Carbohydrate Research, 87 (1980) 257–264
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

STRUCTURAL ANALYSIS OF THE ALKALI-SOLUBLE POLYSACCHARIDE FROM THE SCLEROTIA OF *Grifora umbellata* (Fr.) PILÁT

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(Received June 25th, 1980; accepted for publication, July 18th, 1980)

ABSTRACT

An alkali-soluble polysaccharide (**AP**), $[\alpha]_D$ +14° (*c* 0.5, 5% sodium hydroxide), was isolated from the sclerotia of *Grifora umbellata*. **AP** was a homogeneous β-glucan and the molecular weight was determined by gel filtration to be ~1,200,000. From the results of methylation study, Smith degradation, and enzymic hydrolysis, it was concluded that **AP** is a polysaccharide composed of a backbone of β-(1→3)-linked D-glucopyranosyl residues, and possessed of a single β-D-glucopyranosyl group joined through O-6 of every third D-glucopyranosyl residue of the backbone.

INTRODUCTION

Grifora umbellata is a fungus that belongs to the Polypolaceae basidiomycetes, and is parasitic on animate roots of alder and Japanese oak. The sclerotia of this fungus have been used as a diuretic in Chinese medicine. In 1973, Miyazaki and Oikawa[1] proposed a possible structure for the water-soluble polysaccharides from the sclerotia. Furthermore, they reported that these polysaccharides inhibit the growth of subcutaneously implanted Sarcoma 180 in mice[2,3]. On the other hand, the insoluble residue from the water extraction was found to contain polysaccharides similar to the water-soluble one, and we have already reported the extraction procedure for polysaccharides from the sclerotia and an outline of their structures[4].

This article is concerned with structural analysis of the alkali-soluble polysaccharide in the sclerotia of *G. umbellata*.

RESULTS AND DISCUSSION

The defatted sclerotia of *G. umbellata* were successively extracted with hot water and 10% aqueous zinc chloride solution. The residual material was extracted several times with 10% sodium hydroxide containing 5% of urea at 4°. After neutralization and dialysis, the alkali-soluble polysaccharide (**AP**) was purified through the copper–polysaccharide complex (yield, 21% on the basis of the defatted sclerotia). **AP** was insoluble in water, but soluble in 5% sodium hydroxide, and it was found, by g.l.c. analysis after methanolysis and trimethylsilylation, to be composed of

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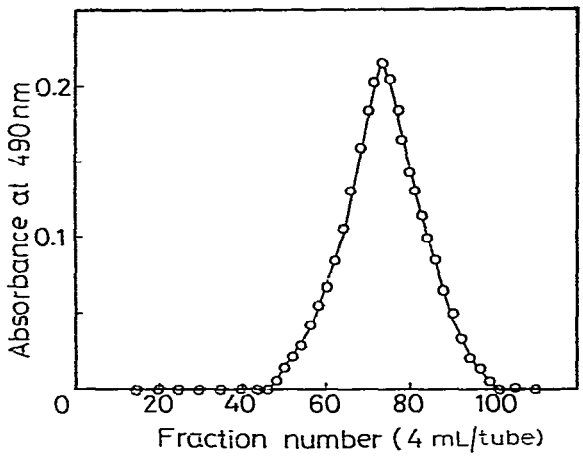


Fig. 1. Gel filtration of AP on a column (2.5 × 95 cm) of Sepharose CL-2B. [AP (2 mg) in 0.2M sodium hydroxide (1 mL) was eluted with 0.2M sodium hydroxide, and fractions (4 mL) were assayed by the phenol–sulfuric acid method.]

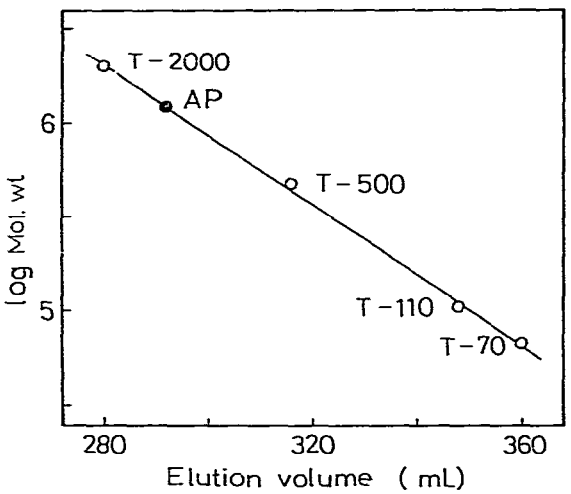


Fig. 2. Determination of molecular weight of AP by Sepharose CL-2B gel-filtration. [The column (2.5 × 95 cm) was standardized with dextrans T-2000 (mol. wt. 2,000,000), T-500 (500,000), T-110 (110,000), and T-70 (70,000). The carbohydrate elution-volume was plotted against the logarithm of the molecular weight of the carbohydrate.]

D-glucose only; it had $[\alpha]_D$ +14° (*c* 0.5, 5% sodium hydroxide). The i.r. spectrum showed an absorption at 895 cm^{-1}, characteristic of the β-glucosidic linkage, indicating that **AP** is a glucan that mainly contains β-glucosidic linkages.

AP was subjected to gel filtration with Sepharose CL-2B (see Fig. 1). The elution profile of **AP** showed a single peak. From the elution volume (see Fig. 2), the molecular weight of **AP** was determined to be ~1,200,000.

AP was methylated by the procedure of Hakomori, and the methylation product

TABLE I

PRODUCTS OBTAINED BY HYDROLYSIS OF METHYLATED AP AND DERIVATIZATION

Alditol acetate corresponding to	*Relative retention time*	*Primary mass fragments*	*Relative peak-area*
2,3,4,6-Tetra-*O*-methyl-D-glucose	**1.00**	205,161,117,45	**1.00**
2,4,6-Tri-*O*-methyl-D-glucose	1.85	233,161,117,45	2.39
2,4-Di-*O*-methyl-D-glucose	4.57	233,189,117	0.89

was hydrolyzed with acid. The hydrolyzate was analyzed by g.l.c. of the derived alditol acetates. Three peaks that appeared in the chromatogram were identified as the corresponding alditols from 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-*O*-methyl-D-glucose, by comparison of their retention times with those of authentic samples under the same conditions. From the areas under the peaks, the molar ratios of these compounds were determined to be 1:2.4:0.9. Furthermore, the compounds were characterized by g.l.c.–m.s. (see Table I). The results of methylation analysis indicated that **AP** contains mainly (1→3)-linkages, and has branching at O-6 of (1→3)-linked D-glucopyranosyl residues.

The glucosidic linkages assigned were also supported by the results of periodate oxidation and Smith degradation. **AP** was oxidized with 0.02M sodium metaperiodate at 4° (see Fig. 3). After complete oxidation (16 days; periodate consumption, 0.45 mol. and formic acid production, 0.25 mol per glucose residue), the oxidized **AP** was reduced with sodium borohydride, to yield the corresponding polyalcohol (**AP-I**). A portion of the resulting **AP-I** was completely hydrolyzed with acid. G.l.c. of the hydrolysis products as the corresponding alditol acetates indicated the presence of glycerol and D-glucose. Smith degradation of **AP-I** afforded a soluble product, the

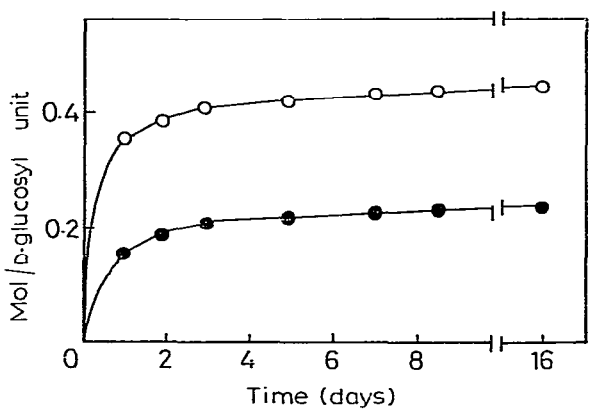
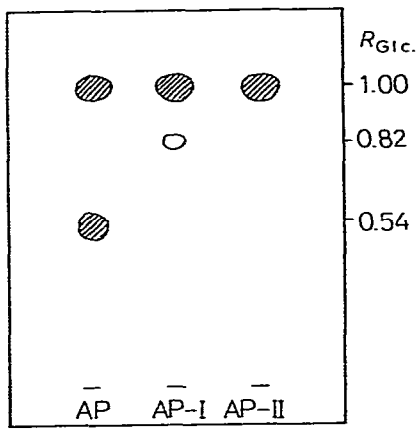


Fig. 3. Oxidation of **AP** in 0.02M sodium metaperiodate at 4°. [Key: ○, periodate consumption; ●, formic acid production.]



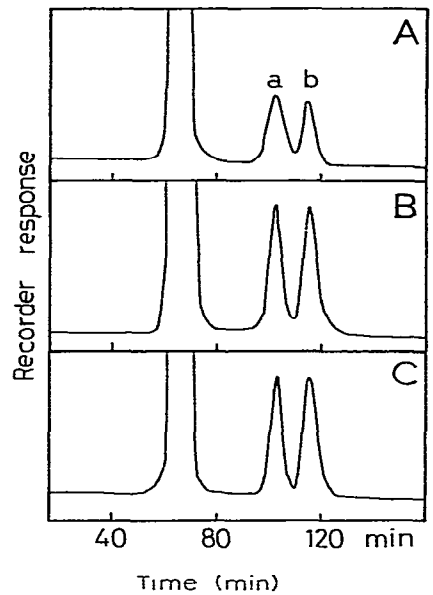


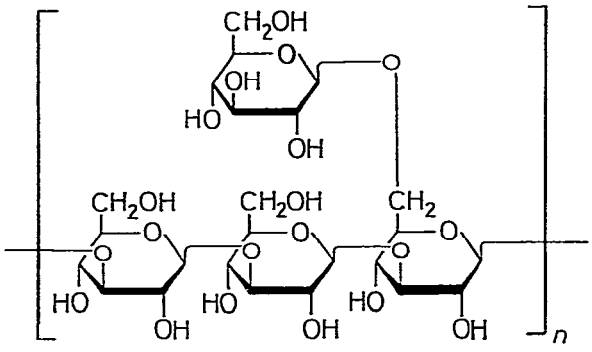
Fig. 4. Paper chromatogram of the enzymic digests of **AP**, **AP-I**, and **AP-II**.

Fig. 5. Elution patterns of the enzymic digests of **AP** at different incubation times. [Incubations were conducted for (A) 30 min, (B) 90 min, and (C) 180 min, respectively. After incubation, each sample was applied to a column (1.5 × 45 cm) of Dowex 50-W X-4 resin, and eluted with water at a flow rate of 0.2 mL per min. Peak a is D-glucose, and peak b is gentiobiose.]

p.c. analysis of which showed that it was glycerol only. Nonhydrolyzed material (**AP-II**), recovered in 60% yield (on **AP-I**), was resistant to further oxidation. These results are in good agreement with those expected from the methylation analysis.

AP, **AP-I**, and **AP-II** were treated with exo-(1→3)-β-D-glucanase; D-glucose and gentiobiose (R_{Glc} 0.54) were liberated from **AP**, and D-glucose and 6-*O*-substituted D-glucose (R_{Glc} 0.82) from **AP-I**. On the other hand, only D-glucose was liberated from **AP-II** (see Fig. 4). The results indicate that **AP** consists of a (1→3)-linked β-D-glucopyranosyl backbone, partially branched on O-6 of D-glucosyl residues of the backbone. When **AP** was completely hydrolyzed by the enzyme, the reducing power was 74% of the total sugar. Moreover, during the enzymic hydrolysis of **AP**, the yields of D-glucose and gentiobiose were constant, in the peak-area ratio of 1:1 (see Fig. 5); this value corresponds to the molar ratio of 2:1 for D-glucose to gentiobiose. These findings indicate that the branching of **AP** occurs regularly at O-6 of every third D-glucopyranosyl residue of the β-(1→3)-linked backbone, and that each side chain is composed of only one D-glucosyl group.

From the foregoing results, the structure of **AP** may represented by the repeating unit shown in formula **1**. This structure is very similar to that observed for sclerotan[5,6] and schizophyllan[7]. However, the molecular weight of **AP** is very much larger than those of these polysaccharides.



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A water-soluble glucan from *G. umbellata* has been reported to possess antitumor activity against Sarcoma 180 in mice[2,3]. Moreover, high antitumor activity against Sarcoma 180 has been demonstrated for a (1→3)-β-D-glucan having β-D-glucopyranosyl groups (1→6)-linked to every third or fourth residue of the main chain[8].

EXPERIMENTAL

Materials. — Sclerotia of *G. umbellata* were collected in Hokkaido, Japan, in the Autumn of 1975. exo-(1→3)-β-D-Glucanase was prepared from a culture of *Basidiomycetes* QM 806 according to the method described by Reese and Mandels[9]. The purified enzyme had 234 units of specific activity per mg as assayed by the method of Houtari *et al.*[10].

General methods. — All evaporations were conducted under diminished pressure at 40–45°. Specific rotations were determined at 20° with an Ohyodenki Model MP-1T automatic polarimeter. Infrared (i.r.) spectra were recorded with a Jasco IRA-1 infrared spectrometer. Paper chromatography (p.c.) was performed on Toyo No. 50 filter paper by the multiple, ascending method, with the following solvent systems (v/v): (*A*) 12:5:4 ethyl acetate–pyridine–water and (*B*) 4:1:1 1-butanol–acetic acid–water. The compounds on the chromatograms were located with the alkaline silver nitrate[11]. Gas–liquid chromatography (g.l.c.) was performed in a Shimadzu GC-4APF apparatus fitted with a flame-ionization detector. The glass column (0.3 × 200 cm) was packed with 3% of ECNSS-M on Neopak 1A (80–100 mesh), and (*1*) operated at 165°, or (*2*) programmed from 120 to 190° at 4°/min, with a gas flow-rate of 40 mL of helium per min. Peak areas were measured with a Shimadzu ITG-4A digital integrator. Gas–liquid chromatography–mass spectrometry (g.l.c.–m.s.) was conducted with a Hitachi Model M-52 apparatus equipped with a glass column (0.3 × 100 cm) packed with 2% of OV-1 on Chromosorb W (80–100 mesh) at 160°, and mass spectra were recorded at an ionizing potential of 20 eV.

Enzymic hydrolysis of the polysaccharides with exo-(1→3)-β-D-glucanase was performed by incubating the sample in 0.05M acetate buffer (pH 4.8) for 18 h at 37°. Total sugars were estimated by the phenol–sulfuric acid method[12]. Reducing sugars were determined by the Somogyi–Nelson method[13].

Preparation of **AP**. — The defatted sclerotia (100 g) were successively extracted 9 times with hot water (1.000 L) and 8 times with 10% aqueous zinc chloride (1.000 L), as previously described[4]. The residual material was suspended in 10% sodium hydroxide containing 5% of urea (1.000 L) for 24 h at 4°. The alkaline suspension was centrifuged, and the resulting, brownish extract was collected. The extraction was repeated (6 times) with the same solution until the extract showed a negative 1-naphthol reaction. The extracts were combined, made neutral with 3M hydrochloric acid at 4°, and de-ionized by dialysis against running tap-water. The gelatinous dialyzate was successively washed with ethanol and ether, and dried *in vacuo*; yield 25 g.

The crude, alkali-soluble polysaccharide was dissolved in 1% sodium hydroxide (2.500 L), and then 7% aqueous copper sulfate (2.500 L) was added dropwise to the solution, with stirring. The resulting copper–polysaccharide complex was collected by centrifugation, and the polysaccharide regenerated by the usual method[14]. Purified, alkali-soluble polysaccharide (**AP**) was thus obtained; yield 21 g.

The sugar composition was determined as follows. **AP** (5 mg) was heated with M hydrogen chloride in methanol (0.2 mL) for 24 h at 100°, the solution evaporated, and the product per(trimethylsilyl)ated with hexamethylsilazane and chlorotrimethylsilane in pyridine. The compounds were determined by g.l.c. (condition *1*). Only peaks corresponding to D-glucose appeared on the chromatogram. **AP** had $[\alpha]_D$ +14° (*c* 0.5, 5% sodium hydroxide), and showed at 895 cm^{-1} an absorption band characteristic of a β-D-glucosidic linkage. The sugar content of **AP** was determined to be 92% (as D-glucose) by the phenol–sulfuric acid method.

Gel filtration. — A solution (1 mL) of **AP** (2 mg) in 0.2M sodium hydroxide was applied to a column (2.5 × 95 cm) of Sepharose CL-2B. The column was equilibrated, and eluted, with 0.2M sodium hydroxide, and the effluent was collected in 4-mL fractions. The carbohydrate content of each fraction was determined by the phenol–sulfuric acid method. The column was calibrated with the following dextrans: T-2000 (mol.wt., 2,000,000), T-500 (500,000), T-110 (110,000), and T-70 (70,000), which are products of Pharmacia Fine Chemicals. The results are given in Figs. 1 and 2.

Methylation analysis. — A portion (10 mg) of **AP** was methylated three times by the Hakomori procedure[15]. The reaction mixture was dialyzed against running tap-water for 3 days, and the nondialyzable fraction was dried. The product showed no absorption for free hydroxyl groups in its i.r. spectrum. The methylated **AP** was hydrolyzed with 90% formic acid for 2 h at 100°, and then with 0.25M sulfuric acid for 12 h at 100°. (After hydrolysis with formic acid, this acid was removed by evaporation before treatment with the sulfuric acid.) The acid was neutralized with barium carbonate, and the neutral hydrolyzate was evaporated to dryness. The sugars thus

obtained were converted into their alditol acetates[16] for g.l.c. analysis (condition *1*) and g.l.c.–m.s. analysis. The results are summarized in Table I.

Periodate oxidation and Smith degradation. — A suspension of **AP** (2.0 g) in 0.02M sodium metaperiodate (500 mL) was kept at 4° in the dark with continuous stirring. At intervals, the periodate consumption was determined by the Fleury–Lange method[17], and, after reduction of the excess of periodate with ethylene glycol, formic acid liberated was titrated with 0.01M sodium hydroxide. **AP** consumed 0.45 mol of periodate and released 0.24 mol of formic acid per D-glucosyl unit (see Fig. 3). After completion of the oxidation, the oxidized **AP** solution was dialyzed against running tap-water for 2 days, and the product was collected by centrifugation, dissolved in 0.1M sodium hydroxide (80 mL), and reduced with sodium borohydride (1 g) for 24 h at room temperature. The excess of borohydride was decomposed by addition of M acetic acid, and the reaction mixture was dialyzed against running tap-water, and then lyophilized. A white powder of **AP** polyalcohol (**AP-I**) was obtained; yield 1.5 g.

A portion (4 mg) of **AP-I** was heated with 90% formic acid (2 mL) at 100° and then with 0.5M sulfuric acid (2 mL) for 3 h at 100°. The acid was neutralized with barium carbonate, and the neutral solution was evaporated to dryness. The sugars thus obtained were converted into their alditol acetates, and these were subjected to g.l.c. (*condition 2*). Two peaks, corresponding to D-glucose and glycerol, were detected.

In another experiment, **AP-I** (100 mg) was subjected to hydrolysis with 0.25M sulfuric acid (50 mL) for 20 h at room temperature. The acid was neutralized with M sodium hydroxide, the solution dialyzed against de-ionized water for 3 days, and the nondialyzable fraction lyophilized. This product was designated Smith-degraded **AP** (**AP-II**); yield 60 mg. The dialyzable fraction was de-ionized with Amberlite IR-120 (H^+) and IRA-410 (OH^-) resins, and the solution was concentrated to a small volume. P.c. with solvent *A* revealed the presence of glycerol only.

Enzymic hydrolysis. — **AP**, **AP-I**, and **AP-II** (5 mg each) were incubated with exo-(1→3)-β-D-glucanase (234 units) in 10 mL of 0.05M acetate buffer (pH 4.8) for 18 h at 37°. After inactivation of the enzyme by heating for 10 min at 100°, each incubation mixture was analyzed by p.c. with solvent *B*. The results are given in Fig. 4.

In another experiment, **AP** (25 mg) was dissolved in 0.1M sodium hydroxide (10 mL), and then the pH was adjusted to 4.8 with 0.1M acetic acid. This solution was incubated with exo-(1→3)-β-D-glucanase (2340 units) in 10 mL of 0.05M acetate buffer (pH 4.8) at 37°, and the reducing power of the reaction mixture was monitored at intervals. The reducing power increased with the incubation time, and reached a maximum value at 24 h (74% of the total sugar). The reaction mixture at 30 min (the reducing sugar was 27.8% of the total sugar), at 90 min (45% of the total sugar), and at 180 min (57.3% of the total sugar) was each applied to a column (1.5 × 45 cm) of Dowex 50-W X-4 resin and eluted with distilled water at a flow rate of[18] 0.2 mL per min, the elution profile being recorded with a Shodex RI SE-11 differential

refractometer. The results are shown in Fig. 5. Definite amounts of D-glucose and gentiobiose were applied to the column in order to calculate their peak-area ratio.

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