Polysaccharides in Fungi. XXVI.¹⁾ Two Branched (1→3)- β -D-Glucans from Hot Water Extract of Yü ěr

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Two water-insoluble glucans, U-3-N ($[\alpha]_D +1.0^\circ$, 0.5 M sodium hydroxide) and U-3-AP₁ ($[\alpha]_D +2.5^\circ$, 1 M sodium hydroxide) were isolated from hot-water extract of the fruiting bodies of Yü ěr (Chinese name) (*Auricularia* sp.). U-3-N and U-3-AP₁ were investigated by a combination of chemical and spectroscopic methods. The results indicated that U-3-N (molecular weight, 6.1×10^5) was similar to β -(1→6)-branched (1→3)- β -D-glucan (N-5P; molecular weight, 5.6×10^5) isolated from the alkaline extract of the fruiting bodies, and U-3-AP₁ (molecular weight, 6.3×10^4) was β -(1→6)-branched (1→3)- β -D-glucan containing β -(1→6)-linked D-glucopyranosyl residues. U-3-N showed potent antitumor activity against the solid form of sarcoma 180, although U-3-AP₁ had little effect on the tumor.

Keywords branched (1→3)- β -D-glucan; Yü ěr (*Auricularia* species); polysaccharide structure; antitumor activity; ¹³C-NMR

We have isolated the acidic polysaccharide (U-3-A)²⁾ from the hot water extract and β -D-glucan (N-5-P)³⁾ from the alkaline extract of the fruiting bodies of Yü ěr (*Auricularia* species) belonging to Auriculariaceae. We reported on the structural characterizations and the biological activities such as antiinflammatory activity²⁾ and antitumor activity.⁴⁾ Since we found the presence of the neutral polysaccharides (U-3-N and U-3-AP₁) together with U-3-A in the hot water extract of the fruiting bodies, the present paper deals with the structural characterization and the antitumor activity of U-3-N and U-3-AP₁.

The fruiting bodies were successively extracted with methanol and 70% aqueous ethanol, in a boiling water bath. The residue was extracted 4 times with water (1 l) in a boiling water bath. The crude polysaccharides obtained from the hot water extract were fractionated with cetyltrimethylammonium bromide (CTAB) to provide the precipitate of the acidic polysaccharide (U-3-A) and the supernatant, as described in a previous paper.²⁾ The supernatant was purified by addition of ethanol and anion-exchange chromatography on diethylaminoethyl (DEAE)-Toyopearl 650 M. The neutral fraction was lyophilized to give a neutral polysaccharide (U-3-N) in 1.24% yield. The fraction eluted by the gradient elution with aqueous sodium chloride afforded a polysaccharide (U-3-AP₁) in 0.63% yield. The polysaccharides thus obtained were insoluble in water, and soluble in an alkaline solution. Each polysaccharide showed a symmetrical elution pattern in gel filtration on Toyopearl HW-65 with 0.5 M sodium hydroxide solution. Gas-liquid chromatography (GLC) and paper chromatography (PPC) showed that both polysaccharides were composed solely of D-glucosyl residues. The polysaccharides showed characteristic absor-

bance at 890 cm^{-1} in the infrared (IR) spectra and had low specific rotations $[\alpha]_D +1.0^\circ$ ($c=0.13$, 0.5 M NaOH) for U-3-N; $[\alpha]_D +2.5^\circ$ ($c=0.13$, 1 M NaOH) for U-3-AP₁, suggesting the presence of the β -D configuration. The molecular weights of U-3-N and U-3-AP₁ were estimated by gel filtration to be *ca.* 6.1×10^5 and *ca.* 6.3×10^4 , in an alkaline solution, respectively.

Each glucan was methylated, and the fully methylated glucans were hydrolyzed, then converted into the partially methylated alditol acetates. As shown in Table I, GLC and GLC-mass spectrometry (GLC-MS) revealed that U-3-N was (1→6) branched (1→3)-linked glucan and U-3-AP₁ was (1→6) branched (1→3)-linked glucan containing (1→6) linked residues. On periodate oxidations, U-3-N and U-3-AP₁ consumed 0.50 and 0.70 mol of periodate per hexosyl residue, respectively. Sequential borohydride reduction of each oxidized polysaccharide, and acid-hydrolysis (Smith degradation) gave glycerol and glucose. The results were consistent with the data obtained from the methylation analysis.

The carbon-13 nuclear magnetic resonance (¹³C-NMR) spectra of U-3-N and U-3-AP₁ were recorded. The spectrum of U-3-AP₁ (see Fig. 1) was very similar to that of U-3-N except for the relative intensities of the signals. The values of chemical shifts and characteristic triplet signals at substituted C-3⁵⁾ showed that U-3-N and U-3-AP₁ were β -(1→6) branched (1→3)- β -D-glucans. β -D-Configurations of glucopyranosyl residues were supported by the ¹J_{CH} value of 163.0 Hz.⁶⁾

The glucan (U-3-N) was digested with *exo*-(1→3)- β -D-glucanase (Lysing enzyme) from *Rhizoctonia solani*, and enzymic degradation products of U-3-N were examined by gel filtration on Bio-gel P-2, only two peaks, corresponding

TABLE I. GLC^{a)} and GLC-MS of Partially Methylated Alditol Acetates

Methylated sugar (as alditol acetate)	Relative retention time ^{b)}	Main mass-fragments (<i>m/z</i>)	Molar ratios		Mode of linkage
			U-3-N	U-3-AP ₁	
2,3,4,6-Me ₄ -Glc	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205	1.00	1.00	[Glc] 1→
2,4,6-Me ₃ -Glc	1.51	43, 45, 87, 101, 117, 129, 161, 233	2.16	1.29	→3 [Glc] 1→
2,3,4-Me ₃ -Glc	1.72	43, 87, 99, 101, 117, 129, 161, 189, 233	Trace	0.38	→6 [Glc] 1→
2,4-Me ₂ -Glc	2.69	43, 87, 117, 129, 189	1.03	0.91	→3 [Glc] 1→
					→6

a) Conditions: 3% ECNSS-M on Gaschrom Q at 170 °C. b) Relative to authentic 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol.

Fig. 1. ^{13}C -NMR Spectrum of U-3-AP₁ in DMSO- d_6 at 60 °C

to mono- and disaccharide, were revealed. The molar ratio of disaccharide to monosaccharide reached a constant value (0.45) at 24 h.

The foregoing data indicated that polysaccharide U-3-N was a branched glucans composed of a (1→3)- β -D-glucopyranosyl main chain, and had single, β -(1→6)-linked D-glucopyranosyl groups attached as side chains. The side chain attached, on average, to every third residue of the backbone. The data also indicated that U-3-AP₁ was β -(1→6)-branched (1→3)- β -D-glucan containing β -(1→6)-linked residues, and higher branched and smaller molecular-weight glucan than U-3-N.

The conformational behavior of linear, and branched (1→3)- β -D-glucans has been discussed in regard to changes in the visible absorption spectra of the complexes formed with Congo red, as previously reported.³⁾ The values of the visible absorption maximum (λ_{max}) of Congo red were largely shifted to a longer wavelength in the presence of U-3-N (501 nm) and U-3-AP₁ (495 nm), at a low concentration (0.1 M) of sodium hydroxide, whereas at high concentrations (0.25 M and 0.50 M) of sodium hydroxide, no λ_{max} shifts were observed. The results suggested that U-3-N has an ordered, triple-helical structure in a weakly alkali solution, and has single chains in a highly alkaline solution, and U-3-AP₁ may have a partially triple-helical structure.

Branched (1→3)- β -D-glucans having side chains of single D-glucosyl group at O-6 have been known to exhibit antitumor activity against sarcoma 180 implanted in mice, as previously reported.³⁾ The antitumor activity of the polysaccharides against sarcoma 180 in mice is listed in Table II. U-3-N showed antitumor activity at doses of 1 and 5 mg/kg, although U-3-AP₁ had little effect on the tumor. The difference of the activity may be due to that of structure. Many (1→3)- β -D-glucans in the fruiting bodies of fungi are usually extracted by an alkaline solution, but the glucans (U-3-N and U-3-AP₁) in *Yü őr* were also extracted with hot water. The reason may be explained by the following results. Each glucan was an alkali-soluble, but the solution neutralized with acid was dialyzed against water to yield a water-insoluble material of the glucan. The neutral polysaccharides (U-3-N and U-3-AP₁) were dissolved, together with the acidic polysaccharide (U-3-A),

TABLE II. Antitumor Activity of the Polysaccharides against Sarcoma 180 Solid Tumor

Sample	Dose (mg/kg/d $\times$ 10)	Mean tumor wt. $\pm$ S.D. ^{a)} (g)	Inhibition ratio (%)	Complete regression
Exp. 1				
U-3-N	0.5	5.29 ± 2.98^b	51	0/8
	1	1.78 ± 1.78^c	84	2/8
	5	3.24 ± 2.21^c	70	1/8
Control	—	10.86 ± 4.79	—	0/8
Exp. 2				
U-3-AP ₁	0.5	6.50 ± 5.16	30	0/10
	1	8.50 ± 6.64	8	0/9
	5	7.78 ± 7.09	16	1/9
PSK	200	1.31 ± 1.24^d	86	0/8
Control	—	9.22 ± 5.51	—	0/10

a) Significant difference from the control [b] $p < 0.05$, [c] $p < 0.01$, [d] $p < 0.005$.

in 0.5 M sodium hydroxide, and the neutralized solution was dialyzed against water. The polysaccharides were yet soluble in water. The results suggested an interaction of the glucan with the acidic polysaccharide in the solution.

As described in the earlier articles,^{2,3)} the residue of hot-water extract was successively extracted with 5% sodium carbonate⁷⁾ and with 1 M sodium hydroxide, at room temperature. Antitumor β -(1→6)-branched (1→3)- β -D-glucan N-5-P was isolated from the sodium hydroxide extract, its properties were as follows (U-3-N in parentheses): $[\alpha]_D + 2.3^\circ$ ($+1.0^\circ$); molecular weight, 5.6×10^5 (6.1×10^5); branched ratio, 1/3 (1/3); max of complex with Congo red, 510 nm (501 nm); yield, 1.2% (1.24%). Thus, U-3-N was similar to N-5-P, although high-order structure may be slightly different. The work revealed that antitumor, branched (1→3)- β -D-glucan (U-3-N) was able to isolate from hot-water extract before alkaline extraction.

These findings are of interest in connection with a physiological role of these polysaccharides in the fruiting body of this fungus. The water-soluble mixture of U-3-N and U-3-AP₁ together with U-3-A may occur as cell-surface glycans, whereas N-5-P extracted with alkaline solution might arise from an intracellular component, or be present as a component of the cell wall.⁸⁾

Experimental

Materials The dried fruiting bodies of *Yü őr* (*Auricularia* sp.) are commercially available in Hong Kong.³⁾ Lysing enzyme was purchased from Sigma Chemical Company. Toyopearl HW-65 and DEAE-Toyopearl 650 M were purchased from Tosoh Industry Co., and standard dextrans and Bio-gel P-2 were purchased from Pharmacia Fine Chemicals and Bio-Rad Laboratories, respectively.

General All evaporations were conducted under diminished pressure at bath temperatures not exceeding 40 °C. Specific rotations were measured with a JASCO DIP-4 automatic polarimeter. IR spectra were recorded with a JASCO A-102 spectrometer. PPC was performed by the double-ascending method, using ADVANTEC TOYO No. 51B filter-paper and the following solvent system (v/v): 1-butanol-pyridine-water (6:4:3). Sugars were detected with an alkaline silver nitrate reagent.⁹⁾ GLC was performed in a Shimadzu GC-4CM apparatus equipped with a flame-ionization detector, using a glass column (0.3 $\times$ 200 cm) packed with 3% ECNSS-M on Gaschrom Q (100–120 mesh). Peak areas were measured with a Shimadzu Chromatopac C-R3A. GLC-MS was conducted with a JEOL JMS-D 300 apparatus equipped with a glass column (0.2 $\times$ 100 cm) packed with 3% ECNSS-M, at 190 °C, at a pressure of helium of 1.2 kg/cm². The MS were recorded at an ionizing potential of 70 eV, an ionizing current of 50 μ A, and a temperature of the ion source

of 210°C.

Isolation of the Polysaccharides After the successive extraction of the dried, pulverized fruiting bodies (85 g) with methanol (1 l, 20 h) and 70% aqueous ethanol (7 times, 2 l, 5 h), in a boiling water bath, the residue was extracted 4 times with water (1 l) for 5 h in a boiling water bath. The extracts were deproteinized, and the solution was precipitated by 2% CTAB to give the acidic polysaccharide (U-3-A), as reported previously.²⁾ The supernatant was precipitated by addition of 3 volumes of EtOH, and the precipitate was dissolved in water, then dialyzed against distilled water. Non-dialyzable solution was applied to a column of DEAE-Toyopearl 650 M (3 × 42 cm). The neutral fraction was precipitated by addition of 1 volume of EtOH. The precipitate was dispersed in water, and lyophilized to afford the purified polysaccharide (U-3-N), in 1.24% yield. The acidic fraction of DEAE-Toyopearl 650 M was dialyzed, lyophilized, and applied to a column of DEAE-Toyopearl 650 M, then the column was eluted by the gradient elution with NaCl solution (0 to 1 M). The peak 1 (No. 31–40) was dialyzed, and lyophilized to afford the purified polysaccharide (U-3-AP₁), in 0.63% yield.

Gel Filtration and Estimation of Molecular Weight Gel filtration of U-3-N and U-3-AP₁ on a column 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 constructed by the use of standard dextrans as previously reported.³⁾

Analysis of Component Sugar The polysaccharides (U-3-N and U-3-AP₁) were hydrolyzed with 2 M trifluoroacetic acid for 8 h at 100°C. The hydrolyzates were evaporated to remove the acid, and analyzed by PPC and GLC of alditol acetates, as previously described.³⁾

Methylation Analysis The polysaccharides (U-3-N and U-3-AP₁) was methylated 4 times by Hakomori's method¹⁰⁾ until they showed no hydroxyl absorption in the IR spectra, as previously described.¹¹⁾ The fully methylated glucans were heated successively with 90% formic acid for 6 h at 100°C, and with 0.25 M sulfuric acid for 15 h at 100°C. After neutralization of the acid with barium carbonate, the partially methylated sugars were converted into the alditol acetates. The resulting, partially methylated alditol acetates were analyzed by GLC and GLC-MS.¹²⁾

Periodate Oxidation and Smith Degradation Each sample (10 mg) was dissolved in 0.5 M NaOH (1 ml) and neutralized with 0.5 M HCl (1 ml), and water (18 ml) and 20 mM NaIO₄ (20 ml) were added. They were kept in the dark, with stirring, for 7 d at 4°C. An aliquot (1 ml) was taken at various times, and the periodate consumption was determined by an arsenite method.¹³⁾ The oxidized polysaccharides were reduced with sodium borohydride to give the polyalcohols as described previously.³⁾ They were treated successively with 90% formic acid for 6 h at 100°C and with 0.25 M sulfuric acid for 15 h at 100°C. The hydrolyzates were analyzed by GLC as the alditol acetates, using a programmed rise in temperature of 6°C/min from 60 to 185°C. The retention times of glycerol acetate and glucitol acetate were 13.8 and 39.5 min, respectively.

¹³C-NMR Spectroscopy The ¹³C-NMR spectra were recorded with a

JEOL-GX 270 spectrometer in the Fourier-transform mode, with complete proton-decoupling for solutions in Me₂SO-*d*₆ (ca. 100 mg/ml) at 60°C.

Enzymic Hydrolysis U-3-N (8.5 mg) was treated with *exo*-(1→3)-β-D-glucanase (Lysing enzyme; 4.0 mg) in 17 mM McIlvaine buffer, pH 4.88 (30 ml), at 37°C.³⁾ A portion (4 ml) of the reaction mixture was taken at various times (5, 10, 24, 48, and 72 h), and each was heated for 15 min at 100°C. Then, the solution was evaporated to ca. 0.5 ml, and applied to a column (1.5 × 97 cm) of Bio-gel P-2. The column was eluted with water, and fractions (1.3 ml each) were collected, and analyzed by the phenol-sulfuric acid method.¹⁴⁾ The molar ratio (*G*₂/*G*₁) of products was calculated from the peak area.

Interaction with Congo Red in Aqueous Sodium Hydroxide Each Sample (0.2 mg/ml) was dissolved in sodium hydroxide (0.10, 0.25, and 0.50 M) containing Congo red (50 μM), and λ_{max} was measured using a Hitachi 323 spectrophotometer.

Antitumor Activity The antitumor activity against the solid form of sarcoma 180 in mice was tested as described in a previous report.³⁾ The inhibition ratios were calculated by use of the formula: inhibition ratio (%) = [(*A* - *B*)/*A*] × 100, where *A* is the average tumor weight of the control group, and *B* is that of the tested group.

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