Polysaccharides in Fungi. XXIX.[1] Structural Features of Two Antitumor Polysaccharides from the Fruiting Bodies of *Armillariella tabescens*

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An antitumor polysaccharide containing peptide moieties AT-HW ($[\alpha]_D+31°$ in water) and an antitumor polysaccharide AT-AL ($[\alpha]_D+209°$ in 1 M sodium hydroxide) were isolated from hot-water extract and the alkaline extract of the fruiting bodies of *Armillariella tabescens*, respectively. Chemical structures of AT-HW and AT-AL were investigated by a combination of chemical and spectroscopic methods. The results indicate that the major constituent of AT-HW (molecular weight, 105000), a heteroglycan, is composed primarily of β-(1→6)-linked D-glucopyranosyl and D-galactopyranosyl residues, and contains their branched residues and terminal sugar (gluco-, manno-, and fucopyranose) residues, in addition to β-(1→3)-linked D-glucopyranosyl residues, while AT-AL (molecular weight, 93000) is chiefly composed of α-(1→3)-linked D-glucopyranosyl residues.

Keywords *Armillariella tabescens*; polysaccharide; chemical structure; methylation analysis; ^{13}C-NMR

Armillariella tabescens (FR.) SING. (Tricholomataceae) is an edible mushroom, and we extracted polysaccharides from the fruiting bodies harvested in Japan. Since biological response modifiers (BRMs) have become of interest,[2] we isolated a polysaccharide (AT-HW) from hot-water extract and another (AT-AL) from 1 M sodium hydroxide extract. Since the structural features of polysaccharides have important bearing on their antitumor and immunomodulating activities, this paper deals with the structural characterizations of these two polysaccharides isolated from the fruiting bodies of *A. tabescens*.

Materials and Methods

Isolation of Two Polysaccharides The fruiting bodies (600 g) of *A. tabescens* were harvested in October, 1988 in Gifu prefecture, Japan. They were homogenized in 0.9% NaCl (2 l), and the residue collected by centrifugation was extracted 10 times with water (1 l) in a boiling water bath. The hot-water extract was dialyzed against distilled water for 4 d. Non-dialyzable fraction was centrifuged, and the supernatant was precipitated by addition of 3 volumes of ethanol. The ethanol precipitate was dissolved in water, and lyophilized to afford the polysaccharide (AT-HW) in 1.6 g yield.

The residue after hot-water extraction was extracted 3 times with 3% sodium carbonate (1 l) for 24 h at room temperature, and then was extracted 4 times with 1 M sodium hydroxide (1 l) containing sodium borohydride (200 mg) for 48 h at room temperature. The crude polysaccharide fraction (AT-CS) was obtained from the former extract in 0.7 g yield. The latter extract was neutralized with 2 M HCl, then dialyzed against distilled water. The insoluble materials in the non-dialyzable fraction were collected by centrifugation, washed with water, dispersed in water, and lyophilized to afford the polysaccharide (AT-AL) in 3.2 g yield. A crude polysaccharide containing protein (AT-ALS) was obtained from the soluble solution in the non-dialyzable fraction in 4.2 g yield.

Gel Filtration and Estimation of Molecular Weight Gel filtration of polysaccharides on a column (1.5 × 91 cm) of Toyopearl HW-65 was performed with 0.5 M sodium hydroxide as the eluent, and the molecular weight was estimated by a calibration curve using standard dextrans T-70, T-110, and T-150 (Pharmacia) as previously reported.[3]

Determination of Components The polysaccharides were hydrolyzed with 2 M trifluoroacetic acid (TFA) for 8 h at 100 °C. After the solutions were evaporated to remove the acid, the hydrolyzates were analyzed by gas chromatography (GC) as alditol acetates and by paper chromatography (PC), as described previously.[1] The amino acid composition was estimated using a Yanaco LC-11 amino acid analyzer.

Methylation Analysis Methylation was performed with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide (DMSO) as described previously.[3] After three repetitions of the procedure, the products showed no hydroxyl absorption in the infrared (IR) spectra. The methylated products were heated successively with 90% formic acid for 6 h at 100 °C, and with 2 M TFA for 6 h at 100 °C. The partially methylated sugars were converted into the alditol acetates. The resulting partially methylated alditol acetates were analyzed by GC and GC-mass spectrometry (MS). GC was performed on a Shimadzu GC-4CM gas chromatograph equipped with hydrogen flame ionization detector, using a glass column (0.3 cm × 2 m) packed with 3% ECNSS-M on Gaschrom Q (100—120 mesh) at 175 °C at a flow rate of 50 ml per min (nitrogen), and also on a Shimadzu GC-15A instrument with a CP-Sil 88 FS-WCOT fused silica capillary column (0.25 mm × 25 m) (Chrompack) at 180 °C at a helium flow of 92 ml per min (splitter vent), with a splitting ratio of 1 : 122. Retention times and peak areas were measured by a Shimadzu C-R5A chromatopac. GC-MS was conducted with a JEOL JMS-D 300 apparatus equipped with a glass column (0.2 cm × 1 m) packed with 3% ECNSS-M as described previously.[3]

Periodate Oxidation and Smith Degradation AT-AL (40.3 mg) was dissolved in 1 M sodium hydroxide (5 ml), and the solution was neutralized with dil. HCl, then water was added to 20 ml, while AT-HW (50.2 mg) was dissolved in water (20 ml). Each sample was oxidized with 20 mM $NaIO_4$ (20 ml) in the dark, with stirring for 8 d at 4 °C. Periodate consumption was determined at various times by an arsenite method.[4] The oxidized polysaccharides were reduced with sodium borohydride to give the polyalcohols (29.3 mg from AT-HW and 32.0 mg from AT-AL). A part of each polyalcohol was hydrolyzed with acid, and hydrolyzates were analyzed as the alditol acetates using a programmed rise in temperature of 6 °C/min from 60 to 185 °C, as described previously.[3]

Carbon-13 Nuclear Magnetic Resonance (^{13}C-NMR) The spectra were recorded on a JEOL-GX 270 FT NMR spectrometer for solutions of AT-HW in DMSO-d_6 at 60 °C and AT-AL in 1 M NaOD at room temperature. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard was used for NaOD solution.

Results and Discussion

The polysaccharides, AT-HW and AT-AL were obtained from hot-water extract and the alkaline extract of the

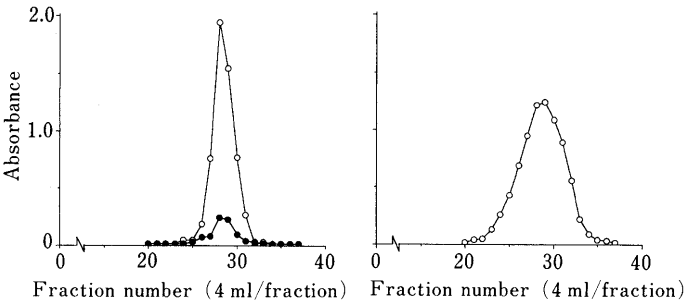

Fig. 1. Chromatograms of AT-HW (Left) and AT-AL (Right) on Toyopearl HW-65

The column was eluted with 0.5 M NaOH, and carbohydrate and peptide were assayed by the phenol–sulfuric acid method (—○—) and 280 nm (—●—), respectively.

TABLE I. Methylation Analysis of AT-HW

Methylated sugar (as alditol acetate)	Relative retention times[a]		Primary mass fragments (*m/z*)	Molar ratio	Mode of linkage
	Column A	Column B			
2,3,4-Me_3–Fuc	0.61	0.66	117, 131, 161, 175	2	[Fuc*p*]1→
2,3,4,6-Me_4–Man	1.00	0.98	45, 117, 161, 205	2	[Man*p*]1→
2,3,4,6-Me_4–Glc	1.00	1.00	45, 117, 161, 205	4	[Glc*p*]1→
2,3,5,6-Me_4–Gal	1.16	1.09	45, 59, 89, 117, 205	1	[Gal*f*]1→
2,4,6-Me_3–Glc	2.03	1.80	45, 117, 161, 233	6	→3[Glc*p*]1→
2,4,6-Me_3–Man	2.03	1.84	45, 117, 161, 233	1	→3[Man*p*]1→
2,3,4-Me_3–Glc	2.49	2.28	117, 161, 189, 233	11	→6[Glc*p*]1→
2,3,4-Me_3–Gal	3.54	3.13	117, 161, 189, 233	11	→6[Gal*p*]1→
2,4-Me_2–Glc	5.28	4.49	117, 189	4	→3,6[Glc*p*]1→
3,4-Me_2–Gal	7.34[b]	6.36[b]	189	3	→2,6[Gal*p*]1→
2-Me –Man			117		→3,4,6[Man*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. *b*) Overlapping peak. Column A, 3% ECNSS-M; column B, CP-Sil 88 (capillary column).

TABLE II. Methylation Analysis of AT-AL

Methylated sugar (as alditol acetate)	Relative retention times[a]		Primary mass fragments (*m/z*)	Molar ratio	Mode of linkage
	Column A	Column B			
2,3,4-Me_3–Xyl	0.64	0.72	117, 161	1	[Xyl*p*]1→
2,3,4,6-Me_4–Glc	1.00	1.00	45, 117, 161, 205	1	[Glc*p*]1→
2,4,6-Me_3–Glc	2.04	1.80	45, 117, 161, 233	19	→3[Glc*p*]1→
2,4,6-Me_3–Man	2.04	1.84	45, 117, 161, 233	3	→3[Man*p*]1→
2,3,6-Me_3–Glc	2.49	1.93	45, 117, 233	1	→4[Glc*p*]1→
2,6-Me_2–Man	3.42	2.99	45, 117	1	→3,4[Man*p*]1→
2,4-Me_2–Glc	5.23	4.49	117, 189	1	→3,6[Glc*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. Column A, 3% ECNSS-M; column B, CP-Sil 88 (capillary column).

fruiting bodies of *A. tabescens*, respectively. AT-HW, $[\alpha]_D$ +31° (*c*=0.15, water), is soluble in water, and AT-AL, $[\alpha]_D$+209° (*c*=0.25, 1 M sodium hydroxide) is insoluble in water but soluble in alkaline solution. Each polysaccharide showed a symmetrical elution pattern in gel filtration as shown in Fig. 1. On the AT-HW elution pattern (left), the absorbance at 280 nm was shown at the same position as carbohydrate, but it is not known whether the peptide moieties are associated with or linked to the polysaccharide; AT-AL did not show the absorbance at 280 nm. The molecular weights of AT-HW and AT-AL were estimated by the calibration curve of gel filtration to be *ca.* 105000 and *ca.* 93000 in an alkaline solution, respectively. It was found by GC and PC of each hydrolysate that AT-HW contained a polysaccharide consisting of glucose, galactose, mannose, and fucose (molar percent: 53.8, 24.7, 12.9, 8.6) and 13% peptide moieties (determined by Lowry method[5]). AT-AL contained no nitrogen and was composed of 77.6% glucose, 14.7% mannose, and 7.7% xylose in molar percentages. Both samples were fully methylated, and the methylated products were hydrolyzed with acid, then the sugars were converted into the partially methylated alditol acetates. Analyses by GC and GC-MS[6] gave the results shown in Tables I and II. The peak corresponding to 3,4-di-*O*-methyl-D-galactopyranose derivative in Table I was shown by the mass fragments of GC-MS to be overlapped with the peak of 2-*O*-methyl-D-mannopyranose derivative from (1→3,4,6)-linked D-mannopyranosyl residues. The results indicate that the sugar polymer of AT-HW is highly branched heteroglycan containing large amounts of (1→6)-linked D-glucopyranosyl and D-galactopyranosyl residues, and that AT-AL is composed chiefly of (1→3)-linked D-glucopyranosyl residues.

On periodate oxidations, AT-HW and AT-AL consumed 1.4 and 0.2 mol of periodate per hexosyl residue, respectively. In sequential borohydride reduction of each oxidized polysaccharide and by acid-hydrolysis (Smith degradation), AT-HW yielded glycerol, glucose and mannose (molar ratio, 9 : 1) as residual sugars, while AT-AL gave a large amount of glucose and a small amount of mannose in addition to trace amounts of glycerol and erythritol. The results were nearly the same as the data obtained from the methylation analysis.

The ^{13}C-NMR spectra of AT-HW and AT-AL are shown in Figs. 2 and 3, respectively. The main signals in AT-HW at 103.11, 76.46, 75.38, 73.22, 69.91, and 68.38 ppm were assigned to the anomeric carbon (C-1), C-5, C-3, C-2, C-4, and C-6 of β-D-glucopyranose and/or β-D-galactopyranose, respectively.[1,7] The signal at 60.99 ppm would correspond to C-6 of residues unsubstituted at C-6. Characteristic triplet signals[8] corresponding to C-3 of β-(1→3)-linked D-glucose residues indicated by the methylation analysis were not clearly observed, but broad signals near 86 ppm were observed. The spectrum of AT-AL in 1 M sodium deuteroxide was similar to that of (1→3)-α-D-glucan[9] (AG-AL from *Agrocybe cylindracea*) except that signals were weak. This suggests that AT-AL is chiefly composed of α-(1→3)-linked D-glucopyranosyl residues.

The foregoing data suggest the following: A heteroglycan, the major constituent of AT-HW isolated from the

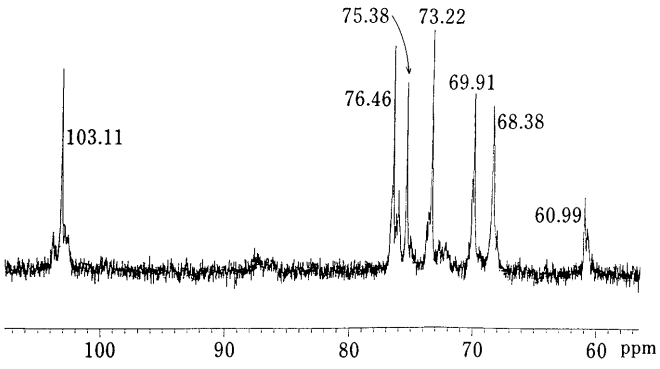

Fig. 2. ^{13}C-NMR Spectrum of AT-HW in DMSO-d_6 at 60 °C

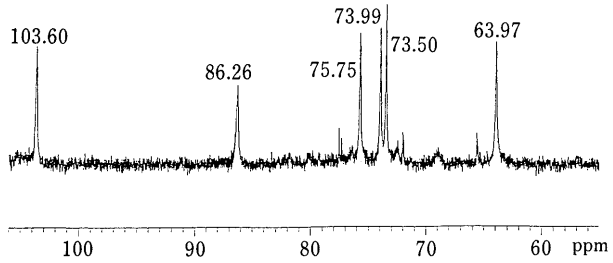

Fig. 3. ^{13}C-NMR Spectrum of AT-AL in 1 M NaOD at 22 °C

TABLE III. Amino Acid Composition of AT-HW

Amino acid	Molar composition (%)	Amino acid	Molar composition (%)
Glycine	15.8	Lysine	3.4
Aspartic acid	12.9	Isoleucine	3.3
Glutamic acid	10.1	Phenylalanine	2.4
Alanine	8.9	Tyrosine	2.2
Methionine	8.0	Arginine	1.6
Threonine	7.7	Histidine	1.6
Serine	7.7	Cystein	1.3
Valine	6.1	Proline	1.2
Leucine	5.9		

hot-water extract, is mainly composed of β-(1→6)-linked D-glucopyranosyl and D-galactopyranosyl residues, and contains their branched residues and 3-types of terminal sugars (gluco-, manno-, and fucopyranose) residues, in addition to β-(1→3)-linked D-glucopyranosyl residues. It has not yet been determined, however, whether each (1→6)-linked glucosyl residue and (1→6)-linked galactosyl residue exist as a block or an alternate. AT-AL also contained 13% peptide moieties consisting of the amino acids listed in Table III. A polysaccharide, AT-AL isolated from the alkaline extract is chiefly composed of a linear (1→3)-α-D-glucan.

The molecular weights of both polysaccharides are closely approximated values, however, AT-HW is a water-soluble polysaccharide containing small amounts of peptide moieties, the main consitituent of AT-HW is β-(1→6)-linked D-glucopyranosyl and D-galactopyranosyl residues, while AT-AL is water-insoluble and alkali-soluble, and could be expressed as (1→3)-α-D-glucan. AT-HW is a structurally new type of polysaccharide, and (1→3)-α-D-glucans such as AT-AL have been isolated from other fungi.[8,10] The presence of the polysaccharides is of chemotaxonomic interest. Since most polysaccharides reported as potent BRMs are (1→3)-β-D-glucans,[8,11] two with differing structural features obtained from the same source are also interesting for their immunomodulating and other biological activities. A following paper[12] will describe these biological activities including antitumor activity. Detailed structural studies of AT-HW and AT-AL are in progress.

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