Carbohydrate Polymers **17** (1992) 177–183

Structural analysis and rheological behaviour of an extracellular polysaccharide from *Drechslera spicifera*

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(Received 26 October 1990; revised version received 4 December 1990; accepted 17 December 1990)

The fungus *Drechslera spicifera* produces a high viscosity extracellular glucan polymer in Galzy and Slonimski medium containing glucose. This polysaccharide submitted to methylation study, Smith degradation and enzymic hydrolysis was concluded to be composed of a backbone of β (1 → 3)-linked D-glucopyranosyl residues and possessed two single branches of a β-D-glucopyranosyl residue joined through C-6 for every five D-glucopyranosyl residues of the backbone as lentinan from *Lentinus edodes*. In water solution, this polysaccharide developed a high viscosity, ten times the viscosity of scleroglucan, and showed good thermal stability. Its behaviour was investigated as a function of the sodium hydroxide concentration. At low NaOH concentration the viscosity rises, due to an increase in solubility and probably to interchain interactions with proteins. When the NaOH concentration increased, there was a rapid and irreversible decrease in the viscosity attributed to a conformational transition.

INTRODUCTION

Extracellular polysaccharides with various degrees of structural complexity frequently occur in fungal cultures. Many of them have been shown to be branched glucans with a (1 → 3)-β-D-glucans linked backbone having single glucopyranosyl units at intervals along the chain in the (1 → 6)-β-D-glucans configuration. The degree of substitution was reported to vary from organism to organism between 10 and 80% (Clarke & Stone, 1963; Berthellet *et al.*, 1984). Because of their functional properties in solution and their antitumour effect, these polysaccharides have attracted much attention and correlations between structure and activities have been attempted (Chihara, 1978; Misaki *et al.*, 1981). In this way, we have investigated different fungi belonging to the mycological collection of the laboratory (Aouadi *et al.*, 1990) and among them, a strain of *Drechslera spicifera* (Bain) v. Arx.

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Drechslera spicifera (Deuteromycotinae) is an agent of phaehyphomycosis, a cutaneous, subcutaneous or systemic disease that develops in tissue in the form of dark-walled, septate mycelium (Polonelli & Morace, 1985). It produces an extracellular viscous polysaccharide. The purpose of this study was to isolate and characterize the exopolysaccharide and to compare its properties with scleroglucan.

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: *Drechslera spicifera* (CMPG 731) was isolated from a sample of crab shell from the North Sea (De Hoog *et al.*, 1985). The strain was kept at +4°C on a gelose medium with malt extract (1·5%). The fungus grown on the same

Carbohydrate Polymers 0144-8617/91/$03.50

medium at 24°C for 8 days was used as an inoculum. Cultivation was performed at 24°C in 250-ml shake flasks containing 100 ml of Galzy and Slonimski medium (1957) with 3% (w/v) glucose. The pH value was 4·2 after autoclaving.

After 5 days of shaking (180 rpm), mycelium was separated from the polysaccharide by dilution of the culture broth and filtration. The glucan was obtained from the clear filtrate by precipitation with 1·5 volumes of ethanol. The filamentous mass was collected, and resuspended in water. After 24 h of shaking the exopolysaccharide (EPS) was reprecipitated as above. The precipitate was washed several times with increasing concentration of ethanol (60 to 95%) and dried *in vacuo* at 25°C for 48 h (98 mg/100 ml). Scleroglucan is manufactured by Mero-Rousselot-Satia (France).

Acid hydrolysis

Ten milligrams of EPS was hydrolysed in 70% sulphuric acid (1 ml) for 30 min at room temperature. The solution was diluted with water (6 ml) and kept at 100°C overnight. The hydrolysate was neutralized and analysed after concentration and filtration.

Enzymatic hydrolysis

Exo (1 → 3)-β-D-glucanase from Basidiomycete QM-806 was used (Peterson & Kirkwood, 1975). A suspension of the glucan (25 mg) in 0·1 M sodium acetate buffer (25 ml, pH 4·6) containing 2·5 mg of enzyme was stored at 50°C for 2 days. After heat inactivation (100°C, 10 min), filtration (Sartorius membrane, 3 μm) and concentration (100 μl), the digest (3 μl) was analysed by HPLC.

HPLC

High performance liquid chromatography (HPLC) was performed on a Waters Ass. equipment (Millford, Mass.) using a CHO-682 column (Interchim) for analysis of mono- and disaccharides. The sugars were monitored with a refractive index detector.

Smith degradation (Goldstein *et al.*, 1965; Kato *et al.*, 1983)

Solutions of samples (100 mg) in 15 mM sodium periodate (200 ml) were stored at 22°C in the dark. The oxidized glucan was reduced by sodium borohydride (160 mg), with stirring, for 20 h at room temperature. The glucan polyalcohol (P-I) was subjected to mild hydrolysis with 0·25 M sulphuric acid for 20 h at 22°C. The mixture was precipitated by addition of an equal volume of ethanol. The supernatant liquor was de-ionized with Amberlite MB3 resin, and subjected to HPLC analysis. The precipitate was incubated with exo-(1 → 3)-β-D-glucanase under the same conditions of hydrolysis as the polysaccharide.

Methylation analysis

The polysaccharide was methylated by the Paz Parente *et al.* method (Paz Parente *et al.*, 1985). EPS (500 μg) was dissolved in dimethyl sulphoxide (150 μl) in a teflon-lined screw-cap tube. Lithium methylsulphinyl carbanion (150 μl) was added under an inert atmosphere and the mixture was sonicated for 60 min then left for 2 h at 20°C. After cooling to −4°C, cold methyl iodide (300 μl) was added. A new sonication was conducted for 60 min. The methylation was stopped by addition of water (2 ml) containing thiosulphate and the permethylated polysaccharide was extracted with chloroform (3 × 2 ml). After washing of the chloroform phase with water (4 × 2 ml), it was dried, filtered, concentrated and freeze dried.

Analysis of methyl ethers

The permethylated EPS was treated with 0·5 M methanolic HCl (500 μl) for 24 h at 80°C. The methyl ethers were analysed after peracetylation in 1:1 pyridine-acetic anhydride (100 μl) overnight at 20°C according to Fournet *et al.* (1981).

Gas liquid chromatography

Methyl sugars were acetylated and analysed by GLC (Girdel 300) using a silicone OV-101 capillary column (25 m × 0·2 mm), a temperature programme from 140 to 225°C at 2°C/min, with flame-ionization detection. The results were confirmed by GLC coupled to a quadripolar R-1010C Nermag mass spectrometer. The temperature programme was slightly modified, 100 to 240°C at 5°C/min.

^{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 central peak of DMSO at 39·5 ppm. High viscosity of solutions at 20 g/litre, have been reduced by ultrasonic depolymerization of polysaccharides.

Viscosity measurements

The viscosities were determined as a function of the shear rate in a low shear viscometer Contraves 30 at a controlled temperature.

Optical rotation

Optical rotation was measured on 0·1% (w/v) polysaccharide solution in water with a Fica spectropolarimeter, model spectropol 1b, operating at 300 nm with a 5 cm thermostated cell. The temperature was controlled by a Haake circulating water bath.

RESULTS AND DISCUSSION

Production and chemical composition

Upon acid hydrolysis, the polysaccharide material isolated from the culture medium by precipitation with ethanol gave only glucose; the nitrogenous materials determined by microanalysis were equivalent to 1·9% nitrogen. In order to ascertain the effect of the carbon source on the extracellular production, *Drechslera spicifera* (731) was grown by flask-shaking cultivation at 24°C for 5 days in Galzy and Slonimski medium (100 ml) containing 3% of D-glucose or other carbohydrates. As shown in Table 1, no difference in composition was observed, best results were obtained with glucose. In the following, only polysaccharide obtained on glucose medium was considered. Effect of medium-pH on the production was studied (Table 2). The amount of polysaccharide secreted was shown to decrease when the pH increased, so pH = 4·5 was chosen.

Structure

The structure of the glucan was elucidated by methylation analysis, Smith degradation and by degradation with exo-(1 → 3)-β-D-glucan hydrolase. Complete degradation of the glucan with an exo-(1 → 3)-β-D-glucanase of Basidiomycete QM 806 (Peterson & Kirkwood, 1975) gave only gentiobiose and D-glucose in the molar ratio 1:1·5. On mild hydrolysis with 0·25 M sulphuric acid, the polyalcohol (P-I) gave the degraded glucan (P-II) with simultaneous liberation of glycerol only. When digested with exo-(1 → 3)-β-D-glucanase, P-II gave only D-glucose. The polysaccharide was methylated by the method of Paz Parente *et al.* (1985) and the fully methylated polysaccharide was methanolized. GLC analysis of the methyl sugars after acetylation revealed the presence of 2,3,4,6-tetra=, 2,4,6-tri=, and 2,4-di-*O*-methyl-D-glucose (molar ratios, 0·8:1·58:1). This result indicated that the D-glucan consisted mainly of (1 → 3)-linked D-glucosyl residues, two out of five (1 → 3)-linked D-glucosyl residues being substituted at O-6. The foregoing data indicate a β-D-glucan with the possible structure as shown in Scheme I.

Table 1. Effects of various carbohydrates as carbon source on the production of extracellular polysaccharide by *Drechslera spicifera*

Carbohydrate	Yield (mg per 100 ml of broth)	Composition of polysaccharide
D-Glucose	98	Glucose
D-Mannose	78	Glucose
D-Xylose	57	Glucose
D-Mannitol	24	Glucose
D-Galactose	15	Glucose
Sucrose	67	Glucose
Maltose	43	Glucose
Lactose	10	Glucose
Trehalose	15	Glucose

Table 2. Effects of the initial pH of medium on the production of extracellular polysaccharide by *Drechslera spicifera*

Initial pH of medium	Yield (mg per 100 ml of broth)
4·5	96
5·5	73
6·5	50

The extracellular polysaccharide secreted by *Drechslera spicifera* is structurally related to many other fungal polysaccharides, in particular, these of *Botrytis cinerea* (Dubourdieu *et al.*, 1981) and *Lentinus edodes* (Chihara *et al.*, 1969). There is a main chain containing (1 → 3)-linked β-D-glucopyranosyl residues and generally a single (1 → 6)-linked β-D-glucopyranosyl group linked to the backbone. The frequency of the side chain depends on the species, and probably also on the culture conditions (Buck *et al.*, 1968).

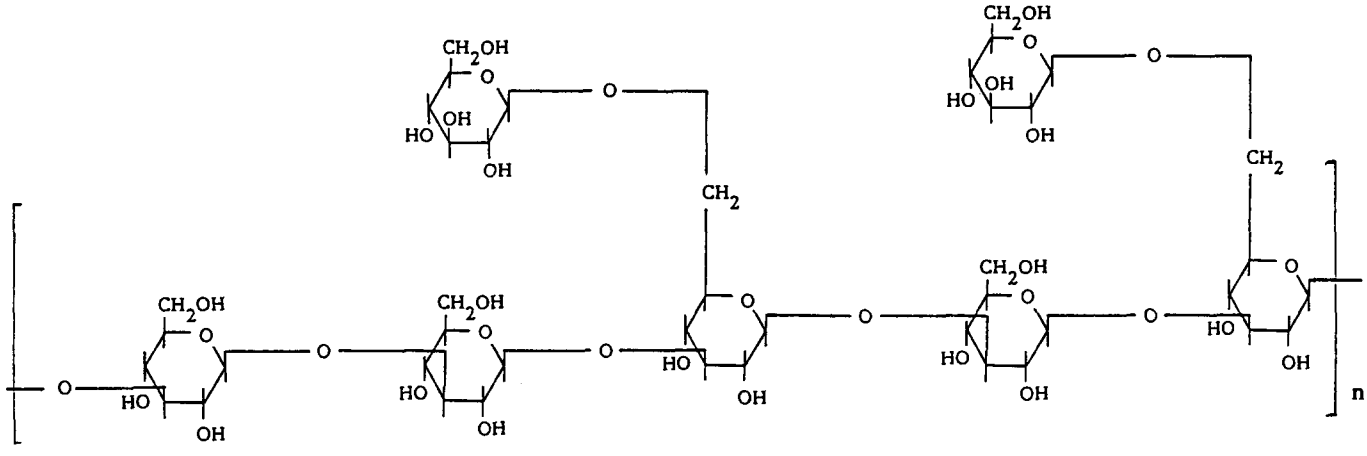
^{13}C-NMR studies

An interesting method for structural characterization of these β-D-glucans is nuclear magnetic resonance (NMR). Rapid information on the degree of branching can be obtained from ^{13}C spectra by comparison with the ^{13}C-NMR spectrum of scleroglucan, a well-known exopolysaccharide produced by *Sclerotium rolfsii* (Rodgers, 1973) with a degree of branching of one pendant glucose unit every three monomeric units of the main chain. Both spectra are represented on Fig. 1 with the assignment of the carbon signals for the scleroglucan according to Rinaudo & Vincendon (1982). An increase in the degree of branching leads to some modifications. In particular in the region of C-3 and C-6 signals with a relative increase on peak heights of the D and C units, a decrease on the signals of the B unit and the appearance of signals due to an E unit with chemical shift closely similar to those of the A unit.

Rheological properties

General behaviour

Like the other 6-*O*-branched (1 → 3)-β-D-glucans, the polysaccharide of *Drechslera spicifera* exhibits a



Scheme 1. Repeating unit proposed for the chain of extracellular polysaccharide of *Drechslera spicifera*

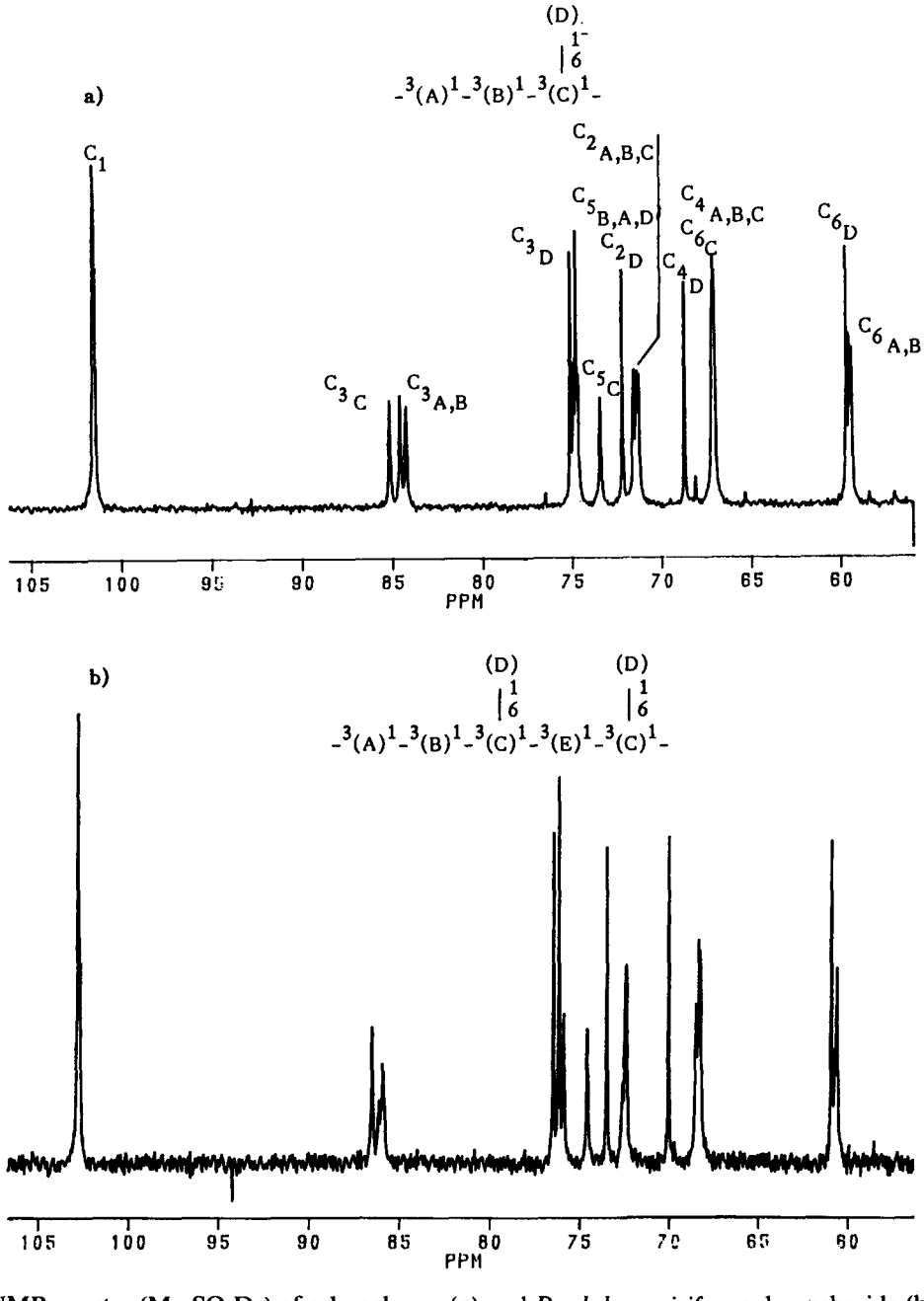


Fig. 1. ^{13}C-NMR spectra (Me_2SO-D_6) of scleroglucan (a) and *Drechslera spicifera* polysaccharide (b) at 60°C.

Table 3. Effect of heating on the relative visocity (η_{rel}) of *Drechslera spicifera* polysaccharide solutions in different conditions of pH ([C] = 0·25 g/litre; γ = 0·15 s^{-1})

Solution	Heating conditions					
	25°C/24 h	40°C/24 h	60°C/5 h	60°C/24 h	80°C/1 h	80°C/24 h
H_2O	43	43	43	43	43	43
2·5 M HCl	80	241	195	Gel	159	Gel
5 × 10^{-2} M NaOH	255	255	—	249	—	193
1 M NaOH	1·7	—	—	—	—	—

temperature-stable high viscosity and a pseudoplastic behaviour over a wide range of pH values. Table 3 shows the relative viscosities measured at 25°C after 24 h of heating at different temperatures under acidic and basic conditions. In 2·5 M HCl, the increase was attributed to a slow hydrolysis of β-(1 → 6) linkages up to formation of a gel due to the obtaining of a linear glucan consisting of β-(1 → 3)-linked D-glucopyranosyl units. In 5 × 10^{-2} M NaOH solution, the increase of viscosity was correlated to an increase in solubility (Bo *et al.*, 1987) and a good stability was observed. On increasing the sodium hydroxide concentration, a rapid and irreversible decrease was observed as obtained for scleroglucan by Bo *et al.* (1987). This point will be discussed later. The effects of ionic strength are negligible and the viscosity was insensitive to the presence of divalent salts such as $CaCl_2$ (Table 4).

Table 4. Relative viscosities of *Drechslera spicifera* polysaccharide solutions in different ionic strengths and salt conditions ([C] = 0·5 g/litre, γ = 0·08 s^{-1})

Solution	η_{rel}
H_2O	750
0·1 M NaCl	662
1·7 M NaCl	739
0·3 M $CaCl_2$	741

Viscosity studies

The viscosities of the exopolysaccharide from *Drechslera spicifera* were measured at various shear rates at different concentrations (0·45–0·02 g/litre) in H_2O, in 5 × 10^{-2} M NaOH and in 10^{-1} M NaOH solutions. Figure 2 shows the general behaviour of a 0·45 g litre solution in the three solvents. Scleroglucan behaved the same way in water and in NaOH while the polysaccharide of *Dreschlera spicifera* behaved differently. In NaOH, its viscosity was six to seven times higher than in water. Moreover its viscosity (0·45 g litre) was more than ten-fold higher than scleroglucan. With very dilute solutions (0·02 to 0·2 g/litre), this difference did not show and the intrinsic viscosities extrapolated from values obtained with these concentrations were very similar to the ones obtained with scleroglucan. As a matter of fact, by extrapolating plots of reduced viscosity, calculated in the Newtonian regime versus the concentration, we obtained in H_2O, $[\eta]$ = 14 100 ml/g and $[\eta]$ = 9700 ml/g in 5 × 10^{-2} M NaOH; the last value is very similar to the one determined by Bo *et al.* (1987) on scleroglucan. We feel that this

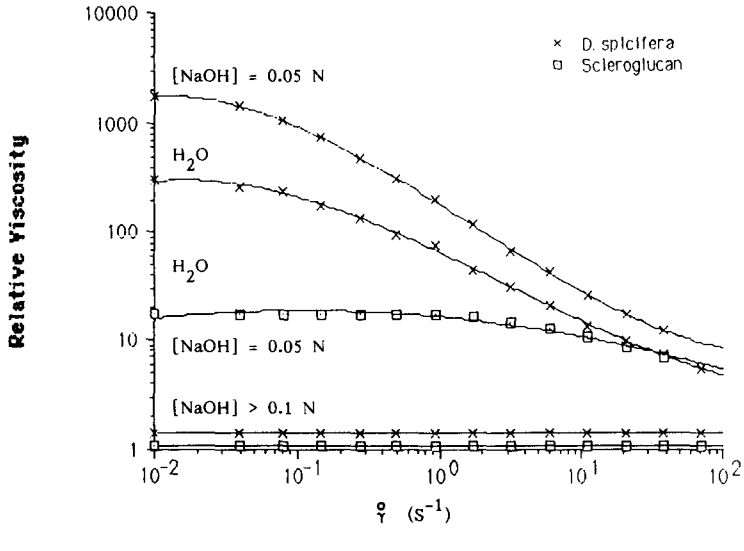


Fig. 2. Relative viscosities versus shear rate for 0·45 g/litre solutions of *Drechslera spicifera* polysaccharide (×) and scleroglucan (□) in different NaOH concentrations.

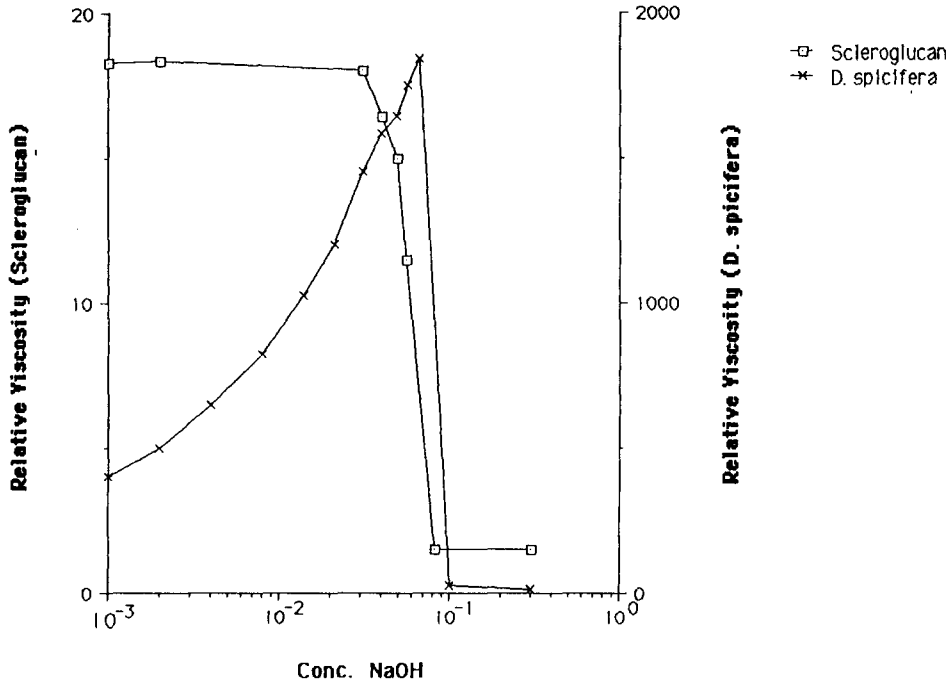


Fig. 3. Effect of the NaOH concentration on the viscosimetric behaviour of 0·45 g litre solutions of scleroglucan (□) and *Drechslera spicifera* polysaccharide (×).

unusual behaviour could be attributed to the presence of proteins (10%) which might allow interactions between different chains.

Weight average molecular weight estimation

Due to the poor filtrability of the water solution, the weight average molecular weight, $\bar{M}_w$, was not measured by the laser light scattering method but estimated by using the $[\eta]$-$\bar{M}_w$ relationship established by Yanaki *et al.* (1980) with schizophyllan. An $\bar{M}_w = 7 \times 10^6$ was found in water; this value is in agreement with literature data on different fungal glucans but this result has to be taken with caution because of questions over the validity of the intrinsic viscosity determination. From the $[\eta]$ value of 9700 in 5×10^{-2} M NaOH and the data of Bo *et al.* (1987), an $\bar{M}_w = 4 \times 10^6$ would be obtained.

Influence of NaOH concentration on the rheological properties at 25°C

In Fig. 3, the relative viscosities of a 0·45 g litre *Drechslera* polysaccharide solution and a 0·45 g litre scleroglucan solution is plotted versus the sodium hydroxide concentration. By increasing the NaOH concentration from 0 to 3×10^{-2} M, no variation was found in the viscosity of the scleroglucan solution while a large increase was recorded for the solutions of *Drechslera* up to 10^{-1} M NaOH concentration. This fact may be attributed either to an increase in the solubility or more probably to specific interactions due to the presence of proteins. Above 3×10^{-2} M NaOH concentration the rapid decrease in viscosity found on the scleroglucan solution must be related to the progressive triple helix-coil transition (Bo *et al.*, 1987). The same phenomenon was also observed above 7×10^{-2} M NaOH concentration with the solution of *Drechslera* polymer. This behaviour seems general with this family of polysaccharides but the transition concentration can change, probably depending on the purity of sample, its molecular weight and maybe its degree of branching.

Influence of temperature on the conformation

Changes in conformation are easily followed by optical rotation measurements. Figure 4 shows the results obtained with a 1 g/litre solution of scleroglucan and *Drechslera* polysaccharide in H_2O and NaOH by increasing and decreasing temperature in the range 5 to 70°C. In water, at temperatures lower than 15°C, a large increase in specific rotary power ($[\alpha]_{300}$) was observed for the two polysaccharides due to the gel formation by interaction of molecules in the stiff triple helix form (Bo *et al.*, 1987). This transition was reversible. In the presence of NaOH, whatever the temperature, the $[\alpha]_{300}$ was higher than in water when the NaOH concentration was below the critical concentration for the conformational transition triple helix → coil. No more gel was formed and the reversible transition observed with temperature has been attributed by Bo *et al.* (1987) to a process of ionization of the hydroxyl groups. For

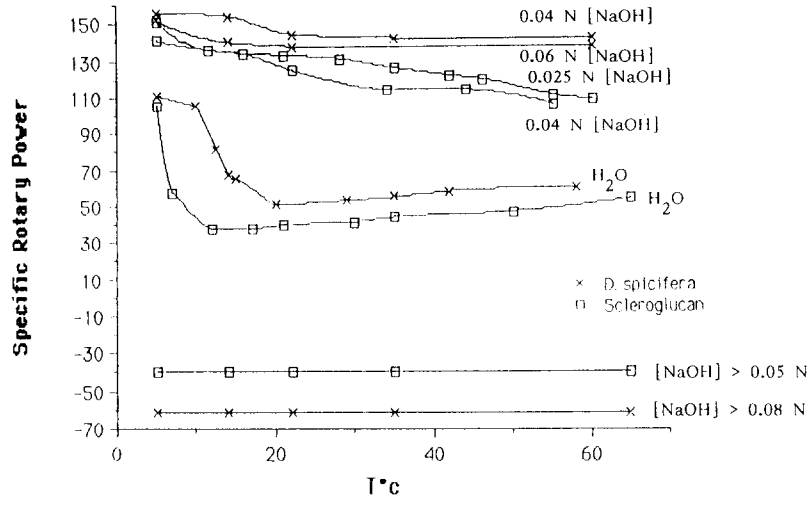


Fig. 4. Effect of the temperature on the specific rotary power ($[\alpha]_{300}$) of solutions of scleroglucan (□) and *Drechslera spicifera* polysaccharide (×).

NaOH concentration around 0·1 M, the transition triple helix → coil observed in viscosities measurement was confirmed with the change of the sign of $[\alpha]_{300}$.

CONCLUSION

As with many fungi, *Drechslera spicifera* produces an extracellular β-D-glucan. This β-D-glucan has a backbone of (1 → 3)-linked D-glucosyl residues, two out of five residues being substituted at O-6 by a single β-D-glucopyranosyl unit, a structure with a degree of branching higher than scleroglucan or schizophyllan, two fungal exopolysaccharides of industrial interest. Similar rheological properties to scleroglucan were observed although rheological studies were made difficult mainly due to problems of solubility related to the high molecular weight of these polysaccharides. Properties of the solutions seemed to be slightly dependent on the degree of branching, while immunomodulating activities, a large field of interest, did not seem to be. The polysaccharide, and partially ultrasonically depolymerized fractions have been compared with other related structures, and these results will appear later in another paper.

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