Carbohydrate Research, 96 (1981) 105–111
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

A NOVEL GLYCOSAMINOGLYCAN FROM THE FUNGUS *Omphalia lapidescence**

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(Received February 3rd, 1981; accepted for publication, March 23rd, 1981)

ABSTRACT

The major, water-soluble polysaccharide isolated from the sclerotia of *Omphalia lapidescence*, and purified by chromatography on DEAE-cellulose and zone electrophoresis, is a heteroglycan (OL-1), $[\alpha]_D^{20}$ +56° (*c* 1, water), which is composed of D-glucose, 2-acetamido-2-deoxy-D-glucose, and D-glucuronic acid in the molar ratios 1.0 : 1.95 : 2.0. The results of periodate oxidation, Smith degradation, and methylation analysis showed that the polysaccharide has a highly branched structure involving 1,3-, 1,4-, 1,6-, and 1,3,6-linked D-glucopyranosyl, 1,3,4-linked 2-acetamido-2-deoxy-D-glucopyranosyl, and (1,3 ?)- and 1,4-linked D-glucopyranosyluronic acid residues. The non-reducing terminal positions are occupied by the three component sugars. The branched glycosaminoglycan OL-1 is the first to be found in the fungi.

INTRODUCTION

Omphalia lapidescence is a fungus that belongs to the Tricholomataceae, and the sclerotia of the fungus have medicinal properties. We have reported[1] on the chemical structure of an alkali-soluble β-D-glucan of the sclerotia and now describe the isolation and characterisation of a water-soluble heteroglycan from this source.

RESULTS AND DISCUSSION

An ethanol-precipitate obtained from the hot-water extract of the sclerotia contained neutral sugar[3] (52.0%, as glucose), amino sugar[4] (19.4%, as 2-amino-2-deoxyglucose), uronic acid[5] (22.5%, as glucuronic acid), and protein[6] (9.3%, as

*Studies on Fungal Polysaccharides, Part XXVIII. For Part XXVII, see ref. 2. A part of this work was presented at the 93rd Annual Meeting of the Pharmaceutical Society of Japan (Tokyo, April 1973).
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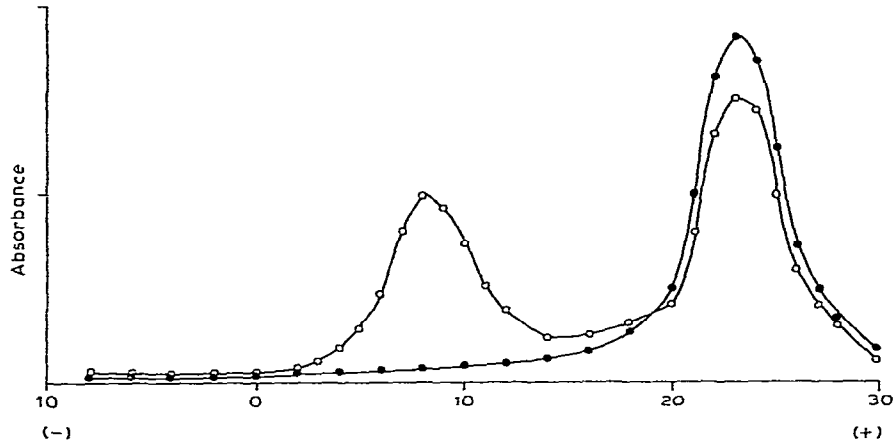

Fig. 1. Zone electrophoresis of the major fraction: —○—, absorbance at 620 nm (anthrone–H_2SO_4); —●—, absorbance at 530 nm (carbazole–H_2SO_4).

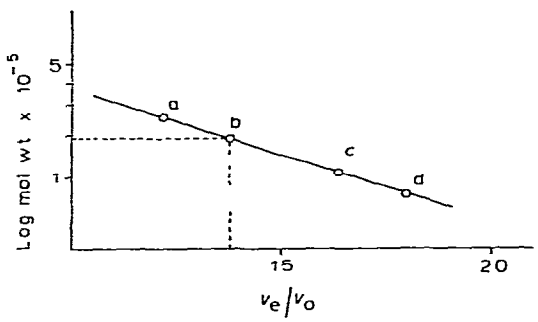

Fig. 2. Determination of molecular weight of OL-1 on Sepharose 6B: a, dextran T-250; b, OL-1; c, dextran T-110; d, dextran T-70.

bovine serum albumin). For the examination of the carbohydrate portion, the precipitate was treated with Pronase E and by the Sevag method, followed by chromatography on DEAE-cellulose. The major fraction (eluted with 0.3M sodium hydrogencarbonate) was further purified by zone electrophoresis in borate buffer (see Fig. 1).

The purified polysaccharide (OL-1) had $[\alpha]_D^{20}$ +56° (*c* 1, water), gave a single spot in electrophoresis on glass-fibre paper in borate buffer (0.026M, pH 9.2) and acetate buffer (0.05M, pH 5.6), and was homogeneous on ultracentrifugation. Gel filtration of OL-1 on Sepharose 6B gave a narrow, single peak with a molecular weight of ~190,000 (see Fig. 2). It contained 2.6% of nitrogen, but no phosphorus (Fiske–SubbaRow method[7]).

Component sugars of OL-1 were identified (p.c.) as D-glucose, 2-acetamido-2-deoxy-D-glucose, and D-glucuronic acid in the molar ratios 1.0:1.95:2.0 (assayed by literature procedures[4,5,9]), and 2-amino-2-deoxy-D-glucose was furthermore identified by h.p.l.c. of the acid hydrolysate, which also contained insignificant

TABLE I

PERIODATE OXIDATION OF OL-1

	Time (h)										
	0.5	1	3	6	9	12	24	36	72	96	120
IO_4^- (mol/unit)	0.05	0 17	0 34	0 43	0 53	0.54	0.62	0.73	0.77	0.79	0.79
HCO_2H (mol/unit)	0.01	0.04	0.13	0 16	0.18	0.19	0 22	0 23	0 23	0 23	0.23

TABLE II

G L.C. DATA FOR ALDITOL ACETATES DERIVED FROM METHYLATED GLUCANS

Component	*Retention time*[a]				*Molar ratio*	
	ECNSS-M column		*OV-225 column*		*OL-1*	*Reduced OL-1*
	OL-1	*Authentic compound*	*Reduced OL-1*	*Authentic compound*		
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol	1.00	1.00	1.00	1.00	2 7	4 1
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol	1.95	1.95	1.83	1.82	2 3	2.7
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol	2.49	2.49	2 21	2.20	2 6	2.6
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol	2.50	2.50	2 32	2 32	1.9	5.9
1,3,5,6-Tetra-*O*-acetyl-2,4-di-*O*-methylglucitol	5.10	5.10	4.22	4.21	1.0	1 0

[a]Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

TABLE III

G.L.C. DATA[a] FOR ALDITOL ACETATES DERIVED FROM METHYLATED OL-1

Component	*Retention time*[b]		*Molar ratio*
		Authentic compound	
2-Acetamido-1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methylglucitol	0 29	0.29	1 0
2-Acetamido-1,3,4,5-tetra-*O*-acetyl-2-deoxy-6-*O*-methylglucitol	0.59	0 59	2 0

[a]Column: neopentylglycol sebacate polyester. [b]Relative to 2-acetamido-1,3,4,5,6-penta-*O*-acetyl-2-deoxy-D-glucitol.

quantities of amino acids. The p.m.r. spectrum of OL-1 contained a signal at δ 2.0 assigned[8] to NAc, and the i.r. spectrum contained bands at 1735, 1630, and 890 cm^{-1} indicative[8,10,11] of carboxyl, acetamido, and β-glycosidic linkages, respectively. The nitrogen content corresponded reasonably to the amino sugar content.

OL-1 consumed 0.7 mol of periodate per "anhydro sugar" unit, releasing 0.23-mol of formic acid, but no formaldehyde (Table I). Some of the three component-sugars of OL-1 survived Smith degradation, but glycerol and erythritol were formed, and detected with alkaline silver nitrate[12].

OL-1 was methylated by the Hakomori[13] and Purdie[14] methods and then hydrolysed, and the products were converted into alditol acetates and analysed by g.l.c., using 3% of ECNSS-M, 5% of OV-225, and 10% of neopentylglycol sebacate polyester. The results, shown in Tables II and III, suggested the presence of 1,3-, 1,4-, 1,6-, and 1,3,6-linked D-glucopyranosyl residues, and non-reducing terminal D-glucopyranosyl and 2-acetamido-2-deoxy-D-glucopyranosyl groups. The formation of 2-acetamido-1,3,4,5-tetra-*O*-acetyl-6-*O*-methyl-D-glucitol suggested the presence of 1,3,4-linked 2-acetamido-2-deoxy-D-glucopyranosyl residues.

When OL-1 was esterified[15] and then reduced with borohydride, D-glucuronic acid could not be detected (p.c.) in an acid hydrolysate. The alditol acetates derived from reduced and methylated OL-1 were analysed by g.l.c. using ECNSS-M (3%) and OV-225 (5%) columns. The results in Table II suggest that glucuronic acid may be present as 1,4-linked and non-reducing end-groups, with a small proportion of 1,3-linked residues, because of the increased amounts of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-, and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitols.

Mass spectrometry of the di-*O*-methyl fraction indicated it to be 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol (*m/z* 43, 87, 117, 129, and 233).

Thus, OL-1 appears to have a highly branched structure involving 1,3-, 1,4-, 1,6-, and 1,3,6-linked D-glucopyranosyl, 1,3,4-linked 2-acetamido-2-deoxy-D-glucopyranosyl, and (1,3 ?)- and 1,4-linked D-glucopyranosyluronic acid residues. Some of the D-glucopyranosyl and D-glucopyranosyluronic acid residues and approximately one-third of the 2-acetamido-2-deoxy-D-glucopyranosyl residues are non-reducing end-groups. The branch points involve D-glucopyranosyl and 2-acetamido-2-deoxy-D-glucopyranosyl residues.

OL-1 is the first branched glycosaminoglycan to be found in a fungus. Details of the chemical structure and biological activity of OL-1 will be reported elsewhere.

EXPERIMENTAL

The glycosaminoglycan from Omphalia lapidescence. — (*a*) *Isolation.* Crushed, dried sclerotia (100 g) of the fungus (pre-extracted with methanol and ethyl acetate) were stirred with distilled water for 8 h at 100°. This procedure was repeated until the extract gave a negative anthrone reaction[16]. After centrifugation, the supernatant solution was dialysed (Visking cellophane tubing) against running water for 2 days

and then concentrated *in vacuo*. The precipitate (1.7 g) formed on the addition of ethanol was collected by centrifugation, washed with ethanol, acetone, and ether, and dried *in vacuo*.

(*b*) *Analysis*. The crude polysaccharide was analysed for neutral sugar[13], hexosamine[4], uronic acid[5], and protein[6].

(*c*) *Protease digestion*. A solution of the crude, water-soluble polysaccharide (1.5 g) in water (300 ml) was adjusted to pH 7.0 with aqueous 1% sodium hydroxide, Pronase E (150 mg, Kaken Kagaku Co. Ltd., Tokyo) was added, and the mixture was stored at 37° for 5 days, and then dialysed against running water for 3 days, concentrated to 100 ml, and shaken vigorously for 0.5 h with chloroform–1-butanol (30 ml, 4:1). The procedure was repeated until the gelatinous precipitate was no longer formed. After centrifugation, the supernatant solution was concentrated under diminished pressure at <35°, and 4 vol. of ethanol were added to the concentrate The resulting precipitate (1.3 g) was collected by centrifugation, and dried *in vacuo*.

(*d*) *Purification*. A solution of the crude polysaccharide (1 g) was applied to a column (3.5 × 50 cm) of DEAE-cellulose (HO^- form). Stepwise elution was performed with water, 0.15, 0.3, and 0.5M sodium hydrogencarbonate, and finally 0.1M sodium hydroxide at 100 ml/h. Each fraction was assayed by using the anthrone reagent[16], dialysed (Visking cellophane tubing) against running water for 3 days and then against distilled water, and concentrated *in vacuo* to a small volume. Ethanol (10 vol.) was added, and each resulting precipitate was collected by centrifugation, and treated as described above. The yields were as follows: water eluate, 7.5 mg; 0.15M salt eluate, 250 mg; 0.3M eluate, 400 mg; 0.5M eluate, 100 mg, and 0.1M sodium hydroxide eluate, 200 mg. A portion (50 mg) of the 0.3M salt eluate was subjected to zone electrophoresis, using "Pevicon" (Polyvinyl resin, M & S Instruments, Japan) as the supporting medium (7 × 40 cm) for 6 h in 0.026M sodium borate (pH 9.2) under an applied current of 40 A. The zone was cut into 40 segments and each was quantitatively extracted with distilled water (10 ml). The sugar content of each segment was determined by using the anthrone[16] and carbazole reagents[5]. The yield of the purified, major fraction (OL-1) was 0.4% of the sclerotia. OL-1 was subjected to chromatography on a column (2.6 × 95 cm) of Sepharose 6B, using water, after calibration with standard dextrans (Fig. 2). The p.m.r. spectrum (D_2O, lock signal DDS, at 28°) of OL-1 was measured by using a Varian NV-14 instrument (60 MHz).

Investigation of OL-1. — (*a*) *Component sugars*. A solution of OL-1 (10 mg) in 0.5M H_2SO_4 (2 ml) was heated at ~100° in a sealed tube for 8 h, and then neutralised ($BaCO_3$), filtered, concentrated, and subjected to chromatography (ascending) on Toyo Roshi No. 50 filter paper with *A*, ethyl acetate–pyridine–water (10:4·3); and *B*, ethyl acetate–pyridine–acetic acid–water (5:5:1:3), and detection with alkaline silver nitrate[12] and *p*-anisidine–HCl[17].

The presence of glucose, 2-amino-2-deoxy-D-glucose, and glucuronic acid, together with small proportions of unidentified oligomers, was revealed. The major oligomer gave 2-amino-2-deoxy-D-glucose on hydrolysis with 2M HCl at 100° for

10 h. Quantification of the component sugars in OL-1 (50 mg) revealed D-glucose, 9.4 mg (D-glucose oxidase method[9]); 2-amino-2-deoxy-D-glucose, 23.3 mg (modified method of Blix[4]); and D-glucuronic acid, 19.7 mg (method of Bitter–Muir[5]). The acid hydrolysate was also analysed by using an Hitachi Model 835 automatic amino acid analyser equipped with a column (4 × 150 mm) of Hitachi 2619 ion-exchanger; a sodium citrate buffer (835 Mitsubishi Chemical Industries Ltd.) was used with a stepwise gradient: pH 1, 2, 3, and 4.

(*b*) *Properties.* Paper electrophoresis of OL-1 in 0.026M borate buffer (pH 9.2) or 0.2M acetic buffer (pH 5.6) showed a single spot (detected with the periodate–Schiff reagent[18]).

(*c*) *Periodate oxidation.* A solution of OL-1 (44.3 mg) in distilled water (20 ml) was made up to 50 ml with 0.22M sodium periodate (4 ml) and distilled water. The mixture was stored in the dark at room temperature. The consumption of oxidant and the amounts of formic acid and formaldehyde released were determined on samples (3 ml) as previously described[1].

(*d*) *Smith degradation.* OL-1 (44.3 mg) was oxidised with periodate as described in (*c*). Excess of periodate was reduced with ethylene glycol (0.4 ml); after 72 h, the solution was dialysed against running water for 24 h and then concentrated to ~20 ml, and sodium borohydride (~100 mg) was added. The mixture was stirred overnight, acidified with acetic acid, dialysed against running water for 2 days, and concentrated. The syrupy residue was hydrolysed with 0.5M H_2SO_4 (3 ml) at ~100° for 6 h. The hydrolysate was neutralised ($BaCO_3$), filtered, concentrated *in vacuo* to a small volume, and subjected to p.c. (solvent *A*).

(*e*) *Esterification and reduction.* To a solution of OL-1 (100 mg) in water (50 ml) was added propylene oxide (20 ml). The solution was stirred at 25° for 14 days, dialysed against running water for 2 days, and concentrated to ~20 ml. A solution of sodium borohydride (5 g) in water (40 ml) was added dropwise during 2 h. The solution was stored overnight and, after decomposition of excess of borohydride with acetic acid, concentrated; methanol (2 × 20 ml) was then evaporated from the residue. Treatment of the residue with ethanol–acetone (5 : 1) gave a precipitate that was collected and dissolved in water (50 ml). The solution was filtered, concentrated, and treated with propylene oxide as described above. A portion of the resulting, reduced material was hydrolysed as described above, and examined by p.c.

(*f*) *Methylation.* Methylation was performed in the usual manner[13,14]. Methylated OL-1 and methylated, reduced OL-1 showed no significant i.r. absorption for hydroxyl at ~3500 cm^{-1}.

Each methylated polysaccharide was heated with 90% formic acid at 100° for 4 h. Formic acid was distilled off, and the residue was hydrolysed with 0.5M H_2SO_4 for 8 h. Reduction and acetylation were then performed as previously described[1]. The resulting alditol acetates were subjected to g.l.c., using a Shimadzu GC-5A unit, equipped with a flame-ionisation detector and a glass column (200 × 0.3 cm) packed with 3% of ECNSS-M on Chromosorb W (aw-dmcs, 60–80 mesh), at 180°; N_2 flow-rate, 50 ml/min. The di-*O*-methyl derivative prepared from OL-1 was dissolved

in acetone and subjected to g.l.c.–m.s , using a Shimadzu Model LKB-9000 mass spectrometer, equipped with a glass column packed with 3% of Silicone OV-225 on Chromosorb W (aw-dmcs, 80–100 mesh), and operated at 170°; 70 eV; trap current, 60 μA; temperature of the ion source, 310°.

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