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Structural features and hypoglycemic activities of two polysaccharides from a hot-water extract of *Agrocybe cylindracea* †,‡

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ABSTRACT

A glucan (AG-HN1, $[\alpha]_D$ +24°) and a heteroglycan (AG-HN2, $[\alpha]_D$ +26°) were isolated from a hot-water extract of the fruiting bodies of *Agrocybe cylindracea*. The structures were investigated by a combination of chemical and spectroscopic methods. The results indicated that high molecular weight glucan AG-HN1 is primarily a β-(1 → 6)-branched (1 → 3)-β-D-glucan containing small amounts of (1 → 4)-linked and (1 → 6)-linked glucopyranosyl residues. Low molecular weight heteroglycan AG-HN2 gives galactose, glucose, fucose, and mannose on hydrolysis and appears to be chiefly composed of (1 → 6)-linked gluco- and galacto-pyranosyl residues, many of them branched, and various nonreducing terminal residues. AG-HN1 showed a remarkable hypoglycemic activity in both normal and streptozotocin-induced diabetic mice by ip administration, and its activity was higher than that of AG-HN2.

INTRODUCTION

Previously, we reported on a linear (1 → 3)-α-D-glucan (AG-AL) from an alkaline extract of the fruiting bodies of *Agrocybe cylindracea* (Fr.) Maire and the antitumor activity of O-(carboxymethyl)ated AG-AL derivatives[2]. It has been reported that polysaccharides from fungi and plants show hypoglycemic activity[3–5], however, the structure-activity relationships among these polysaccharides remain obscure. We have isolated two polysaccharides from a hot-water extract of the fruiting bodies of *A. cylindracea* (an edible mushroom), namely AG-HN1 (a high molecular weight glucan) and AG-HN2 (a low molecular weight heteroglycan), and in this paper describe their structural characterization and their hypoglycemic activities in normal and streptozotocin-induced diabetic mice.

† Polysaccharides in Fungi, Part XXXI. For Part XXX, see ref 1.
‡ Dedicated to Professor C.E. Ballon.
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EXPERIMENTAL

Isolation of the polysaccharides.—Freshly gathered fruiting bodies of *A. cylindracea* (450 g) were homogenized with 0.9% NaCl and the residue obtained by centrifugation was extracted with water (2 × 1 L, heated on a boiling water bath 6 h each time). The extract was dialyzed against distilled water, 3 vol of EtOH was added, and the resulting precipitate collected by centrifugation. The precipitate was purified by chromatography on DEAE-Toyopearl 650M (phosphate form). The non-ionic fraction was dialyzed and freeze-dried to give a crude polysaccharide (AG-HN) in 0.16% yield. This was dissolved in water (200 mL) and then further fractionated by the addition of EtOH (200 mL). The redissolved precipitate and the supernatant fractions were recovered by freeze-drying to give a high molecular weight polysaccharide (AG-HN1) in 0.12% yield and a low molecular weight polysaccharide (AG-HN2) in 0.04% yield, respectively.

Gel filtration.—Gel filtration of polysaccharides on a column (1.5 × 91 cm) of Toyopearl HW-65F, HW-55F (Tosoh), or Sephacryl S-500 (Pharmacia) was performed with 0.1 M NaCl as the eluent. In the case of the product from controlled Smith degradation 0.2 M NaOH was used. Fractions (4 mL) were collected, and analyzed by the phenol–H_2SO_4 method[6]. The molecular weight was estimated from a calibration curve constructed using standard dextrans[7].

Analysis of component sugars.—Polysaccharides were hydrolyzed with 2 M CF_3CO_2H for 8 h at 100°C. CF_3CO_2H was removed by evaporation, and the sugars in the hydrolyzate were analyzed by paper chromatography and by GLC as their alditol acetates, as described previously[5].

Methylation analysis.—The polysaccharides were methylated 4 times by the Hakomori method[8]. The fully O-methylated polysaccharides were hydrolyzed by successive treatments with 90% formic acid for 6 h at 100°C and 2 M CF_3CO_2H for 5 h at 100°C. The partially O-methylated sugars obtained were converted into the alditol acetates, and these were analyzed by GLC and GLC–MS, using 3% of ECNSS-M on Gaschrom Q in a packed column and CP-Sil 88 FS-WCOT in a fused-silica capillary column, as described previously[9,10].

Periodate oxidation and Smith degradation.—AG-HN1 (18 mg) was oxidized with 20 mM sodium metaperiodate for 8 days at 4°C, in the dark. The oxidized polysaccharide was reduced with sodium borohydride to give the polyalcohol (16 mg). Part of the polyalcohol was then hydrolyzed with 2 M H_2SO_4 for 8 h at 100°C, and the components of the hydrolyzate (Smith degradation product) analyzed as their alditol acetates by GLC, using the ECNSS-M column and a temperature program as previously described[7]. A portion of the polyalcohol (13 mg) was partially hydrolyzed with 50 mM H_2SO_4 for 48 h at 20°C, and the insoluble product was collected by centrifugation, dispersed in water, and freeze-dried to give the controlled Smith degradation product (AG-HN1CS) (6 mg). AG-HN1CS was subjected to gel filtration on Toyopearl HW-65F in 0.2 M NaOH.

^{13}C NMR spectroscopy.—^{13}C NMR spectra were recorded with a Jeol GX 270

spectrometer in the Fourier-transform mode for solutions in Me_2SO-d_6 (43 mg/0.5 mL) at 60°C.

Measurement of hypoglycemic activity.—Male mice (ddY strain, 4 weeks old) were obtained from Japan SLC, Inc., Hamamatsu, Japan, and given a standard laboratory diet and water *ad libitum*. Mice used as normals were 5 weeks old. Other 5 week-old mice were made diabetic by intravenous injection of streptozotocin (100 mg/kg) in citrate buffer (pH 4.5). They were considered diabetic if their plasma glucose level 3 weeks after the injection was 400–700 mg/dL. Polysaccharide samples were dissolved in physiological saline solution, and injected intraperitoneally (ip) into normal or streptozotocin-induced diabetic mice. Blood was drawn periodically from the orbital sinus by micro hematocrit tubes and centrifuged to obtain plasma. Plasma glucose was determined by a glucose oxidase procedure using a commercial reagent (Glucose B-test Wako, Wako Pure Chemical Ind., Osaka, Japan). The significance of differences between means was evaluated using Student's *t*-test.

RESULTS AND DISCUSSION

A crude polysaccharide (AG-HN) was obtained from the hot-water extract of the fruiting bodies of *A. cylindracea* was purified by dialysis, ethanol precipitation, and anion-exchange chromatography. Since AG-HN showed two peaks when chromatographed on Toyopearl HW-65F (Fig. 1), it was further fractionated by ethanol precipitation to give AG-HN1 and AG-HN2. The polysaccharides AG-HN1 and AG-HN2 had $[\alpha]_D$ +24° and $[\alpha]_D$ +26°, respectively, in water. AG-HN1 was composed solely of glucose, and AG-HN2 was composed of galactose, glucose, fucose, and mannose (molar ratio, 36:27:17:14). The molecular weights of AG-HN1 and AG-HN2 were estimated by gel chromatography to be 2 000 000 and 55 000, respectively.

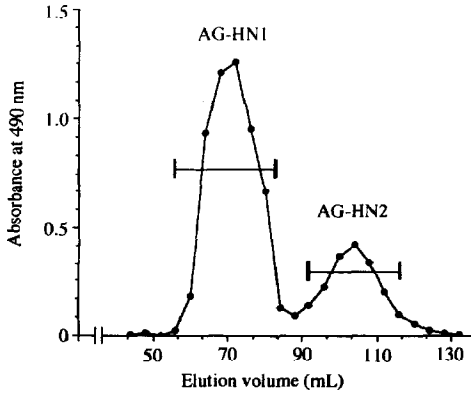

Fig. 1. Chromatogram of AG-HN in 0.1 M NaCl on Toyopearl HW-65F.

TABLE I

Methylation analysis of AG-HN1 and AG-HN2

Methylated sugar	r_r		Primary mass fragments (m/z)	Mol%		Mode of linkage
	(a)	(b)		AG-HN1	AG-HN2	
2,3,4-Me_3-Fuc	0.60	0.66	117, 131, 161, 175		10	Fuc*p*-(1→
2,3,4,6-Me_4-Glc	1.00	1.00	45, 117, 161, 205	16	10	Glc*p*-(1→
2,3,4,6-Me_4-Gal	1.26	1.21	45, 117, 161, 205		5	Gal*p*-(1→
2,4,6-Me_3-Glc	2.07	1.79	45, 117, 161, 233	51	8	→3)-Glc*p*-(1→
2,3,4-Me_3-Glc	2.54	2.28	117, 161, 189, 233	4	27 [b]	→6)-Glc*p*(1→
2,3,6-Me_3-Glc	2.54	2.52	45, 117, 233	13		→4)-Glc*p*-(1→
2,3,4-Me_3-Gal	3.66	3.12	117, 161, 189, 233		20	→6)-Gal*p*-(1→
2,4-Me_2-Glc	5.59	4.49	117, 189	16	7	→3,6)-Glc*p*-(1→
3,4-Me_2-Gal	7.86	6.37	189		13	→2,6)-Gal*p*-(1→

[a] Retention times of the corresponding alditol acetate relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on (a) 3% ECNSS-M at 170°C and (b) CP-Sil 88 (capillary column) at 180°C. [b] Spuriously high value because of overlap with the peak for 2,3,4-Me_3-Man.

The polysaccharides were methylated, and the fully O-methylated polysaccharides were analyzed as described in the Experimental section. Table I shows the results indicating that glucan AG-HN1 is composed of 3-linked and 3,6-linked interior residues (the proportion of branched to unbranched 3-linked glucosyl residues is 1:3), as well as nonreducing terminal residues, and contains 4-linked and 6-linked residues. The heteroglycan AG-HN2 is composed of 6-linked gluco(manno)- and galacto-pyranosyl residues, 3-linked gluco-pyranosyl residues, 2,6-linked galactopyranosyl residues, 3,6-linked glucopyranosyl residues, and nonreducing terminal fuco-, gluco-, and galacto-pyranosyl residues.

The detection of erythritol and glucose in the Smith degradation product of AG-HN1 supports the presence of 4-linked residues and 3- or 3,6-linked glucopyranosyl residues. On gel filtration AG-HN1CS (the controlled Smith degradation product of AG-HN1) showed a more highly dispersed pattern than AG-HN1, a result which suggests that the 4-linked and/or 6-linked residues are not regularly distributed in the 3-linked main chain.

The ^{13}C NMR spectrum of AG-HN1 is shown in Fig. 2. The prominent signals at 102.87, 86.14, 76.21, 72.69, 68.30, and 60.78 ppm were assigned to the anomeric carbon atoms (C-1), C-3, C-5, C-2, C-4, and C-6 of 3-linked β-D-glucopyranosyl residues, respectively. A triplet signal (86.62, 86.14, and 85.91 ppm) is due to substitution at C-3 of β-D-glucosyl units. Other resonances at 75.93 and 74.58 ppm, at 73.54 ppm, and at 70.01 ppm, respectively correspond to C-5, C-2, and C-4 of nonreducing terminal and/or 3,6-branched β-D-glucopyranosyl residues[7]. The weak signal at 98.10 ppm in the anomeric region[11] may be due to C-1 of the (1→4)-linked glucosyl residues indicated by the methylation analysis, and these may be in the α configuration. On the other hand, the signals in the anomeric region (103.75–99.15 ppm) of the spectrum of AG-HN2 suggest the presence of both β-D- and α-D-linkages; however, the individual signals were not assigned.

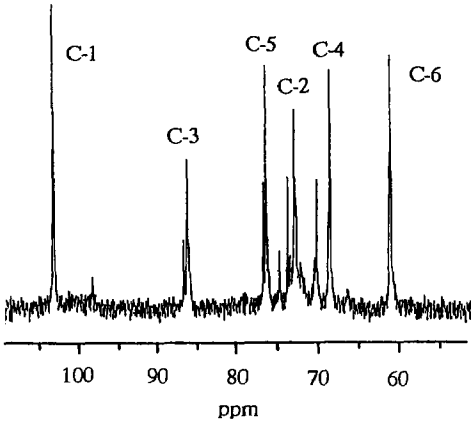

Fig. 2. ^{13}C NMR spectrum of AG-HN1 in Me_2SO-d_6 at 60°C.

The foregoing data indicate that the high molecular glucan AG-HN1 is a β-(1 → 6)-branched (1 → 3)-β-D-glucan having branches on one out of four of the (1 → 3)-linked main-chain residues, and containing small amounts of (1 → 4)-linked and (1 → 6)-linked residues. The low molecular heteroglycan AG-HN2 is chiefly composed of (1 → 6)-linked and branched gluco-, galacto-pyranosyl residues, and three types of nonreducing terminal residues, together with (1 → 6)-linked mannopyranosyl residues.

The unfractionated preparation (AG-HN) consisting of AG-HN1 and AG-HN2 (3:1, w/w) exhibited significant hypoglycemic activity at a dose of 50 mg/kg in normal and streptozotocin-induced diabetic mice (Table II). In tests on normal mice, AG-HN1 had a higher activity than AG-HN2, and its activity was remarkably prolonged (Table III). Furthermore, AG-HN1 and AG-HN2 reduced the plasma glucose level of the diabetic mice to 38% and 70% of control, respectively, at 6 h after the administration although the effect disappeared by 24 h (Fig. 3). In

TABLE II

Effect of AG-HN on plasma glucose level in normal mice and streptozotocin-induced diabetic mice

Group ($n = 5$)	Dose (mg/kg, ip)	Relative plasma glucose levels (%) [a] at times shown				
		0	3	5	7	24 (h) [b]
Normal mice						
Control		100	118.4 ± 9.1	104.4 ± 7.1	104.7 ± 4.2	101.2 ± 4.9
AG-HN	50	100	64.2 ± 6.2 [c]	60.0 ± 9.8 [c]	54.8 ± 8.8 [c]	70.6 ± 4.8 [c]
Diabetic mice						
Control		100	107.7 ± 2.9	113.8 ± 4.8	97.2 ± 1.6	96.8 ± 6.0
AG-HN	50	100	81.3 ± 4.3 [c]	55.7 ± 9.7 [c]	54.8 ± 7.2 [c]	80.3 ± 5.8

[a] The plasma glucose level at 0 h (normal mice 170–200 mg/dL, diabetic mice 400–700 mg/dL) was set at 100. Values represent the mean ± SE. [b] Time after administration. [c] Significant difference from the control, $p < 0.01$.

TABLE III

Effect of AG-HN1 and AG-HN2 on plasma glucose level in normal mice

Group ($n = 5$)	Dose (mg/kg ip)	Relative plasma glucose levels (%) [a] at times shown							
		0	3	5	7	24	48	72	96 (h) [b]
Control		100	98.1 ± 2.4	94.6 ± 1.1	98.0 ± 3.7	85.9 ± 2.2	90.7 ± 4.7	100.1 ± 5.1	97.4 ± 3.9
AG-HN1	50	100	66.0 ± 3.9 [c]	54.5 ± 3.1 [c]	49.5 ± 2.4 [c]	58.6 ± 3.7 [c]	72.3 ± 3.0 [c]	82.4 ± 3.8 [d]	83.5 ± 2.8 [d]
	30	100	68.0 ± 1.8 [c]	61.8 ± 6.1 [c]	64.0 ± 6.2 [c]	79.3 ± 3.1 [c]	86.9 ± 2.3 [d]	83.0 ± 2.7 [c]	86.4 ± 1.0 [d]
	10	100	72.6 ± 5.0 [c]	57.6 ± 2.3 [c]	69.0 ± 4.4 [c]	84.4 ± 2.9 [d]	84.9 ± 2.9 [d]	91.9 ± 4.3	91.9 ± 6.2
Control		100	117.3 ± 4.2	108.5 ± 3.2	110.9 ± 2.3	102.0 ± 3.3			
AG-HN2	50	100	70.3 ± 4.2 [c]	74.5 ± 4.5 [c]	86.5 ± 4.6 [c]	91.8 ± 3.9			

[a] Values represent the mean ± SE. [b] Time after administration. [c] Significant difference from the control, $p < 0.01$, [d] $p < 0.05$.

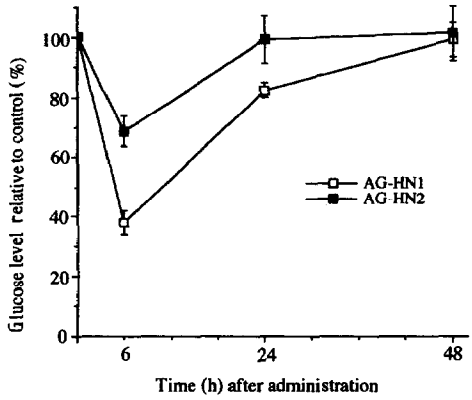

Fig. 3. Effect of the polysaccharides on plasma glucose level in streptozotocin-induced diabetic mice.

contrast, a β-(1 → 6)-branched (1 → 3)-β-D-glucan having a branching ratio of 1:3 (N-5P)[7] and a linear (1 → 3)-β-D-glucan (curdlan, Wako Pure Chemical Ind.) administered ip had little effect on plasma glucose levels in normal mice. These results suggest that (1 → 4)-linked and/or (1 → 6)-linked residues in a β-(1 → 6)-branched (1 → 3)-β-D-glucan are needed for the hypoglycemic effect, and that the high molecular weight of the polysaccharide also contributes to this biological activity.

REFERENCES

1 T. Kiho, Y. Shiose, K. Nagai, and S. Ukai, *Chem. Pharm. Bull.*, 40 (1992) 2110–2114.
2 T. Kiho, I. Yoshida, K. Nagai, S. Ukai, and C. Hara, *Carbohydr. Res.*, 189 (1989) 273–279.
3 M. Tomoda, R. Gonda, Y. Kasahara, and H. Hikino, *Phytochemistry*, 25 (1986) 2817–2820.
4 M. Tomoda, N. Shimizu, R. Gonda, M. Kanari, H. Yamada, and H. Hikino, *Carbohydr. Res.*, 190 (1989) 323–328.
5 T. Kiho, T. Watanabe, K. Nagai, and S. Ukai, *Yakugaku Zasshi*, 112 (1992) 393–400.
6 M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
7 T. Kiho, M. Ito, K. Nagai, C. Hara, and S. Ukai, *Chem. Pharm. Bull.*, 35 (1987) 4286–4293.
8 S. Hakomori, *J. Biochem (Tokyo)*, 55 (1974) 205–208.
9 T. Kiho, Y. Shiose, K. Nagai, M. Sakushima, and S. Ukai, *Chem. Pharm. Bull.*, 40 (1992) 2212–2214.
10 H. Björndal, C.G. Hellerqvist, B. Lindberg, and S. Svensson, *Angew. Chem. Int. Ed. Engl.*, 9 (1970) 610–619.
11 K. Bock, C. Pedersen and H. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 42 (1984) 193–225.