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# Structure and Properties of an Extracellular Polysaccharide from *Laetisaria arvalis*. Evaluation of its Antitumour Activity

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*ABSTRACT*

*A polysaccharide isolated from the culture filtrate of* Laetisaria arvalis *(Basidiomycete) has been submitted to acid hydrolysis, periodate oxidation, Smith degradation, methylation analysis, and treatment with* endo-*(1→3)-β-D-glucanase. A 6-O-branched (1→3)-β-D-glucan with a structure similar to that of scleroglucan has been identified. In aqueous solution, this polysaccharide develops high viscosities and shows pseudoplastic behaviour. Increasing NaOH concentration reduces viscosity, which is due to a similar conformational transition to that found for scleroglucan.*

*The antitumour activity of the glucan was tested against solid Sarcoma-180 in mice. The polysaccharide strongly inhibited tumour growth with an inhibition ratio of almost 100%.*

## INTRODUCTION

In the last two decades, a number of extracellular polysaccharides have been isolated from the culture broth of various fungi and a great variety of neutral and acidic homo- and heteropolymers have been identified (Berthellet *et al.*, 1984; Catley, 1984). Among them (1→3)-β-D-glucans have attracted much attention in view of their functional properties in solution and their inhibitory action on the growth of certain tumours in

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animals (Chihara, 1978). The present authors have isolated a viscous material from the culture filtrate of *Laetisaria arvalis* (Basidiomycete), and it was of interest to investigate its properties. This paper is concerned with the structural analysis of this extracellular polysaccharide: the functional properties of solutions have been compared with that of scleroglucan, a fungal polysaccharide of industrial interest and, in addition, antitumour activity has been tested against solid Sarcoma-180 implanted in mice.

## MATERIALS AND METHODS

### Materials

A strain belonging to the mycological collection of the Laboratory of Cryptogamy (CMPG; Collection Mycology Pharmacy Grenoble) was used in this study: *Laetisaria arvalis* Burdsall (CMPG 934) was isolated from soil samples of Lake Powel (Colorado, USA). The strain was stored at +4°C on a gelose medium with malt extract (1·5%). The fungus was grown on the same medium at 24°C for eight days and used for the inoculum. Cultivation was performed at 24°C in 150 ml shaken flasks containing 100 ml of Galzy & Slonimski (1957) medium with 3% (w/v) glucose and corn steep liquor (1·5%). The pH was 4·1.

After five days shaking (180 rpm) mycelium was separated from polysaccharide by dilution of the culture broth and filtration. The polysaccharide (P-I) was precipitated from the filtrate by adding 1 volume of ethanol, washed with increasing concentration of ethanol (50–95%) and dried *in vacuo* at 25°C (0·2 g/100 ml of culture).

Scleroglucan (SCL) was obtained from Mero-Rousselot-Satia (France).

### General methods

Evaporation was conducted under reduced pressure at temperature not exceeding 45°C.

High-performance liquid chromatography (HPLC) of the products from acid and enzymatic hydrolysis was performed on a Waters Associate equipment using an HPX-87 P column (Biorad) for determination of neutral sugars and an HPX-87 H column (Biorad) for examination of uronic acid content. The sugars were monitored with a refractive-index detector.

Gas-liquid chromatography (GLC) of neutral or methylated sugars after conversion into the corresponding alditol acetates, was usually performed with a Hewlett-Packard gas chromatograph model 5890, on a fused silica wide bore column (0·53 mm × 30 m) packed with SP 2380. A temperature program was used: 165°C for the first 4 min then 1·5°C/min up to 225°C.

**Composition of polysaccharide**

The polysaccharide (10 mg) was hydrolysed with 70% sulphuric acid (1 ml) for 30 min at room temperature. The solution was diluted with water (6 ml) and kept at 100°C for 16 h. After neutralization with barium carbonate, the hydrolysate was filtered, concentrated to 1 ml and filtered again. A portion (500 $\mu$l) was used for analysis of neutral sugars after removal of salt with ion-exchange resin (Amberlite MB-3). Uronic acid was assayed using the other portion.

**Enzymatic hydrolysis with *exo*-(1 → 3)-$\beta$-D-glucanase**

The polysaccharide (P-I) (25 mg) was incubated in 0·1 M acetate buffer (pH 4·6, 25 ml) with *exo*-(1 → 3)-$\beta$-D-glucanase prepared from a culture of Basidiomycete QM-806 (Peterson & Kirkwood, 1975) (2·5 mg) at 50°C. When the reaction was complete (two days), the mixture was heated for 10 min in boiling water and filtered (Sartorius membrane, 3 $\mu$m).

**Methylation analysis**

A sample of P-I (20 mg) was methylated by the method of Hakomori (1964). After methylation, the reaction mixture was diluted with water, dialysed and freeze-dried. The methylation procedure was repreated twice following the method of Purdie & Irvine (1903). Methylated polysaccharide (P-II) was hydrolysed with 90% formic acid at 100°C for 1 h and then heated with 2 M trifluoroacetic acid for 4 h at 100°C. Methylated sugars were converted into their alditol acetates and analysed by GLC.

**Periodate oxidation (Goldstein *et al.*, 1965)**

P-I (100 mg) was oxidized with 0·05 M sodium metaperiodate (200 ml) at room temperature in the dark for 72 h. The reaction was stopped by the

addition of ethylene glycol (8 ml per 100 mg) and the solution was dialysed against water for 24 h. The solution was concentrated and the oxidized polysaccharide was reduced with sodium borohydride (160 mg) with stirring for 15 h at room temperature. Acetic acid (0·1 M, pH = 5·5) was added, the solution dialysed and the polyalcohol P-III was collected by freeze-drying.

The polyalcohol (P-III) (20 mg) was treated with 0·25 M sulphuric acid (40 ml) and kept at 25°C for 24 h. The Smith degraded polysaccharide (P-IV) was recovered by adding ethanol (1·5 volumes) and centrifugation. The supernatant was neutralized, concentrated, filtered, deionized and analysed by HPLC.

P-IV was dialysed against water for 48 h, then freeze-dried. A portion was treated with *exo*-(1 → 3)-β-D-glucanase under the same conditions of hydrolysis of P-I.

## $^{13}$C-NMR spectra

$^{13}$C-NMR spectra were recorded with a Bruker AC 300 spectrometer operating at 75·47 MHz in the pulsed Fourier-transformed mode. All spectra were recorded in dimethylsulphoxide-$d_6$ at 65°C. $^{13}$C Chemical shifts are expressed in ppm downfield from the central peak of DMSO at 39·5 ppm. The solution viscosity was reduced by ultrasonic depolymerization of the polysaccharide.

## Viscosity measurements

Viscosities were determined as a function of the shear rate using a Contraves Low Shear 30 viscometer.

## Assay of antitumour activity

Seven day-old Sarcoma-180 ascites (0·1 ml, $5 \times 10^6$ cells) were transplanted subcutaneously into the right side of female CD1 mice (weight 23 g). The test samples, dissolved in saline solution, were intraperitoneally injected daily for 10 days, starting 24 h after tumour implantation. At days 10, 20 and 30, the tumour area was determined with a caliber square. At day 30, the mice were killed and weighed. The inhibition ratio, expressed in percent, was calculated by comparing the average weight of the tumours of treated mice to that of untreated controls.

## RESULTS AND DISCUSSION

### Structure

The polysaccharide P-I was obtained from the viscous culture as a white fibrous precipitate. After the solvent had been drained off and the fibrous mass dried, the finished product contained not more than 15% moisture and the nitrogenous materials were equivalent to less than 0·6% nitrogen. Only D-glucose was produced on sulphuric acid hydrolysis. GLC of the methylated alditol acetates revealed 2,3,4,6-tetra-*O*-methyl-, 2,4,6-tri-*O*-methyl-, and 2,4-di-*O*-methylglucitol acetates in the approximate molar ratios 1:2:1. P-I is a 6-*O*-branched (1→3)-D-glucan (Table 1).

P-I was incubated with *exo*-(1→3)-β-D-glucanase and the digest analysed for sugar constituents by HPLC. The digest contained only D-glucose and gentiobiose in a molar ratio of about 2:1, indicating that P-I is a D-glucan, composed mainly of β-glucosidic linkages. The periodate-oxidized P-I was reduced to the corresponding polyalcohol P-III, which on acid hydrolysis yielded D-glucose and glycerol. Mild acid hydrolysis of P-III gave an insoluble glucan P-IV and glycerol.

When digested with *exo*-(1→3)-β-D-glucanase, P-IV gave only D-glucose. These findings, as shown in Table 1, were consistent with a single D-glucopyranosyl side branch for each three linear D-glucopyranosyl units. Although it is not possible to deduce the full structure, a repeating unit may be proposed that is very similar to that observed for scleroglucan derived from *Sclerotium rolfsii* (Rodgers, 1973).

Comparison of the $^{13}$C-NMR spectra of P-I and SCL in dimethylsulphoxide-$d_6$ at 65°C is shown in Fig. 1. Assignment of all the carbon signals was achieved by comparison with the results of Rinaudo & Vincendon (1982). A great similarity was demonstrated and confirmed the results of chemical analysis. The polysaccharide of *Laetisaria arvalis* has a regular structure, it is a β-(1→3)-glucan with a degree of branching of one pendant glucose unit on every three monomeric units of the main chain as shown in Fig. 2.

### Rheological properties

6-*O*-Branched (1→3)-β-D-glucans, like scleroglucan or schizophyllan, exhibit functional properties in solution, due to their rigid rod-like structure (Rodgers, 1973; Sandford & Baird, 1983). The main properties of the solutions are a temperature-stable high viscosity and pseudoplastic behaviour. The system is substantially stable between pH 1 and 10 and

**TABLE 1**
Results from Different Methods of Structural Analysis

| *Hydrolysis methods* | *Samples* | | | |
|---|---|---|---|---|
| | *P-I* | *P-II* | *P-III* | *P-IV* |
| Acid hydrolysis | D-Glucose | 2,3,4,6-Tetra-*O*-Me-glucitol (0·8)<br>2,4,6-Tri-*O*-Me-glucitol (2·4)<br>2,4-Di-*O*-Me-glucitol (1) | D-Glucose<br>Glycerol | D-Glucose |
| Enzymatic hydrolysis | D-Glucose (2)<br>Gentiobiose (1) | | | D-Glucose |

(Numbers in parentheses = molar ratios.)

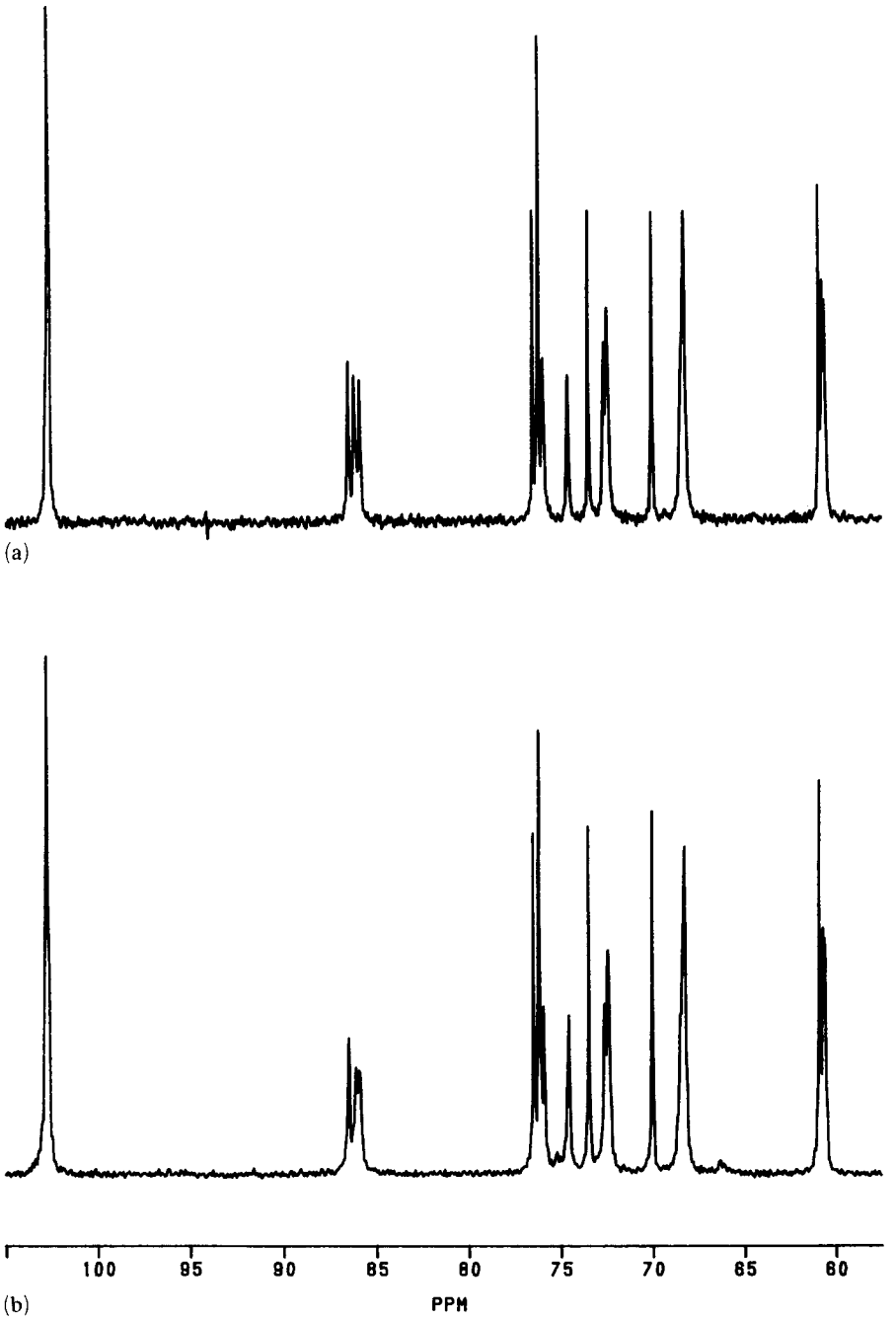


**Fig. 1.** $^{13}$C-NMR spectra of (a) scleroglucan and (b) polysaccharide of *Laetisaria arvalis* in DMSO-$d_6$ solution at 65°C.

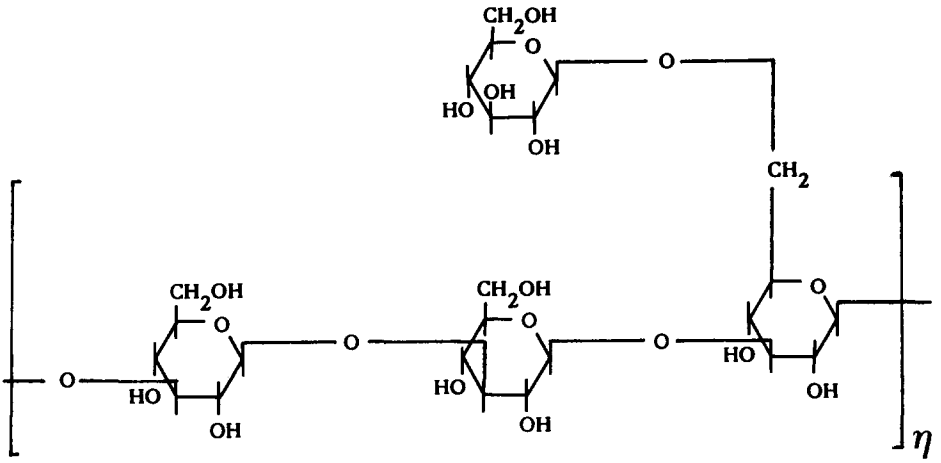


**Fig. 2.** Repeating unit proposed for the chain of extracellular polysaccharide of *Laetisaria arvalis*.

compatible with a variety of electrolytes. Above pH 12, the decrease in viscosity has been attributed to a conformational transition, triple helix to coil form. Such properties make this type of polysaccharide very interesting for numerous applications in food, industrial or pharmaceutical areas, so the rheological properties of the polysaccharide of *Laetisaria arvalis* have been compared with those of a commercial sample of scleroglucan.

The relative viscosities of P-I and SCL (0·5 g/l of each) ($\eta/\eta_0$ with $\eta$ the solution viscosity and $\eta_0$ the solvent viscosity) in water, as a function of the shear rate ($\dot{\gamma}$), are given in Fig. 3. It can be seen that the magnitudes are similar and that, at this concentration, $\eta$ depends markedly on $\dot{\gamma}$ within the range used. Both solutions are found to be pseudoplastic. As seen in Fig. 3, the shear rate dependence of $\eta$ is almost negligible at the lowest shear rate. The measurements of $\eta$ in the Newtonian regime as function of the polymer concentration, enables the intrinsic viscosity, $[\eta]$, to be determined; $[\eta] = 6800$ ml/g for P-I and $[\eta] = 9600$ ml/g for SCL. At this point, it would be important to characterize the polysaccharides by molecular weight, but, unfortunately, it is very difficult to filter P-I solutions through 0·45 $\mu$m Millipore membranes, so light-scattering measurements are difficult to interpret.

Assuming the $[\eta]$–$\overline{M_w}$ relationship established by Yanaki *et al.* (1980) with schizophyllan can be used, viscosity-average molecular weights, $\overline{M_v}$, were estimated for P-I; $\overline{M_v} = 3{\cdot}9 \times 10^6$. With a similar calculation on the scleroglucan data, $\overline{M_v} = 4{\cdot}6 \times 10^6$ is obtained, a value comparable with

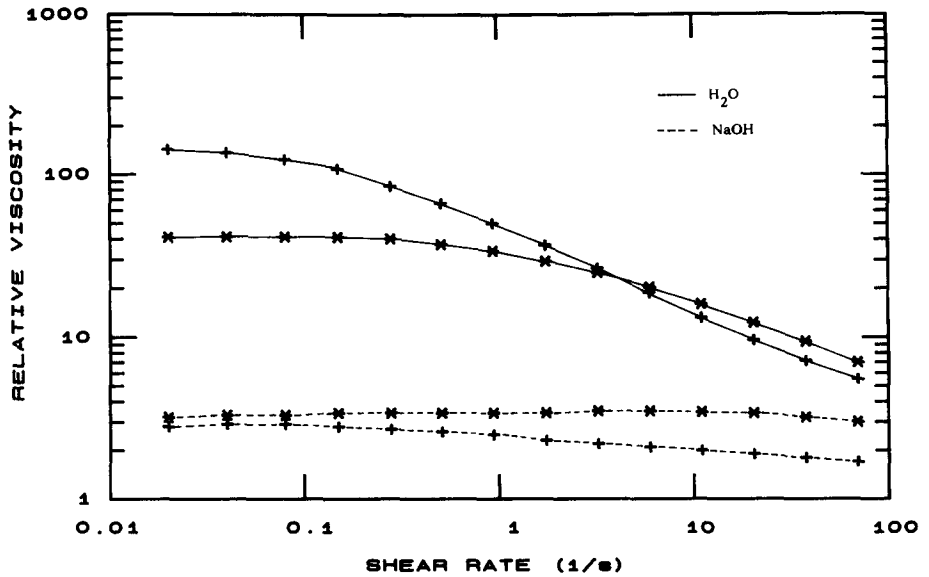


Fig. 3. Relative viscosity versus shear rate of 0·5 g/litre SCL (*) and P-I (+) solutions in water (——) and 0·1 M NaOH (- - - -) at 25°C.

the data published by Bo *et al.* (1987) ($\overline{M_w} = 3{\cdot}9 \times 10^6$ in 0·01 N NaOH) and Lecacheux *et al.* (1986) ($\overline{M_w} = 5{\cdot}7 \times 10^6$ in water).

Scleroglucan and schizophyllan have been shown to be in the triple-stranded helix form in water at 25°C, but in 0·1 M NaOH, the two polysaccharides exist as a single randomly coiled chain (Bo *et al.*, 1987).

Figure 3 illustrates the viscosity dependence on NaOH concentration for samples P-I and SCL. Similar behaviour was observed for P-I and SCL. From these results and literature data it can be concluded that an order–disorder transition from triple-helix to coil occurs with P-I, which is characteristic of branched (1→3)-β-D-glucans (Yanaki *et al.*, 1980; Yanaki & Norisuye, 1983; Bo *et al.*, 1987).

## Antitumour activity

Several (1→3)-β-D-glucans with (1→6)-β-D-glucose side chains are known to exhibit antitumour activity. This antitumour activity is thought to be host-mediated by stimulation of the host's immune system (Hamuro & Chihara, 1984; Furue, 1987). Because of structural similarities with antitumour-active glucans like schizophyllan, lentinan or *Phytophthora* glucan (Kikumoto *et al.*, 1970; Sasaki & Takasaka, 1976; Bruneteau *et al.*, 1988), it is of interest to investigate the antitumour effects of the branched (1→3)-β-D-glucan of *Laetisaria arvalis*.

**TABLE 2**
Antitumour Activity of *Laetisaria arvalis* Polysaccharide Against Sarcoma-180: Evaluation of Tumour Weight at Day 30

| *Substance* | *Dose (mg/kg)* | *Average tumour weight (g)* | *Inhibition*[a] *(%)* | *Complete*[b] *regression* | *Significance*[c] $p <$ |
|---|---|---|---|---|---|
| Control | — | 4·180 | — | 0/15 | — |
| Glucan/934 | 0·2 | 0·220 | 95 | 9/10 | 0·0001 |
| | 1·0 | 0·006 | 99 | 9/10 | 0·0001 |
| | 5·0 | 0·070 | 98 | 9/10 | 0·0001 |

[a] $(C - T/C) \times 100$, where $C$ = average tumour weight of the control group and $T$ = average tumour weight of the treated group.
[b] Number of tumour free mice/number of treated mice.
[c] Evaluated according to Student's $t$-test.

The results of the bioassay are summarized in Table 2, where it is obvious that the glucan from the culture filtrate of *Laetisaria arvalis* exhibits strong antitumour activity. At all concentrations tested the inhibition ratio was higher than 95%, and 9 out of 10 animals showed complete tumour regression. This antitumour effect against Sarcoma-180 is comparable to that of schizophyllan and lentinan. Further studies concerning the antitumour activity and the mode of action of the glucan from *Laetisaria arvalis* are in progress.

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