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Note

Isolation and characterization of a heteroglycan from the fruits of *Astraeus hygrometricus*

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Abstract—A water-soluble polysaccharide was isolated from an alkaline extract of the fruits of the ectomycorrhizal fungus, *Astraeus hygrometricus*. It was found to contain D-mannose, D-glucose, and L-fucose in a molar ratio of 1:2:1. On the basis of total hydrolysis, methylation analysis, periodate oxidation, Smith degradation, and NMR studies (^{1}H, ^{13}C, DQF-COSY, TOCSY, NOESY, ROESY, HMQC, and HMBC) the structure of the repeating unit of the polysaccharide was established as

$$\begin{array}{l}\rightarrow 3)\ \alpha\text{-D-Man}p\ (1\rightarrow 4)\ \beta\text{-L-Fuc}p\ (1\rightarrow 6)\ \beta\text{-D-Glc}p\ (1\rightarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 6 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \uparrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 1 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\ \alpha\text{-D-Glc}p\end{array}$$

Keywords: *Astraeus hygrometricus*; Ectomycorrhiza; Polysaccharide; Structure; NMR spectroscopy

Mushroom polysaccharides have attracted the attention of chemists and immunobiologists in recent years for their immunomodulating, and antitumor properties.[1–4] Several linear[5,6] and branched glucans[7–11] and heteroglucans[12–14] isolated from higher basidiomycetes exert strong immunostimulating and antitumor activity. Other heteropolysaccharides like arabinogalactan,[15] glucogalactan,[16] fucogalactan,[17] fucomannogalactan,[18] mannogalactofucan[19] show similar properties.

Astraeus hygrometricus is an ectomycorrhizal fungus,[20] which grows in association with the roots of Chir Pine (*Pinus roxburghii*) and Sal (*Shorea robusta*) trees. It helps plants in extracting nutrients, especially phosphorus, from very slightly soluble soil minerals and organic substances. During the start of the rainy season (nearly 1st week of June) the fruit bodies of the mushroom develop along the root of these plants just beneath the soil layer in the laterite forest soil of South Bengal, and local people consume them as delicious food. Two fractions were isolated from an aqueous extract of *A. hygrometricus* and detailed structural studies were carried out in our laboratory and reported[21,22] in this journal. In the present study, a heteroglycan (PS) was isolated from an alkaline extract of the mushroom fruits, and its structure is reported therein. Its molecular weight was estimated as 1.6×10^5 Da from the calibration curve prepared with standard dextran,[23] and the specific rotation was determined as $[\alpha]_D^{25}$ −6.0 (*c* 0.66, water). The composition of the PS was identified from the acid-hydrolyzed product by GLC analysis. This analysis showed that PS consists of D-mannose, D-glucose, L-fucose in a molar ratio 1:2:1, respectively. The absolute configuration of the sugars was determined by the method of Gerwig et al.[24]

The mode of linkage of the PS polysaccharide was determined by methylation analysis using the method

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of Ciucanu and Kerek[25] followed by hydrolysis and conversion into alditol acetates. The alditol acetates were analyzed and identified by GLC–MS, which revealed the presence of 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-mannitol, 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-glucitol, and 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-fucitol in a molar ratio 1:1:1:1. These types of linkage were further confirmed by periodate oxidation. The GLC experiment was also carried out on the alditol acetates of the periodate oxidized, reduced, and hydrolyzed product which showed the presence of only D-mannose, thus allowing to conclude that D-glucose, and L-fucose moieties were consumed during oxidation. This result was further confirmed by GLC–MS analysis of the methylated periodate oxidized reduced polysaccharide which showed the presence of 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-mannitol only.

^{1}H (500 MHz) and ^{13}C NMR (125 MHz) experiments were carried out in D_2O at 27 °C. The ^{1}H NMR spectrum (Fig. 1, Table 1) showed three signals in the anomeric region at δ 5.07, 4.99, 4.53 ppm in a ratio of 1:1:2 but expansion of the peak at δ 4.53 ppm showed two overlapped signals at δ 4.53 and 4.52 ppm (Fig. 1). The sugars were assigned as **A**, **B**, **C**, and **D** according to their decreasing value of proton signals. In the ^{13}C NMR spectrum (Fig. 2, Table 1) four anomeric carbon signals were observed at δ 103.4, 103.3, 101.7, 99.1 ppm. All the proton and carbon signals were assigned from TOCSY, DQF-COSY, and HMQC NMR experiments. Since a small $^{3}J_{1,2}$ value for the D-mannosyl residue does not allow to conclude about the anomeric configuration, a proton-coupled ^{13}C NMR experiment was carried out.

The anomeric proton signal of residue **A** at δ 5.07 ppm with low values of $^{3}J_{1,2}$, $^{3}J_{2,3} \sim 3.5$ Hz, and

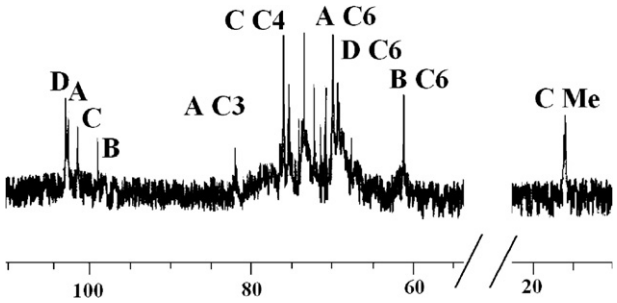


Figure 2. ^{13}C NMR spectrum (125 MHz, D_2O, 27 °C) of PS.

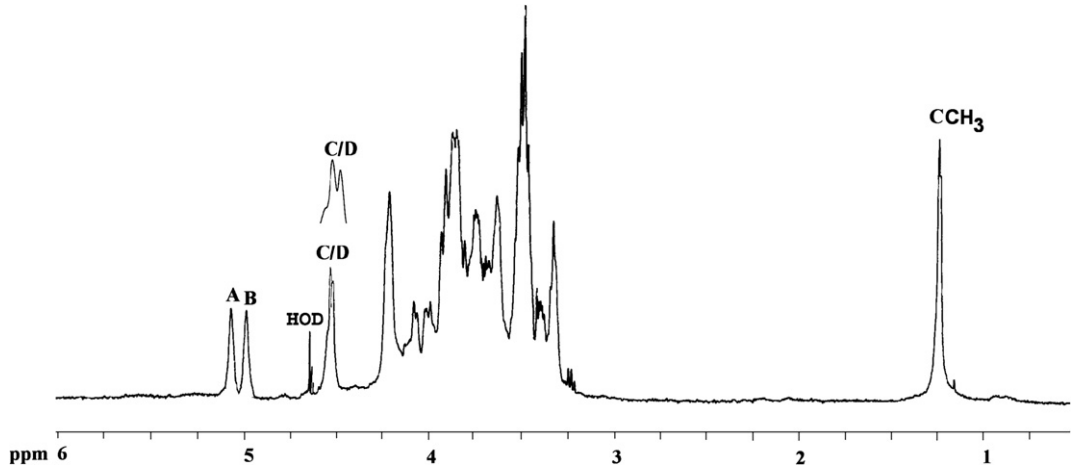


Figure 1. ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) of the PS isolated from *Astraeus hygrometricus*.

Table 1. The ^{1}H and ^{13}C NMR chemical shifts of PS isolated from *Astraeus hygrometricus*

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
→3,6)-α-D-Man*p*(1→ **A**	5.07 103.3	3.81 70.7	3.63 82.0	4.08 67.7	3.90 72.2	3.85 69.9	4.21
α-D-Glc*p*-(1→ **B**	4.99 99.1	3.87 71.1	4.00 74.1	3.77 70.8	3.61 72.7	3.65 61.2	3.25
→4)-β-L-Fuc*p*-(1→ **C**	4.53 101.7	3.51 72.7	3.33 72.9	3.76 76.0	3.93 69.3	1.23 16.0	
→6)-β-D-Glc*p*-(1→ **D**	4.52 103.4	3.33 73.5	3.49 76.0	3.79 69.9	3.93 75.3	4.22 69.3	3.86

$J_{C,H} \sim 171$ Hz clearly indicates that it is an α-linked D-mannosyl moiety. This was further confirmed from the large coupling constant values of $^3J_{3,4}$ (~7.5 Hz) and $^3J_{4,5}$ (~10 Hz). The downfield shifts of C-3 (δ 82.0 ppm) and C-6 (δ 69.9 ppm) signals with respect to standard values for methyl glycosides[26,27] confirm that **A** is (1→3, 6)-linked.

Residue **B** was confirmed as a terminal α-D-glucosyl moiety. The anomeric signals at δ 4.99 ppm, with small coupling constants $^3J_{1,2} \sim 3.2$ Hz and $^2J_{C,H} \sim 171$ Hz supported that it is an α-linked unit. Again large coupling constants for $^3J_{2,3}$ and $^3J_{3,4}$ (~10 Hz) observed in the DQF-COSY spectrum also support that it is an α-D-glucosyl moiety. Thus, from the results of methylation and NMR experiments, it may be concluded that residue **B** is an α-glycosidically linked, terminal α-D-glucosyl moiety.

Residue **C** was assigned as a (1→4)-linked β-L-fucosyl moiety. This is strongly supported by the appearance of a proton signal at δ 1.23 ppm and a carbon signal at δ 16.0 ppm indicating the presence of CH_3 group. The anomeric signal of residue **C** at δ 4.53 ppm, with $^3J_{1,2} \sim 8$ Hz and $^2J_{C,H} \sim 160$ Hz clearly indicates that L-fucose is a β-linked residue. The downfield shift of C-4 (δ 76.0 ppm) with respect to standard values indicates that **C** is (1→4)-linked.

Residue **D** was identified as a (1→6)-linked β-D-glucosyl moiety. The β configuration was confirmed by the appearance of a proton signal at δ 4.52 ppm and large coupling constant value $^3J_{1,2} \sim 9$ Hz and $^2J_{C,H} \sim 160$ Hz. The downfield shift of C-6 (δ 69.3 ppm) indicates that **D** is (1→6)-linked.

The sequences of glycosyl residues for PS were determined from NOESY as well as ROESY experiments (Fig. 3, Table 2). Residue **A** has an interresidue NOE contact from H-1 to H-4 of residue **C**. Hence, residue

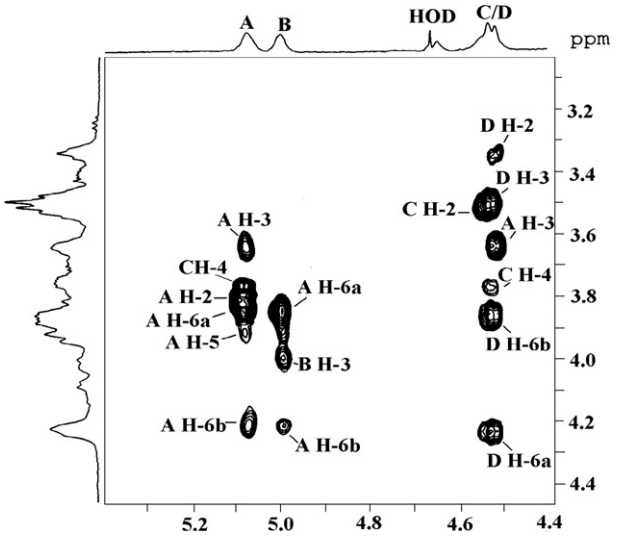


Figure 3. NOESY spectra of PS. The NOESY mixing time was 300 ms.

Table 2. NOE data of the PS isolated from *Astraeus hygrometricus*

Glycosyl residue	δ_{H1}	δ_H	NOE contact protons Residue, atom
→3,6)-α-D-Man*p*-(1→ **A**	5.07	3.81	A H-2
		3.63	A H-3
		3.85	A H-6a
		4.21	A H-6b
		3.76	C H-4
α-D-Glc*p*-(1→ **B**	4.99	4.00	B H-3
		3.85	A H-6a
		4.21	A H-6b
→4)-β-L-Fuc*p*-(1→ **C**	4.53	3.51	C H-2
		3.76	C H-4
		4.22	D H-6a
		3.86	D H-6b
→6)-β-D-Glc*p*-(1→ **D**	4.52	3.33	D H-2
		3.49	D H-3
		3.63	A H-3

A is linked at C-4 of residue **C**, and the sequence is established as

A **C**
α-D-Man*p*-(1→ 4)-β-L-Fuc*p*-(1 →

Residue **B** has a strong NOE contact from H-1 to both H-6a and H-6b of residue **A**. Thus residue **B** is linked at C-6 of residue **A**, and the next sequence is established as

B **A**
α-D-Glc*p*-(1→6)-α-D-Man*p*-(1→

NOE contacts of residue **C** are observed from H-1 to both H-6a and H-6b of residue **D**. Since both **C** and **D** are present in the same signal, therefore, the connectivity between them was finally confirmed by the presence of cross couplings (**C** C-1, **D** H-6a; **C** C-1, **D** H-6b) in the HMBC spectrum. Hence, this indicates that residue **C** is linked at C-6 of residue **D** and the sequence is established as

C **D**
→4)-β-L-Fuc*p*-(1→6)-β-D-Glc*p*-(1→

Residue **D** has a strong NOE contact from H-1 to H-3 of residue **A**. Hence **D** is linked at C-3 of residue **A**, and the following sequence is established as

D **A**
→6)-β-D-Glc*p*-(1→3)-α-D-Man*p*-(1 →

Therefore, residue **A** is a (1→3,6)-linked moiety, and the tetrasaccharide repeating unit of this polysaccharide is established as

A **C** **D**
→3)-α-D-Man*p*-(1→4)-β-L-Fuc*p*-(1→6)-β-D-Glc*p*-(1→
6
↑
1
α-D-Glc*p*
B

The ^{13}C–^{1}H correlations from HMBC experiment (Fig. 4, Table 3) corroborated the assigned tetrasaccharide repeating unit deduced from NOESY experiment. Cross peaks were found between H-1 of residue **A** (δ 5.07 ppm) and C-4 of residue **C** (**A** H-1, **C** C-4), and C-1 of residue **A** (δ 103.3 ppm) and H-4 of residue **C** (**A** C-1, **C** H-4). Cross peaks were also observed between H-1 of residue **B** (δ 4.99 ppm) and C-6 of residue of **A** (**B** H-1, **A** C-6); C-1 of residue **B** (δ 99.1 ppm) and H-6a, H-6b of residue **A** (**B** C-1, **A** H-6a; **B** C-1, **A** H-6b). Cross peaks were also observed between H-1 of residue **C** (δ 4.53 ppm) and C-6 of residue **D** (**C** H-1, **D** C-6); C-1 of residue **C** (101.7 ppm) and H-6a, H-6b of residue **D** (**C** C-1, **D** H-6a; **C** C-1, **D** H-6b). Also cross peaks of H-1 of residue **D** (δ 4.52 ppm) and C-3 of residue **A** (**D** H-1, **A** C-3), C-1of residue **D** (103.4 ppm) and H-3 of residue **A** (**D** C-1, **A** H-3) were observed. Thus from these observations, it is concluded that a tetrasaccharide repeating unit is present in this polysaccharide.

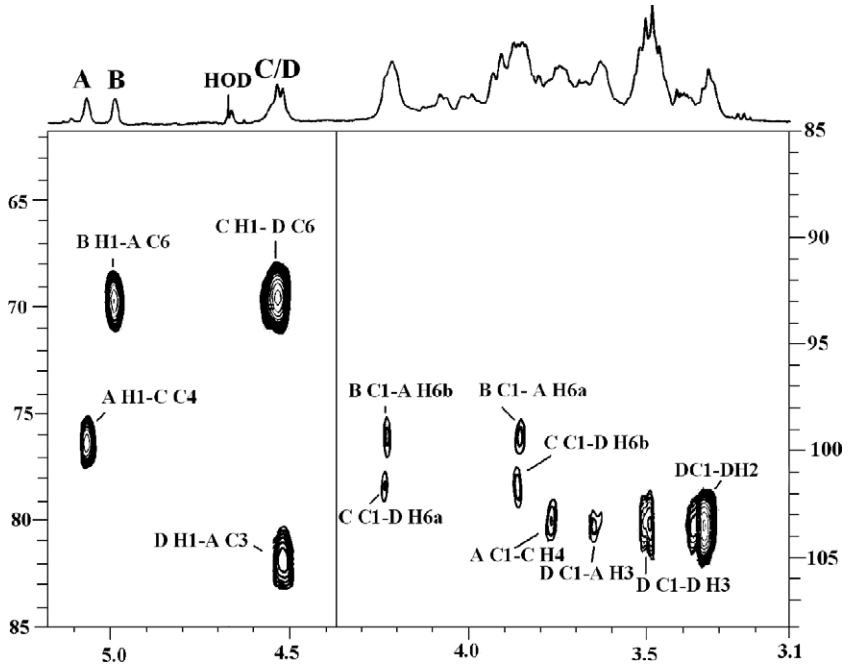


Figure 4. HMBC spectrum of PS. The delay time in the HMBC experiment was 80 ms.

Table 3. The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the PS isolated from *Astraeus hygrometricus*

Residue	Sugar linkage	H-1/C-1	Observed connection		
		δ_H/δ_C	δ_C/δ_H	Residue	Atom
A	→3,6)-α-D-Man*p*-(1→	5.07	76.0	**C**	C-4
		103.3	3.76	**C**	H-4
B	α-D-Glc*p*-(1→	4.99	69.9	**A**	C-6
		99.1	3.85	**A**	H-6a
			4.21	**A**	H-6b
C	→4)-β-L-Fuc*p*-(1→	4.53	69.3	**D**	C-6
		101.7	4.22	**D**	H-6a
			3.86	**D**	H-6b
D	→6)-β-D-Glc*p*-(1→	4.52	82.0	**A**	C-3
		103.4	3.63	**A**	H-3

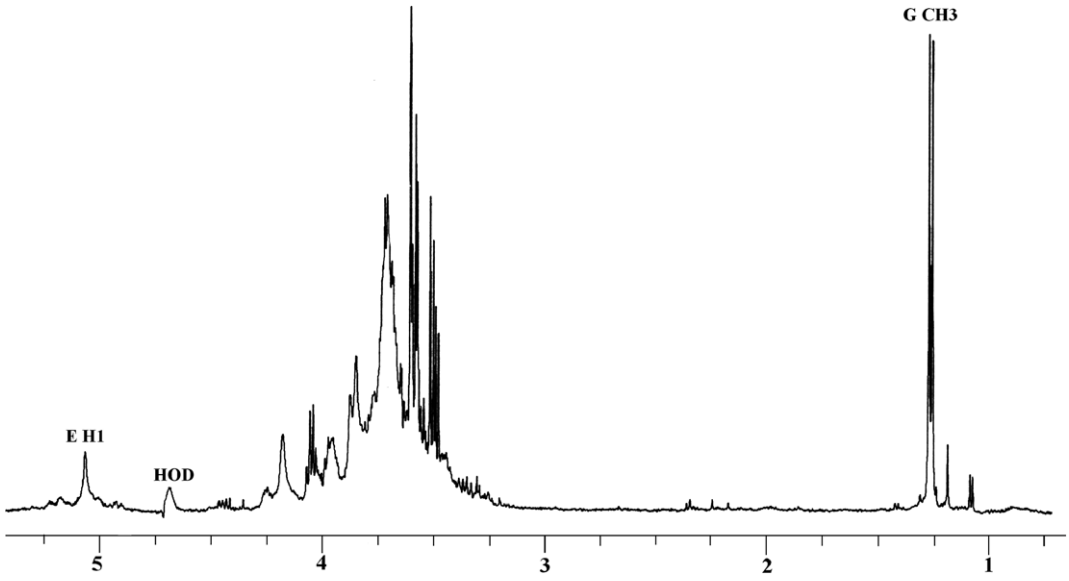


Figure 5a. ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) of the Smith degraded PS (SDPS).

Table 4. The ^{1}H and ^{13}C NMR chemical shifts of Smith degraded product (SDPS)

Residue	Sugar linkage	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
E	α-D-Man*p*-(1→	5.10	4.06	3.88	3.74	3.76	4.21	4.02
		102.3	70.0	71.1	68.0	73.6	61.4	
G	Tetritol-(2→	3.53	3.68	3.61	1.20			
		3.59						
		62.1	74.2	72.1	20.0			

4 3 2 1
G = CH_3–CH(OH)–CH–CH_2(OH)
↑

To further ascertain the linkage sequence of the sugar residues in the repeating unit, PS was subjected to Smith degradation experiments.[28] The Smith degraded PS was separated through a Sephadex G-25 column using water as the eluent, resulting in one fraction. ^{13}C and ^{1}H experiments were carried out on the Smith degraded product (SDPS). Both in the ^{1}H (Fig. 5a, Table 4) and ^{13}C NMR spectra (Fig. 5b, Table 4), the anomeric signals observed at δ 5.1 and 102.3 ppm, respectively, indicates that only an α-D-mannosyl sugar residue is retained after the Smith degradation. The absence of signals at δ 69.9 ppm and δ 82.0 ppm assigned to C-6 and C-3 of the branched D-mannosyl moiety (**A**) indicates that sugars attached to these positions, that is, α-D-glucose (**B**) and β-D-glucose (**D**), respectively, have been consumed during oxidation. Again, the signal for CH_3 of the L-fucosyl moiety (δ 16.0 ppm) shifted to 20.0 ppm indicating that it has been oxidized, and a fragment tetritol (**G**) has appeared due to the oxidative cleavage of the vicinal, C-2(OH)–C-3(OH) group. Hence, fragment **G** originated from the L-fucose moiety. The ^{13}C signals of SDPS, C-1 to C-6, appeared at δ 102.3, 70.0, 71.1, 68.0, 73.6, and 61.4 ppm for the α-D-mannosyl moiety (**E**) and the signals for fragment (**G**), C-1 to C-4, were observed at δ 62.1, 74.2, 72.1, and 20.0 ppm (Fig. 5b, Table 4). The ^{1}H signals (Fig. 5a, Table 4) were assigned from an HMQC experiment. Hence the structure of the Smith degraded product was established as

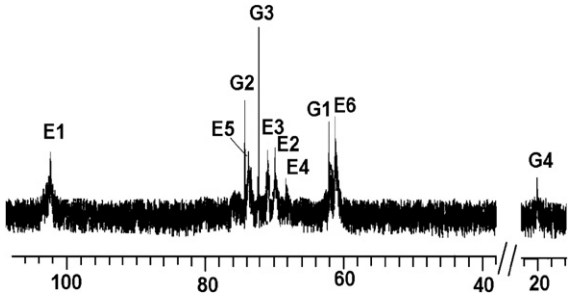
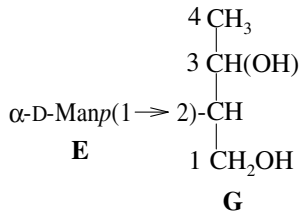
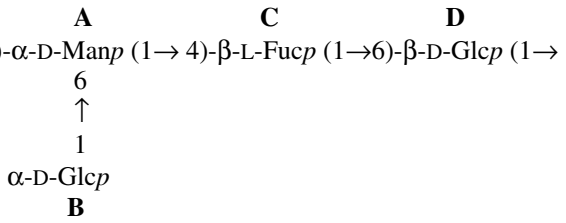


Figure 5b. ^{13}C NMR spectrum (125 MHz, D_2O, 27 °C) of the Smith degraded PS (SDPS).



Therefore, on the basis of the chemical and NMR spectral evidences followed by the analysis of the Smith degraded product, the structure of the polysaccharide PS isolated from the alkaline extract of the fruits of *A. hygrometricus* is



1. Experimental

1.1. Isolation and purification of PS

The fresh fruit bodies of the mushroom (1 kg) were collected from a local market, washed with water several times, crushed, and allowed to warm at 60 °C in 6% aq NaOH. The whole mixture was kept overnight at 4 °C and filtered through a fresh linen cloth. The filtrate was centrifuged at 8000 rpm (using a Heraeus Biofuge stratos centrifuge) for 1 h at 4 °C to obtain a clear soln. The supernatant was collected (250 mL), precipitated in aq EtOH (1:5 v/v) at room temperature, and kept overnight. The precipitated material was collected through centrifugation, dissolved in water, and dialyzed through a cellulose bag for 18 h to remove the low molecular weight materials. The soln was then freeze-dried. This material was again dissolved in water followed by re-precipitation in EtOH and centrifugation. The supernatant, water-soluble part was recovered and freeze-dried (yield, 1.2 gm). PS (30 mg) was purified through gel permeation chromatography on a Sepharose 6B column (65 × 2 cm) using water as an eluent at a flow rate of 11 s/drop. Fractions were monitored by the phenol–H_2SO_4 procedure[29] at 490 nm using Shimadzu UV–vis spectrophotometer, model 1601. Each step resulted in 19 mg of polysaccharide. This process was continued several times and a total of 150 mg was collected.

1.2. Molecular weight determination[23]

The molecular weight of PS was determined by gel-chromatography. Standard dextrans T-200, T-70, and T-40 were passed through a Sepharose 6B column, and then the elution vol was plotted against the logarithm of the respective molecular weight. The elution vol of PS was then plotted in the same graph, and the molecular weight of PS was determined.

1.3. Monosaccharide analysis

PS (1.5 mg) was hydrolyzed by treatment with 2 M TFA (1 mL) for 18 h at 100 °C. The acid in excess was removed by co-distillation with water. The hydrolyzed material was reduced by $NaBH_4$ and acidified with AcOH. It was then co-distilled with MeOH to remove the excess boric acid, and dried over P_2O_5. Thereafter, the whole mass was acetylated with pyridine and acetic anhydride for preparing alditol acetates. GLC–MS analysis was performed on a Hewlett–Packard 5970A automatic system, using a HP-5 capillary column (25 m × 25 m). 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. Quantification was carried out from the peaks area, using response factors from standard monosaccharide.

1.4. Methylation analysis

PS was methylated using the procedure described by Ciucanu and Kerek[25] and the methylated product was isolated by partition in 5:2 $CHCl_3$–water. The organic layer-containing product was washed with water several times and dried. The methylated polysaccharide was hydrolyzed by 1 mL of 90% HCOOH (100 °C, 1.5 h), and the monosaccharides were converted into their corresponding methylated alditol acetates as usual and analyzed by GLC–MS.

1.5. Periodate oxidation

PS (5 mg) was oxidized by 2 mL of 0.1 M $NaIO_4$ in the dark for 48 h at room temperature. The 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. Co-distillation with MeOH led to a residue which was subjected to both hydrolysis and methylation by the same process described earlier and the product was analyzed by GLC–MS.

1.6. Optical rotation

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

1.7. Colorimetric estimations

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

1.8. GLC–MS experiments

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

1.9. Smith degradation[28]

The native PS (24 mg) was oxidized with 0.1 M $NaIO_4$ (5 mL) at 25 °C in the dark for 48 h. The oxidation was stopped by the addition of ethylene glycol, and the soln was dialyzed using water. The dialyzed material was reduced with $NaBH_4$ for 13 h at room temperature, neutralized with 50% AcOH, dialyzed against distilled water, and freeze-dried. This product was hydrolyzed using 0.5 M TFA for 18 h at 25 °C to remove the oxidized sugar attached to the PS chain (Smith degradation). The excess acid was eliminated after repeated addition and evaporation of water by freeze-drying and further purified by passing through a Sephadex G-25 column, resulting in one fraction (10 mg). ^1H and ^{13}C NMR experiments were carried out on this Smith degraded material.

1.10. NMR spectroscopy

^1H and ^{13}C NMR experiments were carried out at 500 MHz and 125 MHz, respectively, using a Bruker Avance DPX-500 spectrometer and a 5 mm broad band probe. For NMR studies PS was dried in vacuum over P_2O_5 for several days, and then exchanged with deuterium[30] by lyophilization with D_2O three times. The deuterium-exchanged polysaccharide (4 mg) was dissolved in 0.7 mL D_2O (99.96% atom ^2H, Aldrich). The ^1H and ^{13}C NMR spectra (both ^1H coupled and decoupled) were recorded at 27 °C. Acetone was used as an internal standard (δ 31.05 ppm) for ^{13}C spectra. The ^1H NMR spectrum was recorded (HOD signal at δ 4.67 ppm) at 27 °C using the WEFT pulse sequence.[31] 2D (DQF-COSY) NMR experiments were performed using the standard Bruker software. The TOCSY experiment was recorded at a mixing time of 150 ms, and complete assignment required several experiments having mixing times ranging from 60 to 300 ms. The NOESY and ROESY mixing delay was 300 ms. The delay time in the HMBC experiment was 80 ms.

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