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Note

Structural investigation of a heteroglycan isolated from the fruit bodies of an ectomycorrhizal fungus *Astraeus hygrometricus*

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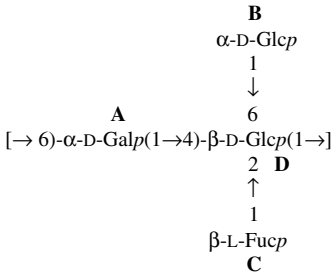
Abstract—A polysaccharide fraction (AQS-II) has been isolated from the hot aqueous extract of the fruits of an ectomycorrhizal fungus *Astraeus hygrometricus*. It was found to contain 63% polysaccharide and 35% protein. The polysaccharide part contains glucose, galactose, and fucose in a 2:1:1 molar ratio. On the basis of total acid hydrolysis, methylation analysis, periodate oxidation, and NMR studies (^{1}H, ^{13}C, DQF-COSY, TOCSY, NOESY, HMBC, and HSQC) the structure of the repeating unit of the polysaccharide was established as



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

Astraeus hygrometricus is an ectomycorrhizal fungus[1] that grows in association with the roots of Chir Pine (*Pinus roxburghii*) and Sal (*Shorea robusta*) trees in the laterite forest of South Bengal during the rainy season. A water-soluble fraction was previously isolated from the hot aqueous extract of this mushroom by our group, which on fractionation through gel permeation chromatography gave two homogeneous fractions. Fraction I (AQSI) was identified as a α-(1→4),β-(1→6)-linked glucan.[2] Fraction II (AQS-II) was subsequently found to be a heteropolysaccharide containing a certain amount of protein. A detailed structural study is now reported for Fraction II.

AQS-II showed $[\alpha]_{D}^{25}$ +23.2 (*c* 0.87, water). The molecular weight of this fraction was determined by gel filtration[3] using different carbohydrate markers and a Sepharose 6B column and found to be 22,000 Da. The total sugar content estimated by the phenol–sulfuric acid method[4] was found to be 63%. The protein content, measured by the Lowry method,[5] was 35%. On total acid hydrolysis with 2 M trifluoroacetic acid, followed by alditol acetate conversion and analysis through

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GLC using columns A (3% ECNSS-M) and B (1% OV-225), AQS-II was found to contain glucose, galactose, and fucose in a 2:1:1 molar ratio. The absolute configuration[6] of each monosaccharide was determined by GLC of the (+)-2-butyl 2,3,4,6-tetra-*O*-trimethysilylglycosides, which showed that glucose and galactose have the D configuration and fucose has the L configuration. AQS-II was then methylated using the Ciukanu and Kerek method,[7] and then by the Purdie method[8] followed by hydrolysis and conversion into alditol acetates. These were then analyzed through GLC using columns A and B and also by GLC-MS using a HP-5 fused silica capillary column. The presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (*m*/*z* 43, 71, 87, 101, 117, 129, 145, 161, 205), 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-L-fucitol (*m*/*z* 43, 59, 72, 89, 101, 115, 117, 131, 161), 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol (*m*/*z* 43, 58, 71, 87, 99, 101, 117, 129, 161, 173, 189, 233), and 1,2,4,5,6-penta-*O*-acetyl-3-*O*-methyl-D-glucitol (*m*/*z* 43, 85, 87, 99, 127, 129, 189, 201, 261) in a molar ratio of nearly 1:1:1:1 was detected. This result indicates that nonreducing D-glucopyranosyl, and L-fucopyranosyl, (1→6)-linked D-galactopyranosyl, and (1→2,4,6)-linked D-glucopyranosyl moieties are present in AQS-II. Thereafter, a periodate oxidation experiment was carried out with AQS-II. The periodate-oxidized and reduced material upon hydrolysis with TFA followed by GLC analysis showed only the presence of glucose. The periodate-oxidized and reduced, methylated material showed on GLC analysis the presence of 1,2,4,5,6-penta-*O*-acetyl-3-*O*-methyl-D-glucitol. Thus the retention of 1,2,4,5,6-penta-*O*-acetyl-3-*O*-methyl-D-glucitol during the periodate oxidation study confirms the mode of linkages of the sugar moieties of AQS-II.

The 500-MHz ^{1}H NMR spectrum of AQS-II (Fig. 1) at 27 °C showed four anomeric proton signals at δ 5.01, 4.92, 4.46, and 4.44 ppm in a 1:1:1:1 molar ratio. The ^{13}C spectrum (Fig. 2) showed three anomeric signals δ 102.96, 101.41, and 98.19 ppm in a molar ratio of nearly 2:1:1. All the ^{1}H and ^{13}C signals were assigned using DQF-COSY, TOCSY, and HSQC NMR experiments. The coupling constants were measured from the COSY spectrum. The four sugar moieties were designated as residues **A**–**D** according to their decreasing chemical shifts (Table 1) in the ^{1}H NMR spectrum.

Residue **A** was assigned as α-(1→6)-linked D-galactopyranose. The α-configuration of residue **A** (δ 5.01) was deduced from the very small value of the $^{3}J_{1,2}$ coupling constant (unresolved). The chemical shifts of all the carbons (Table 1) of residue **A** were assigned from the

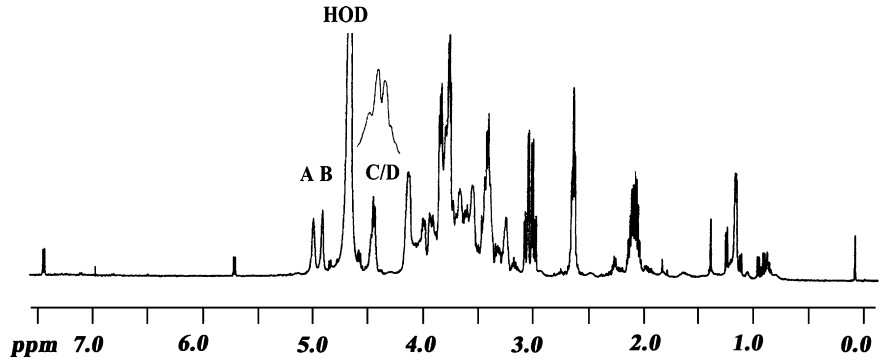

Figure 1. ^{1}H NMR (500 MHz, D_2O, 27 °C) spectrum of AQS-II.

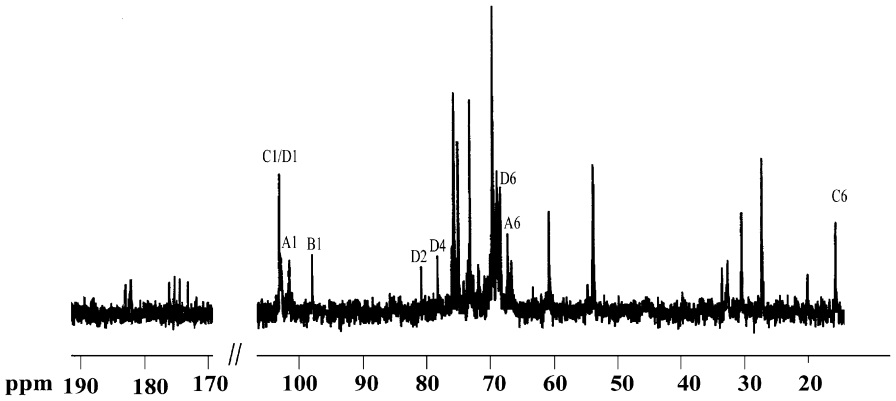

Figure 2. ^{13}C NMR (125 MHz, D_2O, 27 °C) spectrum of AQS-II.

Table 1. ^{1}H and ^{13}C NMR chemical shifts of AQS-II in D_2O at 27 °C

Atoms	Residue			
	→6)-α-D-Gal*p*-(1→ **A**	α-D-Glc*p*-(1→ **B**	β-L-Fuc*p*-(1→ **C**	→2,4,6)-β-D-Glc*p*-(1→ **D**
H-1	5.01	4.92	4.46	4.44
H-2	3.74	3.76	3.45	3.26
H-3	3.77	3.81	3.57	3.42
H-4	3.85	3.62	3.81	3.56
H-5	4.01	3.87	4.14	3.79
H-6a	3.68	3.95	1.18	4.00
H-6b	3.71	4.16		4.15
C-1	101.41	98.19	102.96	102.96
C-2	68.26	71.76	72.84	80.90
C-3	69.95	74.90	73.45	75.56
C-4	68.81	69.45	72.84	79.00
C-5	69.45	72.84	69.93	75.56
C-6	67.18	60.68	15.66	68.12

Values of the ^{1}H chemical shifts recorded with respect to the HOD signal fixed at δ 4.69 ppm at 27 °C.
Values of ^{13}C chemical shifts recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm at 27 °C.

HSQC spectrum. The carbon signal at 101.41 ppm was assigned to C-1 of residue **A**. The C-6 peak showed a downfield shift of 4.98 ppm due to the α effect of glycosylation.[9–11] So, residue **A** is an α-(1→6)-linked D-galactopyranosyl moiety.

Residue **B** was assigned as nonreducing end D-glucopyranosyl component. The appearance of an anomeric proton signal (unresolved) at δ 4.92 ppm, indicates that it is α-linked. Large coupling constant values, $^{3}J_{2,3}$ and $^{3}J_{3,4}$ (8–9 Hz) were observed for residue **B**, supporting that it is a D-glucosyl moiety. The carbon signal at 98.19 ppm was assigned to C-1 of residue **B**. The carbon signals from C-1 to C-6 of residue **B** were identified from the HSQC spectrum, and these correspond nearly to the standard values of methyl glycosides.[10] Thus considering the results of methylation analysis and NMR spectroscopy, it is concluded that residue **B** is an α-linked, nonreducing D-glucopyranosyl moiety.

Residue **C** was assigned as a nonreducing L-fucopyranosyl unit. This is strongly supported by the appearance of a proton signal at 1.16 ppm and a carbon signal at 15.66 ppm for a CH_3 group. From the appearance of the anomeric proton signal for residue **C** at δ 4.46 and the coupling constant value of $^{3}J_{1,2} \sim 8$ Hz, it is clear that L-fucose is β-linked. The C-1 signal of residue **C** appeared at 102.96 ppm. The chemical shifts from the C-1 to C-6 for residue **C** were assigned from the HSQC spectrum, and these correspond nearly to the standard values of methyl glycosides[10] of L-fucose. Thus the results of methylation analysis and NMR experiments clearly indicate that residue **C** is a β-linked L-fucose unit.

Residue **D** was assigned as (1→2,4,6)-linked D-glucopyranose. The carbon signal at 102.96 ppm was assigned as C-1 of residue **D**. Appearance of an anomeric proton signal at δ 4.44 ppm, and a large coupling constant value, $^{3}J_{1,2} \sim 9$ Hz indicate that it is a β-linked moiety. The signals (Table 1) from C-1 to C-6 for residue **D** were identified from the HSQC spectrum, and these values correspond nearly to the standard values for methyl glycosides.[10] The 6.80 ppm downfield shifts of C-2 (80.90 ppm), 8.40 ppm of C-4 (79.00 ppm), and 6.32 ppm of C-6 (68.12 ppm) indicate that residue **D** is linked at C-2, C-4, and C-6 positions.

The cross peaks of both anomeric protons and carbons from HMBC experiment (Table 2, Fig. 3) of each of the sugar moieties were examined, and intra and inter-residual connectivities were observed from the HMBC experiment. Cross peaks were found between H-1 of residue **A** (δ 5.01) with C-4 of residue **D** (**A** H-1, **D** C-4); C-1 of residue **A** (δ 101.41) and H-4 of residue **D** (**A** C-1, **D** H-4) along with intraresidual coupling between H-1 of residue **A** with its own C-2 (**A** H-1, **A** C-2). Similarly cross peaks were found between H-1 of residue **B** (δ 4.92) and C-6 of residue **D** (**B** H-1, **D** C-6); C-1 of residue **B** (98.19) and H-6a and H-6b of residue **D** (**B** C-1, **D** H-6a; **B** C-1, **D** H-6b) along with intraresidual cross couplings between H-1 of residue **B** with C-2 and C-3 (**B** H-1, **B** C-2; **B** H-1, **B** C-3). Cross peaks were also observed between H-1 of residue **C** (δ 4.46) and C-2 of residue **D** (**C**-H1, **D** C-2); C-1 of residue **C** (δ 102.96) and H-2 of residue **D** (**C** C-1, **D** H-2) along with intraresidual coupling between H-1 of residue **C** with C-3 and C-4 (**C** H-1, **C** C-3; **C** H-1, **C** C-4); C-1 of residue **C** with H-2 (**C** C-1, **C** H-2). Cross peaks between H-1 of residue **D** (δ 4.44) and C-6 of residue **A** (**D** H-1, **A** C-6); C-1 of residue **D** (δ 102.96) and H-6a and H-6b of residue **A** (**D** C-1, **A** H-6a, **D** C-1, **A** H-6b) were observed along with intraresidual cross coupling between C-1 of residue **D** and H-3 (**D** C-1, **D** H-3). The appearance of these cross peaks in the HMBC spectrum firmly supports the presence of a tetrasaccharide repeating unit in the AQS-II polysaccharide.

Thus, based on all these chemical and spectroscopic evidences, the structure of repeating unit of the AQS-II polysaccharide is established as in Chart 1.

Table 2. The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of AQS-II

Residue	Sugar linkage	H-1/C-1	Observed connectivities		
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	→6)-α-D-Gal*p*-(1→	5.01	79.00	**D**	C-4
			68.26	**A**	C-2
		101.41	3.56	**D**	H-4
B	α-D-Glc*p*-(1→	4.92	68.12	**D**	C-6
			71.76	**B**	C-2
			74.90	**B**	C-3
		98.19	4.15	**D**	H-6b
			4.00	**D**	H-6a
C	β-L-Fuc*p*-(1→	4.46	80.90	**D**	C-2
			72.84	**C**	C-4
			73.45	**C**	C-3
		102.96	3.45	**C**	H-2
			3.26	**D**	H-2
D	→2,4,6)-β-D-Glc*p*-(1→	4.44	67.18	**A**	C-6
		102.96	3.71	**A**	H-6b
			3.68	**A**	H-6a
			3.42	**D**	H-3

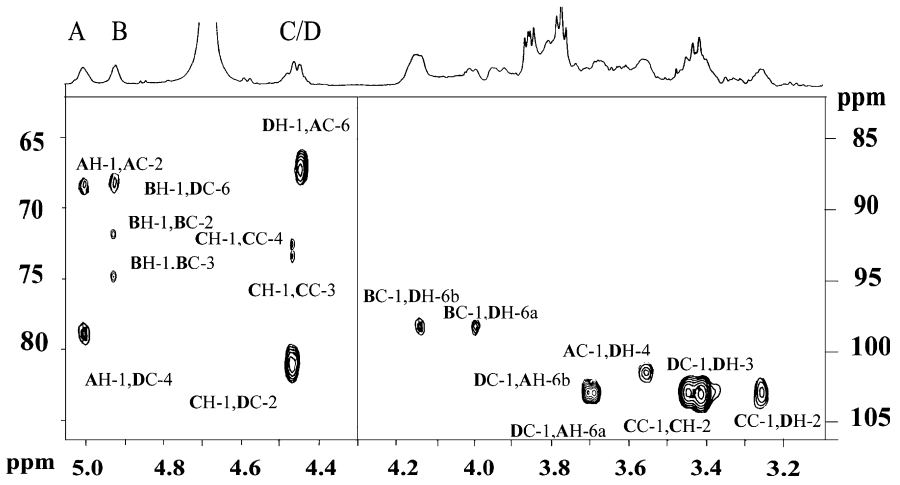

Figure 3. HMBC spectrum (anomeric proton region in the left panel and anomeric carbon region in right panel) of AQS-II.

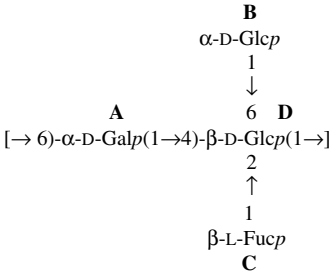

Chart 1. Proposed structure for the repeating unit of the AQS-II polysaccharide of Astraeus hygrometricus.

1. Experimental

1.1. General

Paper chromatography was performed on Whatmann No. 1 and 3 mm sheets. Solvent systems used were (X) 4:1:5 BuOH–AcOH–water (upper phase) and (Y) 8:2:1 ETOAc–pyridine–water. The spray reagent used was alkaline silver nitrate.[12] Optical rotation was measured on a Perkin–Elmer model 241 MC spectropolarimeter at 25 °C. Colorimetric estimations were carried out on a Shimadzu UV–vis spectrophotometer model 1601. All gas–liquid chromatography 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. All the 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. For NMR studies, the freeze-dried

material was kept over P_2O_5 in vacuum for several days and then deuterium exchanged three times, followed by lyophilization[13] with D_2O. The ^1H, TOCSY, DQF-COSY, NOESY, HMBC, and HSQC NMR spectra were recorded with a Bruker Avance DPX-500 spectrometer in D_2O at 27 °C. Chemical shifts were referred to the residual signal of HOD at δ 4.69 ppm. The TOCSY spectrum was recorded at a mixing time of 150 ms and complete assignment required several TOCSY experiments requiring several mixing times ranging from 60 to 300 ms. The NOESY mixing delay was 200 ms. The DQF-COSY spectrum was obtained by using standard pulse sequences. The ^{13}C spectrum of the native polysaccharide solution in D_2O was recorded with a Bruker Avance DPX-500 instrument at 27 °C. Acetone was used (δ 31.05 ppm) as an internal standard for the ^{13}C spectrum. The delay time in the HMBC experiment was 80 ms.

1.2. Polysaccharide isolation and purification

AQS-II Fraction was obtained from the fruit body of *A. hygrometricus* as described.[2] From 2 kg of fruits, 513.3 mg of AQS-II was obtained.

1.3. Molecular weight determination

The molecular weight of AQS-II was determined by gel exclusion chromatography. Standard dextrans, T-200, T-70, and T-40 were passed through a Sepharose 6B column, and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution vol of AQS-II was then plotted in the same graph, and the molecular weight of AQS-II was deduced.

1.4. Total acid hydrolysis

AQS-II (2 mg) was hydrolyzed with 2 M TFA (1 mL) at 100 °C for 16 h in a boiling water bath. The hydrolyzate was then converted into alditol acetates and analyzed by gas–liquid chromatography (GLC) using a Hewlett–Packard model 5730 instrument equipped with a flame-ionization detector. Peaks were identified and estimated with inositol as the internal standard. The alditol acetates were analyzed on glass columns (1.8 m × 6 mm) containing 3% ECNSS-M (A) and 1% OV-225 (B) on Gas Chrom Q (100–120 mesh) at 170 °C. Gas–liquid chromatography-mass spectrometric (GLC-MS) analysis was also performed on Hewlett–Packard 5970 A 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.

1.5. Determination of the absolute configuration

The method used was based on Gerwig et al.[6] After trifluoroacetic acid hydrolysis of 1 mg of AQS-II, the acid was removed by co-distillation with water. A soln 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)trifluroacetamide (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 obtained were identified by comparison with those prepared from the D and L enantiomers of the monosaccharides.

1.6. Methylation analysis

AQS-II was methylated using the method of Ciucanu and Kerek,[7] and the product was isolated by partition between $CHCl_3$ and water. It was methylated again by the Purdie method.[8] The product showed no band in the region 3600–3300 cm^{-1}. It was then hydrolyzed with 90% HCO_2H for 1 h. Excess HCO_2H was evaporated by co-distillation with distilled water. The hydrolyzate was then reduced with $NaBH_4$, and the alditol acetates were prepared as usual. Alditol acetates of the methylated sugar were analyzed by GLC (using columns A and B), and by GLC-MS using an HP-5 fused silica capillary column using the temperature program as stated above. Quantitations were carried out from peak areas.

1.7. Periodate oxidation study

AQS-II was added to 0.1 M $NaIO_4$, and the mixture was kept at 4 °C for 48 h in the dark. The excess of periodate was destroyed by ethylene glycol, and the soln was dialyzed against distilled water. It was then freeze-dried. This material was divided into two portions. One portion was hydrolyzed with 2 M CF_3CO_2H for 16 h, and alditol acetates were prepared. Another portion was methylated by the Ciucanu and Kerek method,[7] and the alditol acetates of the methylated product were prepared. Alditol acetates were analyzed by GLC using columns A and B.

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