Available online at www.sciencedirect.com
SCIENCE DIRECT
Carbohydrate Research 341 (2006) 878–886
Carbohydrate RESEARCH

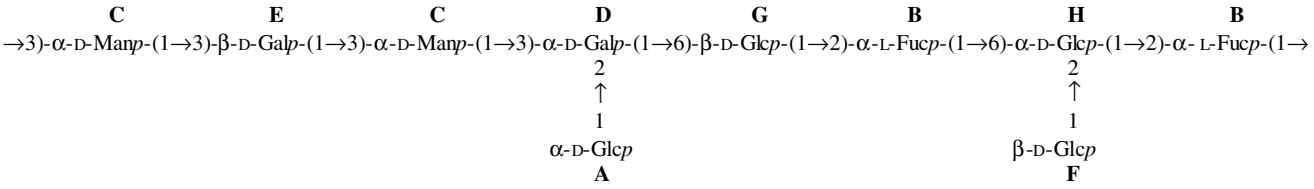
Isolation and structural elucidation of a water-soluble polysaccharide (PS-I) of a wild edible mushroom, *Termitomyces striatus*

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

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

Received 2 January 2006; received in revised form 27 January 2006; accepted 7 February 2006
Available online 6 March 2006

Abstract—A heteropolysaccharide (PS-I), isolated from the hot aqueous extract of an edible mushroom, *Termitomyces striatus*, is composed of D-glucose, D-galactose, D-mannose and L-fucose in a molar ratio 2:1:1:1. Structural investigation of the native as well as the Smith-degraded polysaccharide was carried out using methylation analysis, periodate oxidation studies and 1D and 2D NMR spectroscopy, and the repeating unit of the polysaccharide is established as follows:



Keywords: Wild edible mushroom; *Termitomyces striatus*; Polysaccharide; Smith degradation; Structure; NMR spectroscopy

1. Introduction

Different mushrooms of the genus *Termitomyces* like *Termitomyces striatus*, *T. eurhizus*, *T. microcarpus*, *T. robustus* and *T. clypeatus* have been identified as wild edible mushrooms with high nutritive value and grow abundantly in the termites guts† of the laterite forest soil in Southwest Bengal, India during September–October every year. Local people consume them as delicious food material. *T. robustus* and *T. clypeatus* contain[1] 31% protein, 32% carbohydrate and 10–14% ascorbic acid. The nutrient content of *T. robustus*[2,3] has been analyzed and found to contain free amino acids, protein, glycogen, lipid, sugar and ascorbic acid and traces minerals in varying proportions. Several enzymes of high therapeutic values have been reported from the species *T. microcarpus*[4] and *T. clypeatus*.[5–9] Water-soluble glucans from the species *T. eurhizus*[10] have been reported. Moisture, carbohydrates, proteins, fat, ash, fibre, tannins, phytate, Mg, Fe, Ca, Zn and K were determined[11] in *T. striatus* and *T. robustus*. *T. striatus* contained[12] between 17.13 and 22.93 ppm of iron, and 90.45 ppm of zinc. The biological value[13] of *T. robustus* and *T. striatus* were determined using weanling rats. No work has been reported on the polysaccharide isolated from the aqueous extract of this mushroom. In the present study, a polysaccharide fraction isolated from *T. striatus* showed splenocyte activation. Splenocytes are present in the spleen and contain T-cells, B-cells, macrophages, etc., which are responsible for enhancing the immune system of living organism. Splenocyte activation has been observed in case of a highly branched β-glucan[14] and also

* Corresponding author. Tel.: +91 03222 276558x437/03222 268387; fax: +91 03222 275329; e-mail: sirajul_1999@yahoo.com

† Termite gut is the towering structure made up of soil, fecal matter and undigested cellulose that protrudes from the soil above a termite nest. For details see Supplementary data.

0008-6215/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2006.02.004

a linear glucan.[15] In recent years mushroom polysaccharides have drawn the attention of chemists and immunobiologists on account of their immunomodulation and antitumor[16–18] properties. Therefore, a detailed investigation was carried out on the polysaccharide of this mushroom, and the results are reported herein.

2. Results and discussion

The fresh edible mushrooms, *T. striatus*, were extracted with hot water, and the supernatant was collected by centrifugation and freeze dried. The material was then precipitated in ethanol and the residue was lyophilized. It was dissolved in 4% sodium hydroxide solution and again precipitated in ethanol. The precipitated material was collected through centrifugation and dissolved in water and dialyzed through a DEAE cellulose bag for 24 h to remove alkali and low-molecular-weight materials. The whole solution was then centrifuged at 10,000 rpm at 10 °C. The residue was rejected, and the filtrate (water soluble part) was freeze dried, yielding 1.5 g of crude polysaccharide. The water-soluble polysaccharide (40 mg) was fractionated through a Sepharose 6B column in aqueous medium in several lots, and two fractions (PS-I, 18 mg and PS-II, 20 mg) of polysaccharide were obtained by monitoring the fractions via a spectrophotometer at 490 nm using the phenol–sulfuric acid method.[19] The molecular weights of both PS-I and PS-II were estimated as ~70,000 and ~30,000, respectively, from a calibration curve prepared with standard dextrans.[20] We report herein the structural characterization of PS-I along with its splenocytes activation studies.

The pure polysaccharide (PS-I) has $[\alpha]_D^{25}$ +1.85 (*c* 0.86, water). Total neutral sugars were estimated as 99.2% by the phenol–sulfuric acid method.[19] Hydrolysis of PS-I was carried out with 2 M CF_3COOH for 18 h. The analysis by PC and GLC showed the presence of L-fucose, D-mannose, D-galactose and D-glucose. The relative proportions were estimated by GLC as 1:1:1:2. The absolute configurations of the sugars were determined by the method of Gerwig et al.[21] The PS-I was methylated twice by the Ciucanu and Kerek method[22] and then hydrolyzed. The alditol acetates of methylated products were analyzed by GLC using column A and Column B (see Section 3), and they were identified by GLC–MS analysis using an HP-5 capillary column. PS-I showed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol; 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol; 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-D-glucitol; 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-mannitol; 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol; 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methylD-galactitol; 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl D-fucitol in a molar ratio 2:1:1:2:1:1:2 (Table 1). These linkages were further confirmed by a periodate oxidation experiment. The GLC analysis of alditol acetates of periodate-oxidized, reduced, and hydrolyzed product showed the presence of D-mannose and D-galactose in a molar ratio 1:1, indicating that the D-glucose and L-fucose moieties are consumed during oxidation. The methylated product of periodate-oxidized and reduced polysaccharide was hydrolyzed and acetylated. The GLC analysis of periodate-oxidized, reduced, and methylated PS-I showed the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol; 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-mannitol; 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methyl-D-galactitol in a molar ratio 1:2:1. This observation clearly indicates that the D-glucosyl moieties present in the terminal, linear chain and branch point along with L-fucose are consumed during oxidation.

The 500-MHz ^{1}H NMR spectrum (Fig. 1, Table 2) of PS-I showed eight anomeric proton signals at δ 5.17, 5.10, 5.03, 4.96, 4.94, 4.58, 4.51 and 4.48 in a molar ratio 1:2:2:1:1:1:1:1. The sugar residues were designated **A**–**H** according to their decreasing anomeric chemical shifts.

Residue **A** has an anomeric signal at δ 5.17 and $J_{H\text{-}1,H\text{-}2} \sim 3.25$ Hz, indicating that it is an α-linked residue. The proton chemical shifts from H-1 to H-6 for residue A were assigned from 2D-DQF-COSY and TOCSY spectra. The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling

Table 1. GLC and GLC–MS data for the alditol acetates derived from the methylated PS-I and Smith degraded polysaccharide (SDPS)

Compound	Methylated sugar (as alditol acetate)	T^a	T^b	Molar ratio	Mode of linkage
PS-I	2,3,4,6-Me_4-Glc*p*	1.00	1.00	2	Glc*p*-(1→
	2,3,4-Me_3-Glc*p*	2.49	2.22	1	→6)-Glc*p*-(1→
	2,4,6-Me_3-Man*p*	2.09	1.90	2	→3)-Man*p*-1→
	2,4,6-Me_3-Gal*p*	2.28	2.03	1	→3)-Gal*p*-(1→
	3,4-Me_2-Fuc*p*	0.63	0.59	2	→2)-Fuc*p*-(1→
	3,4-Me_2-Glc*p*	5.27	4.26	1	→2,6)-Glc*p*-(1→
	4,6-Me_2-Gal*p*	3.64	3.13	1	→2,3)-Gal*p*-(1→
SDPS	2,3,4,6-Me_4-Man*p*	1.00	1.00	1	Man*p*-(1→
	2,4,6-Me_3-Man*p*	2.09	1.90	1	→3)-Man*p*-1→
	2,4,6-Me_3-Gal*p*	2.28	2.03	2	→3)-Gal*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 Chrom 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 Chrom Q at 170 °C.

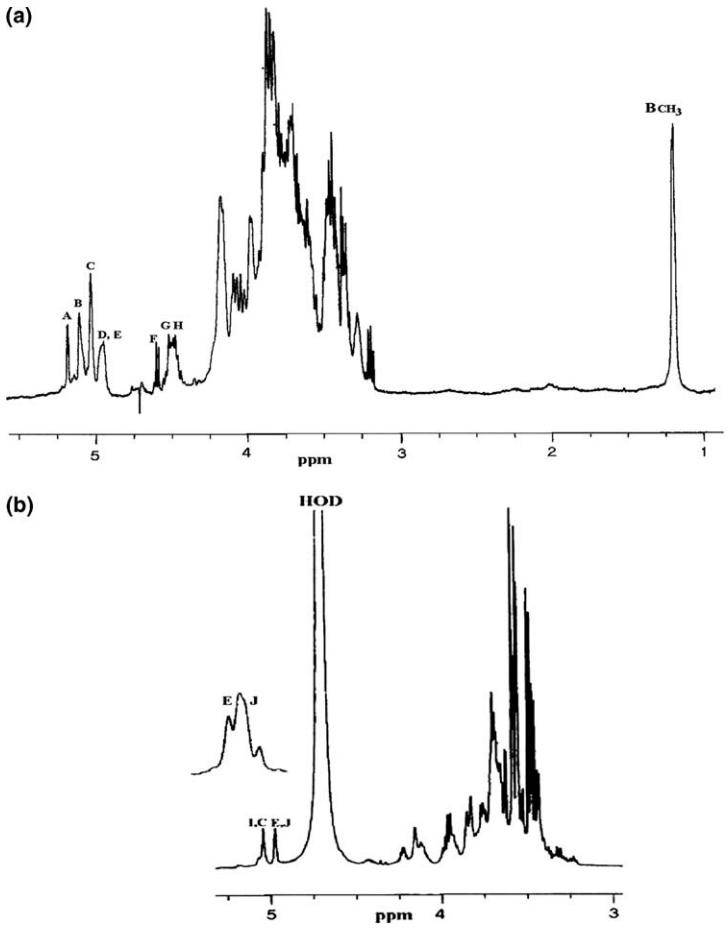


Figure 1. (a) ^{1}H NMR (500 MHz, D_2O, 27 °C) spectrum of polysaccharide, PS-I, isolated from *T. striatus*. (b) ^{1}H NMR (500 MHz, D_2O, 27 °C) spectrum of the Smith-degraded polysaccharide, SDPS-I.

Table 2. ^{1}H and ^{13}C NMR chemical shifts of polysaccharide recorded in D_2O at 27 °C for PS-I[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/C-6	H-6b
α-D-Glc*p*-(1→	5.17	3.47	3.35	3.78	3.66	3.71	3.91
A	100.01	72.14	73.93	69.92	73.15	61.15	
→2)-α-L-Fuc*p*-(1→	5.10	4.06	4.47	3.97	4.15	1.19	
B	98.29	78.49	69.23	72.14	67.40	16.11	
→3)-α-D-Man*p*-(1→	5.03	3.76	3.56	3.84	4.08	3.81	4.01
C	102.59	69.62	81.12	66.54	75.30	61.33	
→2,3)-α-D-Gal*p*-(1→	4.96	3.81	4.15	3.96	3.59	3.42	3.70
D	102.16	78.49	80.03	68.69	75.99	61.33	
→3)-β-D-Gal*p*-(1→	4.94	3.81	3.86	3.96	3.83	3.64	3.67
E	103.75	71.95	84.02	69.23	75.99	61.33	
β-D-Glc*p*-(1→	4.58	3.19	3.43	3.32	3.36	3.69	3.83
F	102.63	73.93	75.33	69.92	73.49	61.15	
→6)-β-D-Glc*p*-(1→	4.51	3.54	3.27	3.89	3.61	4.07	4.17
G	103.32	74.54	76.34	70.00	73.49	67.40	
→2,6)-β-D-Glc*p*-(1→	4.48	3.68	3.28	3.89	3.60	3.98	4.17
H	103.00	78.92	71.95	68.69	70.00	67.30	

[a,b]Interchangeable.

[c] Values of proton chemical shifts were taken with respect to the HOD signal fixed at δ 4.67 ppm, and carbon chemical shifts were recorded with reference to acetone as internal standard, fixed at δ 31.05 ppm at 27 °C.

constants (9–10 Hz) were observed for residue **A** and indicated that it is an α-D-glucosyl residue.

The anomeric signal for residue **B** is δ 5.10 ($J_{H\text{-}1,H\text{-}2} \sim$ 2.7 Hz) showing that it is an α-linked residue. The proton chemical shifts from H-1 to H-6 for residue **B** were assigned from 2D-DQF-COSY and TOCSY spectra. A proton chemical shift for the methyl group at δ 1.19 and the relatively small $J_{H\text{-}3,H\text{-}4}$ coupling constant (<3 Hz) indicate that it has an α-L-fuco-configuration.

Residue **C** has an anomeric signal at δ 5.03. The proton chemical shifts from H-1 to H-6 for residue **C** were assigned from 2D-DQF-COSY and TOCSY spectra. A relatively small coupling constant value of H-1, H-2 and H-2, H-3 and a large coupling constant value of H-3, H-4 indicate that residue **C** is of the α-D-mannosyl configuration.

The anomeric chemical shift for residue **D** is δ 4.96 ($J_{H\text{-}1,H\text{-}2} \sim$ 3.35 Hz), indicating that it is an α-linked residue. The proton chemical shifts from H-1 to H-6 for residue **D** were assigned from 2D-DQF-COSY and TOCSY spectra. The relatively small coupling constant $J_{H\text{-}3,H\text{-}4}$ value for residue **D** shows that it is an α-D-galactosyl residue.

Residue **E** has an anomeric chemical shift at δ 4.94 and a large coupling constant $J_{H\text{-}1,H\text{-}2}$ value (~8.4 Hz), indicating that it is a β-linked residue. The chemical shifts from H-1 to H-6 for residue **E** were assigned from 2D-DQF-COSY and TOCSY spectra. The $J_{H\text{-}3,H\text{-}4}$ value (<5 Hz) for residue **E** indicates that it is a β-D-galactosyl residue.

The anomeric chemical shift for residue **F** is δ 4.58, and it has a large coupling constant for $J_{H\text{-}1,H\text{-}2}$ (7.9 Hz), indicating that it is a β-linked residue. The proton chemical shifts from H-1 to H-6 for residue **F** were assigned from DQF-COSY and TOCSY spectra. The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ values (9–10 Hz) for residue **F** were observed, indicating that it is a β-D-glucosyl residue.

Residue **G** has an anomeric chemical shift at δ 4.51 ($J_{H\text{-}1,H\text{-}2} \sim$ 7.5 Hz) showing that it is a β-linked residue. The proton chemical shifts from H-1 to H-6 for residue **G** were assigned from DQF-COSY and TOCSY spectra. Large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ values (9–10 Hz) for residue **G** were observed, indicating that it is a β-D-glucosyl residue.

The anomeric chemical shift for residue **H** is δ 4.48, and a large coupling constant $J_{H\text{-}1,H\text{-}2}$ (~8 Hz) indicates that it is a β-linked residue. The proton chemical shifts from H-1 to H-6 for residue **H** were assigned from DQF-COSY and TOCSY spectra. The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ values (9–10 Hz) for residue **H** were observed, indicating that it is a β-D-glucosyl residue.

The 125-MHz ^{13}C NMR spectrum (Fig. 2) of PS-I exhibited eight anomeric carbon signals at δ 103.75, 103.32, 103.00, 102.63, 102.59, 102.16, 100.01 and 98.29. All of the carbon signals of the sugar residues of PS-I (Table 2) were assigned by comparing their spectra with those of standard methyl glycosides,[23] and these assignments were also corroborated by an HSQC experiment.

The sequence of glycosyl residues for PS-I were determined from a NOESY experiment (Table 3). Residue **A** has a NOE contact from H-1 to H-2 of residue **D**, in addition to intraresidue NOE contacts to H-3, H-5, H-6a and H-6b and an interresidue contact to H-3 of residue **D**. Since residue **A** is linked at the 2-position of residue **D**, the following sequence is established:

α-D-Glc*p*-(1→2)-α-D-Gal*p*-(1→
A **D**

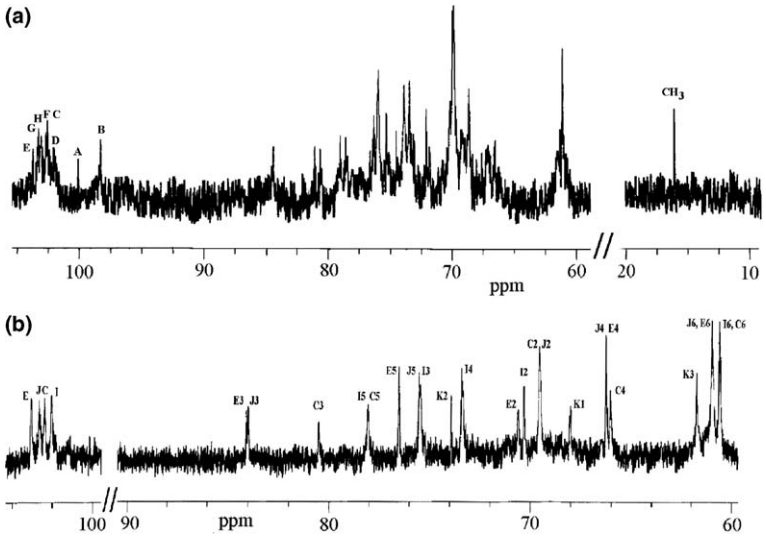


Figure 2. (a) ^{13}C NMR (125 MHz, D_2O, 27 °C) spectrum of polysaccharide, PS-I, isolated from *T. striatus*. (b) ^{13}C NMR (125 MHz, D_2O, 27 °C) spectrum of the Smith-degraded polysaccharide, SDPS-I.

Table 3. NOE effects of PS-I, observed in the NOESY spectrum recorded in D_2O at 27 °C

Unit	NOE signals		
	From	Intraunit	Interunit
A	**A** H-1	**A** H-4, **A** H-5, **A** H-6	**D** H-2, **D** H-3
B	**B** H-1	**B** H-2, **B** H-4, **B** H-5	**C** H-3, **C** H-2, **H** H-6a, **H** H-6b
C	**C** H-1	**C** H-2, **C** H-3, **C** H-5, **C** H-6	**D** H-3, **D** H-4, **E** H-3, **E** H-2
D	**D** H-1	**D** H-3, **D** H-4	**G** H-6a, **G** H-6b
E	**E** H-1	**E** H-2, **E** H-6a, **E** H-6b	**D** H-3
F	**F** H-1	**F** H-3, **F** H-5, **F** H-6	**H** H-2, **H** H-3
G	**G** H-1	**G** H-2, **G** H-4, **G** H-6a, **G** H-6b	**B** H-2
H	**H** H-1	**H** H-2, **H** H-6a, **H** H-6b	**B** H-2, **B** H-4

Residue **B** has strong NOE contacts from H-1 to H-3 of residue **C** and H-6 of residue **H** in addition to intraresidue NOE contacts to H-2, H-4, H-5 and a medium interresidue contact to H-2 of residue **C**. So residue **B** is linked at the 3-position of residue **C** and at the 6-position of residue **H**, indicating the following sequences:

α-L-Fuc*p*-(1→3)-α-D-Man*p*-(1→
B **C**

and

α-L-Fuc*p*-(1→6)-β-D-Glc*p*-(1→
B **H**

Two NOE contacts of residue **C** are observed from H-1 to H-3 of residue **D** and H-3 of residue **E**, with intraresidue NOE contacts to H-2, H-6 and interresidue contacts to H-2 of both residues **D** and **E**. From this point of view it is indicated that residue **C** is linked to the 3-position of residue **D** and 3-position of residue **E**, and the following sequences are established:

→3)-α-D-Man*p*-(1→3)-α-D-Gal*p*-(1→
C **D**

and

→3)-α-D-Man*p*-(1→3)-β-D-Gal*p*-(1→
C **E**

Residue **D** has a NOE contact from H-1 to H-6a and H-6b of residue **G**, in addition to intraresidue NOE contacts to H-2, H-4, H-6a and H-6b, indicating that the residue **D** is linked at the 6-position of residue **G**. Thus the following sequence is established:

→2, 3)-α-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→
D **G**

An NOE contact of residue **E** is observed from H-1 to H-3 of residue **C** in addition to intraresidue contact to H-3. Since residue **E** is linked at the 3-position of residue **C**, and the following sequence is established:

→3)-β-D-Gal*p*-(1→3)-α-D-Man*p*-(1→
E **C**

Now residue **F** has an NOE contact from H-1 to H-2 of residue **H** in addition to an intraresidue contact to H-6. So, residue **F** is linked at the 2-position of residue **H**, indicating the following sequence:

β-D-Glc*p*-(1→2)-β-D-Glc*p*-(1→
↑6
F **H**

Residues **G** and **H** both have a strong NOE contact from H-1 to H-2 of residue **B**, which indicate that both residues **G** and **H** are linked at the 2-position of residue **B**. Hence the following sequences are assigned:

→6)-β-D-Glc*p*-(1→2)-α-L-Fuc*p*-(1→
G **B**

and

→6)-β-D-Glc*p*-(1→2)-α-L-Fuc*p*-(1→
↑2
H **B**

In order to obtain information on the sequence of the sugar residues in the repeating unit, the PS-I was subjected to Smith degradation[24] studies, and the products were separated on a Sephadex G-25 column using water as the eluant, resulting in one fraction (SDPS). GLC analysis of the alditol acetates of the acid-hydrolyzed product from SDPS showed the presence of D-mannose, D-galactose and glycerol in a molar ratio 2:2:1. The alditol acetates of the methylated product from SDPS were analyzed by GLC using column A and column B, and these methylated sugars were also identified by GLC–MS analysis using an HP-5 capillary column. SDPS showed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol; 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-mannitol; 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol in a molar ratio 1:1:2 (Table 1). The 500-MHz ^{1}H NMR spectrum (Table 4, Fig. 1) of SDPS showed three anomeric signals at δ 5.03, 4.97 and 4.95 in a molar ratio 2:1:1. The proton signals from H-1 to H-6 of SDPS were assigned from DQF-COSY and TOCSY NMR experiments. The anomeric signals at δ 5.03 ($J_{\text{H-1,H-2}} < 2$ Hz) correspond to both α-D-Man*p*-(1→ (residue **I**) and →3)-α-D-Man*p*-(1→ (residue **C**). The anomeric signals at 4.97 ($J_{\text{H-1,H-2}} \sim 3$ Hz), and

Table 4. ^{1}H and ^{13}C NMR chemical shifts of Smith degraded polysaccharide (SDPS) of PS-I recorded in D_2O at 27 °C[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/C-6	H-6b
α-D-Man*p*-(1→	5.03	3.76	3.56	3.96	4.15	3.81	4.08
I	102.05	70.44	75.51	73.42	78.07	60.60	
→3)-α-D-Man*p*-(1→	5.03	3.76	3.56	3.96	4.15	3.81	4.08
C	102.40	69.54	81.25	66.04	78.07	60.60	
→3)-α-D-Gal*p*-(1→	4.97	3.80	3.89	3.97	3.84	3.64	3.67
J	102.66	69.54	84.04	66.26	75.51	60.97	
→3)-β-D-Gal*p*-(1→	4.95	3.80	3.86	3.97	3.84	3.64	3.67
E	103.08	70.51	84.16	66.26	76.52	60.97	
	4.21	3.73	3.67				
Gro-(3→	3.95		3.52				
K	67.98	73.93	61.86				

[a,b]Interchangeable.

[c] Values of proton chemical shifts were taken with respect to the HOD signal fixed at δ 4.67 ppm and values of carbon chemical shifts were recorded with reference to acetone as internal standard, fixed at δ 31.05 ppm at 27 °C.

4.95 ($J_{H\text{-}1,H\text{-}2} \sim 7$ Hz) correspond to →3)-α-D-Gal*p*-(1→ (residue **J**) and →3)-β-D-Gal*p*-(1→ (residue **E**), respectively. The 125-MHz ^{13}C NMR experiment (Table 4, Fig. 2) showed four anomeric carbon signals at δ 102.05, 102.40, 102.66 and 103.08 corresponding to α-D-Man*p*-(1 →; →3)-α-D-Man*p*-(1 →; →3)-α-D-Gal*p*-(1→ and →3)-β-D-Gal*p*-(1→ residues, respectively. The carbon signals C-1, C-2 and C-3 of the glycerol moiety were assigned as 67.98, 73.93 and 61.86 ppm, respectively. The NOESY spectrum of SDPS showed that the signal at δ 5.03 (residues **I** and **C**) have two strong interresidue NOE contacts from H-1 to H-3 of both the residues **E** and **J**. The residue **E** showed one strong NOE contact from H-1 to H-3 of residue **C**. Residue **J** has NOE contact from H-1 to H-3a and H-3b of the glycerol moiety (Gro, residue **K**). From these observations one concludes that the nonreducing end α-D-Man*p* unit (residue **I**) is produced during Smith degradation and is generated from (1→3)-α-D-Man*p* (residue **C**) due to the total consumption of the (1→2)-α-L-Fuc*p* (residue **B**). And also (1→3)-α-D-Gal*p* (residue **J**) is produced from the (1→2,3)-α-D-Gal*p* (residue **D**) due to oxidation, followed by Smith degradation of the nonreducing end of the α-D-Glc*p* moiety (residue **A**). The (1→3)-α-D-Gal*p* residue (residue **J**) is attached to a glycerol moiety (residue **K**), which is generated from the (1→6)-β-D-Glc*p* (residue **G**) after IO_4^- oxidation, followed by Smith degradation. But the moieties (1→3)-α-D-Man*p* (residue **C**) and (1→3)-β-D-Gal*p* (residue **E**) of the native polysaccharide (PS-I) are retained during the Smith degradation experiment. Hence Smith degradation results in the formation of one oligosaccharide unit (SDPS) from the parent polysaccharide PS-I, and the structure is established as

I **E** **C** **J** **K**
α-D-Man*p*-(1→3)-β-D-Gal*p*-(1→3)-α-D-Man*p*-(1→ 3)-α-D-Gal*p*-(1→3)-Gro

Therefore, on the basis of above results, it is concluded that PS-I has the following structure:

C **E** **C** **D** **G** **B** **H** **B**
→3)-α-D-Man*p*-(1→3)-β-D-Gal*p*-(1→3)-α-D-Man*p*-(1→3)-α-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→2)-α-L-Fuc*p*-(1→6)-α-D-Glc*p*-(1→2)-α-L-Fuc*p*-(1→
2 2
↑ ↑
1 1
α-D-Glc*p* β-D-Glc*p*
A **F**

The splenocyte activation test was carried out in mouse cell culture medium with the polysaccharide fraction PS-I by the MTT method.[14] Different concentrations (1, 10, 100 ng/mL, 1 and 10 μg/mL) of the PS-I were prepared with PBS solution, and the experiment was performed (see Section 3.9). The percentage (%) viability was calculated in comparison to control as 100% viable from the observed optical density at 570 nm. It was found that 1, 10, 100 ng/mL, 1 and 10 μg/mL concentration of the polysaccharide showed 122.4%, 126%, 133%, 251% and 255% viable activation, respectively. It was observed that the native polysaccharide, PS-I, showed strong splenocyte activation at a dose of 10 μg/mL. It is noteworthy to mention here that several

mushroom polysaccharides[25–27] have also shown a similar kind of splenocyte activation.

3. Experimental

3.1. General methods

All evaporations were carried out at 40 °C. Paper chromatography was performed on Whatman 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 silver nitrate solution.[28] All solvents (except D_2O) were distilled before use. Optical rotations were measured with a Jasco P 1020 automatic polarimeter at 25 °C. Gel filtrations were performed on columns of Sepharose 6B and Sephadex G-25 using a Redifrac fraction collector. Colorimetric estimations were conducted with a Shimadzu UV–vis spectrophotometer, model 1601. Gas–liquid chromatography (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 program from 150 °C (2 min) to 200 °C (5 min) at 2 °C min^{-1}.

3.2. Isolation and purification of the polysaccharide

The fruit body of the mushroom, *T. striatus* (2.0 kg) was collected from the local forest and washed with water. It was swollen in 250 mL of distilled water and boiled for 5 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 100 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 EtOH (1:5, v/v). It was kept overnight at 4 °C and then centrifuged at 10,000 rpm at 10 °C for 1 h. The precipitated material (polysaccharide) was washed with EtOH four to five times and then dried. This process was carried out thrice for better purification of polysaccharide. The dried polysaccharide was then dissolved in 4% NaOH solution and dialyzed through a DEAE cellulose bag against distilled water for 24 h to remove alkali and low-molecular-weight materials. The solution was centrifuged at 10,000 rpm at 10 °C for 45 min, and the water-soluble part was lyophilized (yield, 1.5 g).

The water-soluble polysaccharide (40 mg) was purified through a Sepharose 6B gel-permeation column (90 × 2.1 cm) using water as the eluant with a flow rate of 0.5 mL min^{-1}. A total of 120 test tubes (2 mL each) were collected and monitored spectrophotometrically at 490 nm using the phenol–sulfuric acid method.[19] Two homogeneous fractions, PS-I (test tubes 34–56, yield 18 mg) and PS-II (test tubes 65–95, yield 20 mg) were collected and freeze dried. This purification process was carried out in ten lots, and each individual fraction was again purified and collected; PS-I (yield, 144 mg) and PS-II (yield, 160 mg).

3.3. Determination of molecular weight[20]

The molecular weights of PS-I and PS-II were determined by a gel chromatographic technique. Standard dextrans T-70, T-40 and T-10 were passed through a Sepharose 6B 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 polysaccharide PS-I (1.5 mg) was hydrolyzed separately with 2 M CF_3COOH (1 mL) 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 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 HOAc and evaporation. Pure CH_3OH was then repeatedly evaporated from the residue. 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).

3.5. Absolute configuration of the monosaccharides

The method used was based on that of Gerwig et al.[21] After trifluoroacetic acid hydrolysis of 1.5 mg of the native polysaccharide (PS-I), the acid was removed by co-distillation with water. A solution of 250 μL of 0.625 M HCl in (+)-2-butanol was added to it, and the mixture was heated at 80 °C for 16 h. The reactants were then evaporated, and per-*O*-TMS-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) with a temperature program (3 °C/min) from 150 to 210 °C. The (+)-2-butyl 2,3,4,6-tetra-*O*-TMS-glycosides thus obtained were identified by comparison with those prepared from the D- and L-enantiomers of the monosaccharides.

3.6. Methylation analysis

PS-I (4.0 mg) was methylated using the method of Ciucanu and Kerek.[22] The methylated products were isolated by partition between $CHCl_3$ and H_2O (5:1, v/v). The product showed no band in the region 3600–3300 cm^{-1} in the IR spectrum. The product was then hydrolyzed with 90% HCO_2H for 1 h, and excess HCO_2H 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 (using columns A and B) and GLC–MS (using HP-5 fused silica capillary column).

3.7. Periodate oxidation study

PS-I (5 mg) was added to 1.5 mL 0.1 M $NaIO_4$ 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 HOAc and evaporation. The residue was 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,[22] and the alditol acetates of the methylated product were prepared and analyzed by GLC using columns A and B.

3.8. Smith degradation[24]

The native polysaccharide (PS-I, 28 mg) was oxidized with 0.1 M $NaIO_4$ (5 mL) at 25 °C in the dark during 72 h. The excess periodate was destroyed by adding ethylene glycol, and the solution was dialyzed against distilled water for 45 min. The dialyzed material was reduced with $NaBH_4$ (20 mg) for 16 h at 25 °C, neutralized with 50% HOAc and again dialyzed against distilled water and freeze dried. This portion was subjected to mild hydrolysis with 0.5 M trifluoroacetic acid for 18 h at 25 °C to eliminate the residues of oxidized sugars attached to polysaccharide chain (Smith degradation). The excess acid was removed after repeated addition and evaporation of water. It was further purified by passing through a Sephadex G-25 column, and one fraction was obtained and freeze dried; yield 9 mg.

3.9. NMR studies

^1H NMR and ^{13}C NMR experiments were performed with a Bruker Avance DPX-500 instrument at 27 °C. The native polysaccharide (PS-I) and Smith-degraded polysaccharide (SDPS) were dried over P_2O_5 in vacuum for several days and then deuterium exchanged[29] four times, followed by lyophilization with D_2O (99.96% atom ^2H, Aldrich). The ^1H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.67 ppm) using the WEFT pulse sequence.[30] The 2D-DQF-COSY experiment was carried out using standard pulse sequences at 27 °C. The TOCSY experiment was recorded at mixing time 60 to 300 ms, and the NOESY mixing delay was 200 ms. The ^{13}C NMR experiments of PS-I and SDPS were carried out taking acetone as the internal standard, fixing the methyl carbon signal at δ 31.05 ppm using D_2O as the solvent.

3.10. Splenocyte activation test by the MTT method[14]

A homogeneous spleen cell proliferation in RPMI (Roswell Park Memorial Institute) complete medium was prepared. The cell concentration was adjusted to $10^6 \times$ cells/mL. The above cell suspension (180 mL) was plated in a 96-well flat-bottom cell-culture plate. Sample (20 μL, concentration range of 1 ng–10 μg/mL) was added to each cell. All samples were checked in triplicate. The plate was incubated for 72 h in a 5% CO_2, 37 °C humidified CO_2 incubator. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was prepared in PBS (phosphate buffer solution) at 5 mg/mL concentration. After 72 h of incubation, 20 μL of the above MTT solution was added to each well including the control. It was further incubated for 8 h as above. Thereafter the supernatant was aspirated carefully from each well, and the fromazan crystals were dissolved in 100 μL of Me_2SO. The plate was read at 570 nm. Then the % viability was calculated in comparison to control, taking control as 100% viable.

Acknowledgements

The authors are grateful to Dr. S. Roy, Director, IICB and Dr. S. Lahiri, IACS, Kolkata, for providing instrumental facilities and discussion. Mr. B. Majumder of Bose Institute, Kolkata is acknowledged for technical assistance in running the NMR experiments. The authors further acknowledge Dr. T. K. Maity, IIT, Kharagpur, for studying the biological activity. Soumitra Mondal (one of the authors) thanks CSIR, New Delhi, for providing a senior research fellowship.

Supplementary material

Pictures of termite guts and examples of the mushrooms are provided in Supplementary data. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2006.02.004.

References

1. Ogundana, S. K.; Fagade, O. E. *Food Chem.* **1982**, *8*, 263–268.
2. Alofe, F. V. *J. Food Compos. Anal.* **1991**, *4*, 167–174.
3. Fasidi, I. O.; Kadiri, M. *Nahrung* **1990**, *34*, 415–420.
4. Skeeleton, G. S.; Matanganyidze, C. *Lett. Bot.* **1981**, *3*, 143–149.
5. Khowala, S.; Sengupta, S. *Enzyme Microb. Technol.* **1992**, *14*, 144–149.
6. Rouland, C.; Matoub, M.; Mora, P.; Petek, F. *Carbohydr. Res.* **1992**, *233*, 273–278.
7. Khowala, S.; Ghosh, A. K.; Sengupta, S. *Appl. Microbiol. Biotechnol.* **1992**, *37*, 287–292.
8. Sengupta, S.; Ghosh, A. K. *Biochim. Biophys. Acta* **1991**, *1076*, 215–220.
9. Ghosh, A. K.; Sengupta, S. *J. Biol. Sci.* **1987**, *11*, 275–285.
10. Mondal, S.; Chakraborty, I.; Pramanik, M.; Rout, D.; Islam, S. S. *Carbohydr. Res.* **2004**, *339*, 1135–1140.
11. Ola, F. L.; Oboh, G. *Nahrung* **2001**, *45*, 67–68.
12. Alofe, F. V.; Odeyemi, O.; Oke, O. L. *Plant Foods Human Nutr.* **1996**, *49*, 63–73.
13. Adewusi, S. R. A.; Alofe, F. V.; Odeyemi, O.; Folabi, A. O.; Oke, O. L. *Plant Foods Human Nutr.* **1993**, *43*, 115–121.
14. Ohno, N.; Saito, K.; Nemoto, J.; Kaneko, S.; Adachi, Y.; Nishijima, M.; Miyazaki, T.; Yadomae, T. *Biol. Pharm. Bull.* **1993**, *16*, 414–419.
15. Chakraborty, I.; Mondal, S.; Pramanik, M.; Rout, D.; Islam, S. S. *Carbohydr. Res.* **2004**, *339*, 2249–2254.
16. Borchers, A. T.; Stern, J. S.; Hackman, R. M.; Keen, C. L.; Gershwin, M. E. *Proc. Soc. Exp. Biol. Med.* **1999**, *221*, 281–293.
17. Wasser, S. P.; Weis, A. L. *Crit. Rev. Immunol.* **1999**, *19*, 65–96.
18. Leung, Y. M.; Fung, K. P.; Choy, Y. M. *Immunopharmacology* **1997**, *35*, 255–263.
19. York, W. S.; Darvill, A. K.; McNeil, M.; Stevenson, T.T.; Albersheim, P. *Methods Enzymol.* **1985**, *118*, 33–40.
20. Hara, C.; Kiho, T.; Tanaka, Y.; Ukai, S. *Carbohydr. Res.* **1982**, *110*, 77–87.
21. Gerwig, G. J.; Kamerling, J. P.; Vilegenthart, J. F. G. *Carbohydr. Res.* **1978**, *62*, 349–357.
22. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.
23. Agrawal, P. K. *Phytochemistry* **1992**, *31*, 3307–3330.
24. Abdel-Akher, M.; Hamilton, J. K.; Montgomery, R.; Smith, F. *J. Am. Chem. Soc.* **1952**, *74*, 4970–4971.
25. Kim, H. M.; Han, S. B.; Oh, G. T.; Kim, Y. H.; Hong, D. H.; Hong, N. D.; Yoo, I. D. *Int. J. Immunopharmacol.* **1996**, *18*, 295–303.
26. Leung, M. Y.; Fung, K. P.; Choy, Y. M. *Immunopharmacology* **1997**, *35*, 255–263.
27. Liu, F.; Ooi, V. E.; Fung, M. C. *Life Sci.* **1999**, *64*, 1005–1011.
28. Hoffman, J.; Lindberg, B.; Svensson, S. *Acta Chem. Scand.* **1972**, *26*, 661–666.
29. Dueñas Chasco, M. T.; Rodriguez-Carvajal, M. A.; Mateo, P. T.; Franko-Rodriguez, G.; Espartero, J. L.; Iribas, A. I.; Gil-serrano, A. M. *Carbohydr. Res.* **1987**, *303*, 453–458.
30. Hård, K.; Zadelhoff, G. V.; Moonen, P.; Kamerling, J. P.; Vilegenthart, J. F. G. *Eur. J. Biochem.* **1992**, *209*, 895–915.