMR chemical shifts of PS-I isolated from *Termitomyces microcarpus* in D_2O at 27 °C

Sugar 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
→ 4)-α-D-Glc*p*-(1→	98.0	71.40	71.70	75.70	69.60	60.59
	5.14	3.44	3.62	3.42	3.60	3.80, 4.12
A						
→ 3)-β-D-Glc*p*-(1→	102.40	72.28	85.06	68.93	75.90	60.74
	4.55	3.15	3.39	3.31	3.64	3.79, 4.05
B						

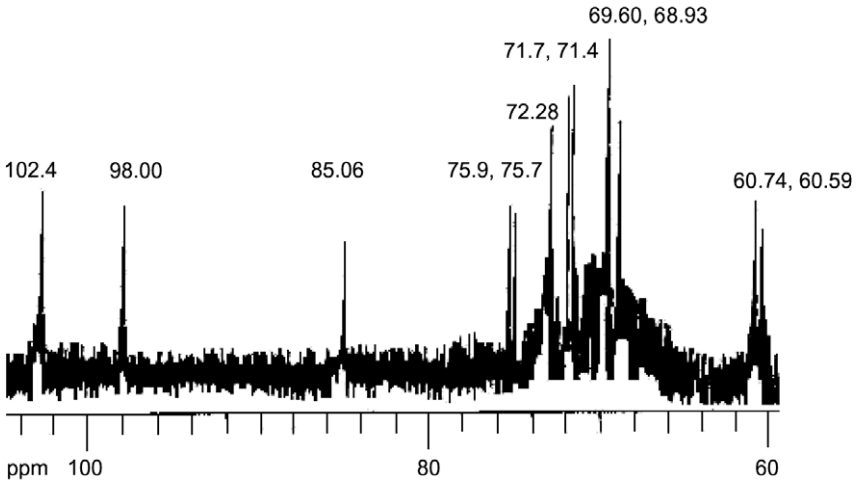

Figure 2. ^{13}C NMR spectrum (500 MHz, D_2O, 27 °C) of the polysaccharide (PS-I) isolated from *Termitomyces microcarpus*.

and δ 102.4 ppm. These anomeric carbon signals were assigned for the α-linked residue A (δ 98 ppm) and the β-linked residue B (δ 102.4 ppm), and these assignments were also corroborated by HSQC experiment. All the carbon signals (Table 2) of residue A and residue B were assigned with the help of the HSQC spectrum, and these values nearly correspond to the standard methyl glycosides.[31,32] The carbon signal at δ 71.4, 71.7, 75.7, 69.60 and 60.59 ppm correspond to the C-2, C-3, C-4, C-5 and C-6 of residue A (Table 2). The C-4 signal of residue A at δ 75.7 ppm is shifted to 5.1 ppm downfield compared to the standard methyl glycosides due to the α-glycosylation[31,32] effect. The C-3 (71.7 ppm) and C-5 (69.60 ppm) signals of residue A showed an upfield shift of 2.4 and 2.9 ppm, respectively, compared to the literature value of methyl glycosides due to the β-glycosylation effect.[31,32]

For residue B (δ 102.4 ppm), the carbon signals at δ 72.28, 85.06, 68.93, 75.9 and 60.74 ppm correspond to C-2, C-3, C-4, C-5 and C-6, respectively (Table 2). The α effect of the glycosylation of the C-3 signal of residue B appears at δ 85.06 ppm, which is shifted 8.26 ppm downfield, compared to that of standard methyl glycopyranosides.[31,32] The C-3 and C-5 signals of residue B showed an upfield shift of 1.82 and 1.73 ppm, respectively, due to the β-glycosylation effect.

The sequence of glycosyl residues was confirmed on the basis of the 2D-NOESY NMR spectra (Table 3, Fig. 3). Since the anomeric proton of residue A has a strong interresidual NOE contact with H-3 of residue B and intraresidual NOE contacts with H-2, H-3, H-6a and H-6b, it is confirmed that residue A is linked at the 3-position of residue B. On the other hand, the anomeric proton of residue B has strong NOE contact with H-4 of residue A and also intraresidual NOE contact with H-2, H-4 and H-6a. These results confirm that residue B is connected with residue A at the 4-position.

These results conclude that residue A is a (1→4)-linked α-D-glucopyranosyl residue and residue B is a (1→3)-linked β-D-glucopyranosyl residue.

Table 3. NOE effect of PS-I isolated from *Termitomyces microcarpus*, observed in the NOESY spectrum recorded in D_2O at 27 °C

Anomeric proton glycosyl residue	δ	NOE contact protons	
		δ	Residue
→ 4)-α-D-Glc*p*-(1→	5.14	3.44	A H-2
		3.62	A H-3
A		3.80	A H-6a
		4.12	A H-6b
		3.39	B H-3
→ 3)-β-D-Glc*p*-(1→	4.55	3.15	B H-2
		3.31	B H-4
B		3.79	B H-6a
		3.42	A H-4

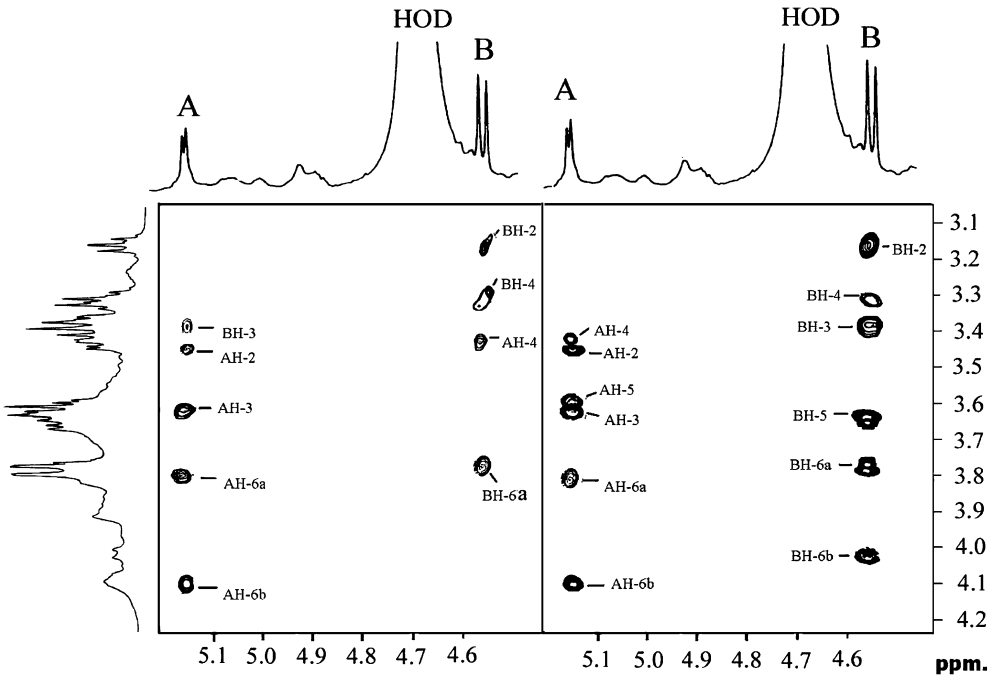

Figure 3. The NOESY (left panel) and TOCSY (right panel) spectra of PS-I isolated from *Termitomyces microcarpus.* The mixing time for the TOCSY spectrum was 150 ms. Complete assignment required several TOCSY experiments having mixing times ranging from 60 to 300 ms. The NOESY mixing time was 300 ms.

Since, the residues A and B are present in 1:1 molar ratio, the repeating unit present in PS-I of *T. microcarpus* is established as

$$\underset{\text{A}}{\rightarrow 4)\text{–}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 3)}\text{-}\underset{\text{B}}{\beta\text{-D-Glc}p\text{-}(1\rightarrow}$$

1. Experimental

1.1. Isolation and purification of the polysaccharide

The fresh fruiting bodies of *T. microcarpus* (1 kg) collected from the local market, were washed with water and allowed to boil in distilled water (250 mL) for 12 h. The mixture was kept overnight at 4 °C and filtered through fresh linen cloth. The filtrate was then centrifuged at 8000 rpm (using a Heraeus Biofuge stratos centrifuge) for 45 min at 6 °C to obtain a clear solution. The supernatant clear solution was collected (200 mL) and precipitated in ethanol (1:5, v/v) at room temperature. After keeping the precipitate at 4 °C overnight, it was collected by centrifugation at 6 °C for 1 h. Then it was dissolved in a minimum quantity of water and dialyzed through dialysis tubing (cellulose membrane, Sigma–Aldrich) for 6 h to remove low-molecular-weight materials. The solution was then freeze dried. The water-soluble polysaccharide (30 mg) was purified by gel-permeation chromatography on a Sepharose-6B column (65 × 2 cm) using water as eluant. A total of 100 test tubes containing 2 mL of eluant were collected in a Redifrac fraction collector at a flow rate of 10 s/drop. These were monitored by the phenol–sulfuric acid procedure[27] at 490 nm using a Shimadzu UV–vis Spectrophotometer, model 1601. Two fractions, test tubes number (18–42) and (50–80) were obtained. These test tubes were collected together separately and freeze dried. Fr-I was collected, yielding 9 mg. This process was repeated several times, and every time two fractions of same test tube numbers were collected.

1.2. Molecular weight determination[27]

The average molecular weight of the polysaccharide was determined by a gel-chromatographic technique performed on a Sepharose-6B gel filtration column (65 × 2 cm) eluting with distilled water at a flow rate of 10 s/drop in a Redifrac fraction collector. The elution volume of PS-I was plotted on the standard calibration curve prepared by plotting the elution volume of standard dextrans (T-40, T-70 and T-200) against the logarithm of their respective molecular weights.

1.3. Paper chromatographic studies

Paper partition chromatographic studies were performed on Whatmann nos. 1 and 3MM sheets. Solvent systems were used as (X) BuOH–HOAc–H_2O (v/v/v, 4:1:5, upper phase) and (Y) EtOAc–pyridine–H_2O

(v/v/v, 8:2:1). The spray reagent used was alkaline silver nitrate solution.[33]

1.4. Monosaccharide analysis

The polysaccharide PS-I (1.5 mg) of *T. microcarpus* was hydrolyzed by treatment with 2 M CF_3COOH (1 mL) for 18 h at 100 °C. The excess acid was removed by co-distillation with water. The hydrolyzed material was then divided into two parts: one part was set for paper chromatographic analysis, and the other part was reduced by $NaBH_4$, followed by acidification with HOAc. It was then co-distilled with MeOH to remove excess boric acid, and dried over P_2O_5. Thereafter, the whole mass was acetylated with pyridine and Ac_2O for preparing alditol acetates, and analyzed by GLC performed with a Hewlett–Packard 5810 gas chromatography equipped with a flame-ionization detector. The instrument was fitted with a glass column (1.8 m × 6 mm) packed with 3% ECNSS-M on Gas Chrom Q (100–120 mesh) at 170 °C and 1% OV-225 on Gas Chrom Q (100–120 mesh) at 170 °C.

1.5. Methylation analysis

The polysaccharide PS-I of *T. Microcarpus* was methylated using the procedure described by Ciucanu and Kerek,[30] and the product was isolated by partitioning between 5:2 $CHCl_3$–H_2O. The product contained in the organic layer was washed with water several times and dried. The methylated polysaccharide was hydrolyzed by treatment with 1 mL of 90% HCOOH (100 °C, 1.5 h), and the monosaccharides were converted into their corresponding methylated alditol acetates in the usual manner. The sugar linkages of the constituent methylated alditol acetates were analyzed by GLC using columns A and B as above and also by GLC–MS analysis, performed on a Hewlett–Packard 5988A automatic GLC–MS system 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}.

1.6. Periodate oxidation study

The PS-I (7 mg) was oxidized by 2 mL of 0.1 M $NaIO_4$ (sodium meta periodate) in the dark for 48 h at room temperature. Excess $NaIO_4$ was destroyed by addition of ethylene glycol, and the solution was dialyzed against distilled water for 3–4 h. The dialyzed product was reduced by $NaBH_4$ overnight, then neutralized with CH_3COOH. The residual material was obtained by repeated addition of CH_3OH to that solution, followed by distillation. The residue was subjected to both hydrolysis and methylation by the same process described earlier, and the product was analyzed by GLC using columns A and B.

1.7. Optical rotation

Optical rotation was measured on a Perkin–Elmer model-241 MC spectropolarimeter at 25 °C.

1.8. Colorimetric estimations

Colorimetric estimations were carried out on a Shimadzu UV–vis spectrophotometer, model 1601.

1.9. GLC experiments

All gas-liquid chromatographic analyses (GLCs) were performed on a Hewlett–Packard Model 5730 A gas chromatograph having a flame-ionization detector and glass columns (1.8 m × 6 mm) packed with 3% ECNSS-M (A) on Gas Chrom Q (100–120 mesh) and 1% OV-225 (B) on Gas Chrom Q (100–120 mesh). All GLC analyses were performed at 170 °C.

1.10. GLC–MS experiments

All GLC–MS experiments were carried out in a Hewlett–Packard 5970 MSD instrument using an 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.

1.11. NMR spectroscopy

The ^1H and ^{13}C NMR experiments were recorded at 500 and 125 MHz, respectively, on a Bruker Avance DPX-500 spectrometer using a 5-mm broad-band probe. For NMR measurements PS-I was dried in vacuum over P_2O_5 for several days, and was then exchanged with deuterium[34] by lyophilizing with D_2O for several times. The deuterium-exchanged polysaccharide (5 mg) was dissolved in 0.7 mL of D_2O (99.96% atom ^2H, Aldrich). The ^1H and ^{13}C NMR spectra were recorded at 27 °C. Acetone was used as the internal standard (δ 31.05 ppm) for the ^{13}C spectrum. The ^1H NMR spectrum was recorded by fixing the HOD signal at δ 4.67 ppm. The DQF-COSY NMR experiment was performed using standard Bruker software. The mixing times in the TOCSY and NOESY experiments were 150 ms and 300 ms, respectively.

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