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Isolation and physicochemical characterization of soluble scleroglucan from *Sclerotium rolfsii*. Rheological properties, molecular weight and conformational characteristics

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Abstract

Scleroglucan produced by *Sclerotium rolfsii* ATCC 201126 exhibited pseudoplastic behaviour and an exponential relationship between apparent viscosity and polymer concentration. Viscosity showed strong heat-stability (100°C/60 min). Tolerance up to 20% w/v NaCl and over a wide pH range (0–13) was also found. A sharp decrease on viscosity at NaOH concentrations higher than 0.15 M was observed due to dissociation of the triple-helix. According to intrinsic viscosity determinations and gel filtration chromatography on Sepharose CL-4B, the weight-average molecular weight of native polysaccharide was about 5×10^6 Da for the triplex and 1.6×10^6 Da for the random coil. These values, in addition to the change in complexation with Congo Red, support the idea of the triple-helical conformation in neutral or slightly alkaline solutions (<0.15 M NaOH), and single chains in a highly alkaline environments.

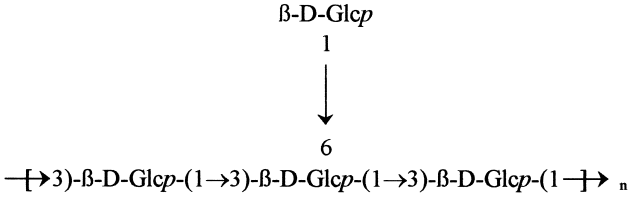
Keywords: *Sclerotium rolfsii*; Rheological properties; Conformation

1. Introduction

Although several polysaccharides of fungal origin are known, the world of fungi has not been exploited enough in this area (Gorin & Spencer, 1968). Among the potential applications of fungal polysaccharides, particular attention has been paid in recent years to the antitumour effects ascribed to the (1,3)-β-D-glucans as well as to their use as biomedical drugs against infections.

These biopolymers usually exhibit immunostimulatory activities, thus belonging to the group of biological response modifiers (BRM), though the mechanism has not been established (Pretus, Ensley, McNamee, Jones, Browder & Williams, 1991; Singh, Whistler, Tokuzen & Nakahara, 1974). Therapy with these immunomodulators has the advantage of naturally strengthening the immune system. Likewise, branched (1,3)-β-D-glucans have been considered for their anti-inflammatory activities (Hara, Kiho, Tanaka & Ukai, 1982). As evidenced by the increase in tumour-necrosis-factor-α release and superoxide-anion production activities, scleroglucan produced by *Sclerotium rolfsii* gave much better results than other glucans tested and exhibited high immunostimulatory activity (Kulicke, Lettau & Thielking, 1997).

The chemical structure of scleroglucan from *Sclerotium rolfsii* was recently confirmed (Fariña, 1997) as a regular polymer corresponding to a tetrasaccharide repeating unit:



The solution of this polysaccharide shows remarkable rheological behaviour over a wide range of pH, temperature and electrolyte concentration, which make it useful for many industrial purposes including enhanced oil recovery and food application (Brigand, 1993; Zentz, Verchère & Muller, 1992). These and the above mentioned properties have stimulated research on this type of β-D-glucans.

There is much controversy regarding the macromolecular and structural characteristics required for these polymers to be biologically effective with no concensus on the influence of molar mass, helical structure and (1,6)-β-branches on the

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antitumoral and immunostimulatory properties of β-D-glucans (Kulicke et al., 1997; Stokke, Elgsaeter, Hara, Kitamura & Takeo, 1993). Another important topic is the solubility in aqueous media, which depends on the (1,6)-β-D-branching in the molecule, and allows the biopolymer to become clinically applicable since this form can be safely administered via the systemic route with no toxicity and other complications (Williams et al., 1991).

For scleroglucan and other related (1,3)-β-D-glucans it has been reported that their molecular weight does not depend on the culture conditions used for polysaccharide production, for example when studying the influence of cultivation time (Lecacheux, Mustiere, Panaras & Brigand, 1986).

The present paper deals with the isolation and physical characterization of the scleroglucan produced by *Sclerotium rolfsii* ATCC 201126, a strain isolated from rotten red pepper. The water-soluble polysaccharide was produced at fermenter scale under optimized culture conditions and particular attention has been paid to the incidence of the cultivation time on the physical and conformational properties of the biopolymer produced.

2. Materials and methods

2.1. Inoculum preparation and scleroglucan production

The polymer used in our studies was produced by the fungus *Sclerotium rolfsii*, isolated from rotten red pepper in Uruguay (generously provided by Dr L. Betucci, from Universidad de la República, Montevideo, Uruguay). This strain, now classified as *Sclerotium rolfsii* ATCC 201126, does not produce conidia but only mycelium and it was preserved following the protocol previously described (Fariña, Siñeriz, Molina & Perotti, 1996).

After activation of the strain on Czapek malt agar, seed cultures were prepared in PM_{20} liquid medium according to Fariña, Siñeriz, Molina and Perotti (1998). These inocula were first blended under aseptic conditions (CB-6 Waring blender, minimum output) and then, they were used for inoculation at 10% v/v of a 10-l stirred-tank reactor fitted with baffles and six-flat bladed Rushton turbine impellers (MicroFerm, New Brunswick Scientific Co.) with a working volume of 8 l.

The optimized culture medium (MOPT) for polysaccharide production contained (in g/l): $NaNO_3$, 2.25; $K_2HPO_4 \cdot 3H_2O$, 2; sucrose, 150; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.05; yeast extract, 1; citric acid$\cdot H_2O$, 0.7 (initial pH adjusted to 4.5). The following conditions were maintained throughout the experiment: air flow rate, 0.5 vvm; stirrer speed, 400 rpm; temperature, 30°C, pH uncontrolled.

For the study of the incidence of the cultivation time on the physicochemical properties of the exopolysaccharide (EPS), two fermentations were carried out under the conditions mentioned above, for 48 and 72 h. At the end of each fermentation, the EPSs were purified for their subsequent characterization.

2.2. Scleroglucan recovery and purification

The culture broth was homogenized in a CB-6 Waring blender, neutralized, and diluted three-fold with distilled water. After heating at 80°C for 30 min, the broth was centrifuged in a 6-l capacity continuous centrifuge (Typ 61-763 Lahr/Schwarzwald) at 17,000 rpm. The EPS from the clear supernatant was cooled at 5°C and precipitated by adding an equivalent volume of ethanol 96°. This mixture was allowed to stand at 5°C for 8 h to complete EPS precipitation.

The precipitate was recovered with a fine sieve (Macotest A.S.T.M. No. 60) and then redissolved in distilled water. This crude EPS was further purified by ethanol 96°-reprecipitation (two times). Finally, the precipitated polymer was freeze-dried and milled to a whitish glucan powder, giving a purified preparation of the polysaccharide. The EPSs corresponding to 48 and 72 h of cultivation (see above), were identified as EPS I and EPS II, respectively, for subsequent experiments. Glucan powder was analysed for protein content by the Folin–Lowry method using bovine serum albumin as standard. Reducing sugars were measured according to the Somogyi–Nelson method (Hodge & Hofreiter, 1962) with glucose as standard. Total carbohydrates were determined by the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) with dextran as standard.

2.3. Rheology measurements

The polymer solutions were prepared by complete dissolution of the freeze-dried EPS in distilled water or dimethylsulfoxide (DMSO), using a magnetic stirrer at a dissolution temperature (T_D) of 20°C. Rheological properties were measured wing a rotational viscometer with narrow gap concentric cylinders or spindles (Cannon LV 2000) equipped with a temperature-controlled unit TCU 1000 and a small sample adapter, at a measurement temperature (T_M) of 25°C unless otherwise stated. Polymer concentration, rotors and rotor speeds used in each experiment are described in the tables and figures. The sample volume was 8 ml, readings were taken after rotation for 2 min, and the data presented are averages of at least three measurements.

The limiting viscosity number (intrinsic viscosity $[\eta]$) was obtained by extrapolation to infinite dilution as defined by the Huggins ($\eta_{sp}/c = [\eta] + k'[\eta]^2 c$, with η_{sp} (specific viscosity) $= (\eta - \eta_0)/\eta_0$, η, the viscosity of solution and η_0, the viscosity of solvent) or Kraemer equations ($(\ln \eta_r)/c = [\eta] + k''[\eta]^2 c$, with η_r (relative viscosity) $= \eta/\eta_0$).

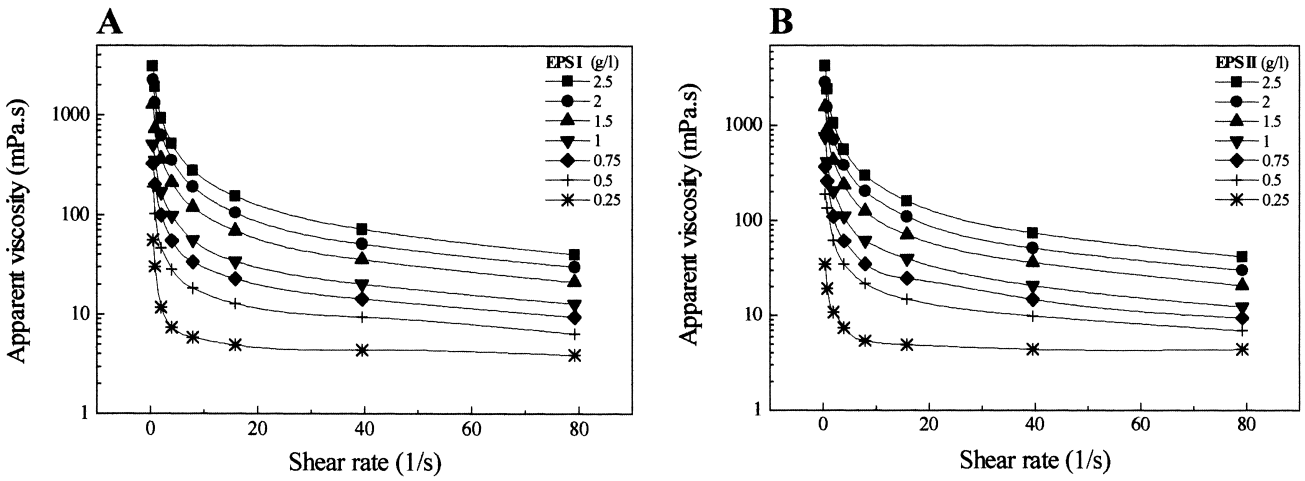

Fig. 1. Dependency of the apparent viscosity on the shear rate for scleroglucan aqueous solutions, EPS I (A) and EPS II (B). $T_M = 25°C$, TL-5 spindle.

2.4. Helix–coil transition analysis

The conformational structure of scleroglucan in solution was established by characterising the Congo Red-EPS complexes. The formation of these complexes and the resulting shift of the maximum adsorption wavelength (λ_{max} value) is a rapid method for detecting helical structures. The greater the effect in the λ_{max} shift, the higher the helical structures content is. Helices can be destroyed by adding sodium hydroxide, which rapidly causes a loss of the interaction of the polysaccharide with Congo Red. Thus, polymers which exist in an ordered conformation (triplex) can form a complex with Congo Red in diluted aqueous NaOH solutions, determining a shift in λ_{max}. On the other hand, polymers which have a random coil structure, for example, when helices are destroyed by high alkali concentrations, cannot form these complexes (Kulicke et al., 1997). In this way, a shift in the visible absorption maximum of Congo Red induced by the presence of a polysaccharide can be used for conformational studies according to the method of Ogawa and Hatano (1978). The transition of the triple-helical arrangement to the single-stranded conformation was studied by measuring the λ_{max} for Congo Red–EPS solutions at NaOH concentrations ranging from 0.05 to 0.4 M.

Polymer aqueous solutions (1 g EPS/l) containing 91 μM Congo Red were subjected to the different concentrations of NaOH and, after 3 h of interaction, the visible absorption spectra were recorded with a Beckman DU 640 UV/visible spectrophotometer at each concentration of alkali.

2.5. Gel chromatography on Sepharose CL-4B with aqueous sodium hydroxide

Gel chromatography on Sepharose CL-4B (Pharmacia Fine Chemicals) was carried out with 0.25 M NaOH as the eluent. Different standard dextrans (Sigma) and the EPS (1.0 mg) were each dissolved in 0.25 M NaOH (500 μl), and applied to a column (1.0 × 64 cm) of Sepharose CL-4B. The column was eluted with 0.25 M NaOH at a flow rate of 0.1 ml/min. Fractions (5.0 ml each) were collected (Pharmacia LKB GradiFrac System), and an aliquot of each fraction was analysed for carbohydrates by the phenol–sulphuric acid method (Dubois et al., 1956).

The weight-average molecular weight (M_w) was estimated from a calibration curve constructed, with: Dextran $M_w \sim 73,000$ (from *Leuconostoc mesenteroides* B-512, Sigma), Dextran $M_w \sim 515,000$ (from *L. mesenteroides* B-512, Sigma) and Dextran $M_w \sim 2,000,000$ (from *L. mesenteroides* B-512, Sigma).

3. Results and discussion

3.1. Scleroglucan production and polysaccharide purification

The yield of the recovery process after purification compared with the theoretical maximum was estimated as almost 20%. In both cases (EPS I and II), losses will occur in most of the eleven steps (homogenization, dilution, heating, centrifugation, precipitation, redissolution and reprecipitation- × 2, drying and milling). For both EPS the presence of reducing sugars was not detected, whilst the total sugars content was about 98% w/w, indicating a high level of purity as compared to other commercial scleroglucans (Wang & McNeil, 1996). The Folin–Lowry determination revealed 1.9 and 1.6% w/w of protein content for EPS I and II, respectively.

There was no advantage in biopolymer concentration by increasing the cultivation time from 48 to 72 h. When polysaccharide production was carried out in shaken flasks, a similar concentration could be attained just after 72 h of cultivation (Fariña et al., 1998). The choice of the optimum cultivation time is important, since one of the fundamental

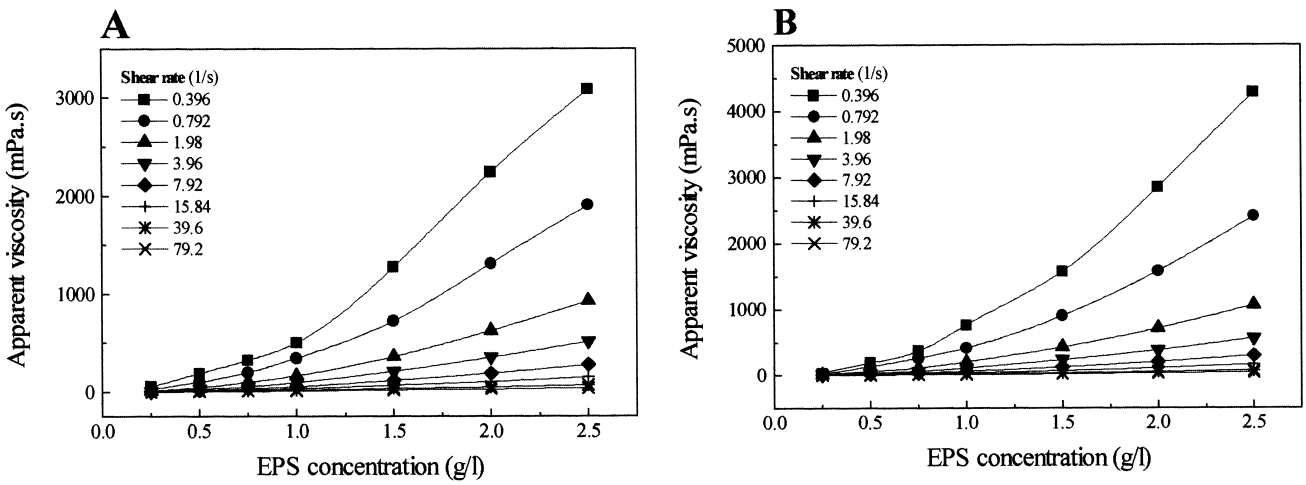

Fig. 2. Correlation between apparent viscosity and EPS concentration—EPS I (A) and EPS II (B). $T_M = 25°C$, TL-5 spindle.

drawbacks is the lengthy scleroglucan production process (Wang & McNeil, 1995).

From the production point of view, the above results might be critical in order to select appropriate operational conditions. However, in this particular case, the aim of our work was not only to achieve a high polysaccharide yield but also the preservation of the physicochemical and conformational properties.

3.2. Rheological properties of scleroglucan

The application of scleroglucan in diverse areas such as thickening drilling muds, fracturing and completion fluids, enhanced oil recovery (EOR), depends to a greater or lesser extent on the ability of the polysaccharide to retain stable rheological characteristics, for example, against hydrolysis, heating and addition of salts. In particular, for EOR, the polymer flooding process requires that the polymer is able to maintain viscosifying properties in sea water at low concentrations and high temperatures for years (Brigand, 1993). Consequently, this important topic was studied.

Table 1
Scleroglucan rheological parameters

Polymer	Concentration (g/l)	Consistency index K (mPa s^n)[a]	Flow behaviour index n
EPS I	0.25	9.7 ± 0.6	0.78 ± 0.03
	0.5	55 ± 4	0.49 ± 0.04
	0.75	165 ± 2	0.26 ± 0.01
	1	272 ± 7	0.31 ± 0.03
	1.5	612 ± 3	0.21 ± 0.01
	2	1073 ± 7	0.20 ± 0.01
	2.5	1538 ± 23	0.24 ± 0.02
EPS II	0.25	19 ± 1	0.38 ± 0.07
	0.5	104 ± 4	0.32 ± 0.04
	0.75	194 ± 8	0.28 ± 0.05
	1	349 ± 4	0.16 ± 0.01
	1.5	738 ± 4	0.18 ± 0.01
	2	1281 ± 5	0.13 ± 0.00
	2.5	1928 ± 16	0.13 ± 0.01

[a] Viscosity measurements at $T_M = 25°C$ with TL-5 spindle and γ ranging from 0.396 to 79.2 l/s.

3.2.1. EPS concentration and viscosity

Aqueous solutions of scleroglucan (EPS I and II) showed non-Newtonian pseudoplastic behaviour, where the viscosity decreased with increasing shear rates. This feature was observed at different polymer concentrations ranging from 0.25 to 2.5 g/l, and shear rates varied from 0.396 to 79.2 l/s (Fig. 1A and B). Similar behaviour was previously reported for other homo- and heteropolysaccharides such as schizophyllan (Rau, Muller, Cordes & Klein, 1990; Steiner, Divjak, Steiner, Lafferty & Esterbauer, 1988), barley (1,3),(1,4)-β-D-glucan (Gómez, Navarro, Manzanares, Horta & Carbonell, 1997), *Bacillus circulans* polysaccharide (Isobe, Endo & Kawai, 1992) and glomerellan (Sarkar, Hennebert & Mayaudon, 1985). Pseudoplasticity might be related to the anisotropy of the rigid triple-helical structure frequently adopted in solution by this kind of polysaccharide (Steiner et al., 1988; Zentz et al., 1992).

An exponential relationship between viscosity and polymer concentration was found (Fig. 2A and B). The high viscosity of this biopolymer at low concentrations is due to both high molecular weight and high structural chain rigidity (Stokke, Elgsaeter, Bjørnestad & Lund, 1992). The viscoelastic characteristics of scleroglucan solutions at low concentrations (0.025–0.25% w/v) or the gelation properties at higher levels of polymer (0.25–2.0% w/v) could be explained as a consequence of the intermolecular association energetically driven by unfavourable polymer–solvent interactions and interstrand hydrogen bonds which determine cross-linking events (Stipanovic & Giammatteo, 1989).

These findings have a significant impact in view of the application of scleroglucan in EOR, mainly for oil reservoirs with offshore location, when a polymer capable to retain viscosifying properties at low concentrations is

Table 2
Relative viscosity of 2 g/l aqueous solutions of EPS I and II after heating at different temperatures and incubation times, according to determinations at $T_M = 25°C$ and $\gamma = 3.96$ l/s with TL-5 spindle

Polymer	Temperature (°C)	Time (min)	Relative value of viscosity ± S.E.M. (%)[a]
EPS I	50	15	96.8 ± 0.4
		30	97.1 ± 0.8
		60	97.1 ± 0.8
	80	15	94.0 ± 4.4
		30	94.1 ± 3.3
		60	90.8 ± 5.5
	100	15	92.3 ± 6.1
		30	91.8 ± 6.8
		60	93.2 ± 4.6
EPS II	50	15	96.2 ± 0.8
		30	96.2 ± 0.2
		60	96.8 ± 0.2
	80	15	96.4 ± 0.2
		30	96.1 ± 0.0
		60	95.2 ± 4.2
	100	15	97.4 ± 1.4
		30	98.5 ± 0.8
		60	96.8 ± 1.8

[a] Controls: EPS I: 100 ± 0.9%, EPS II: 100 ± 0.1%.

particularly desirable since the transport, handling and storage of the mobility control agent is one of the tutors determining operating costs (Brigand, 1993).

To gain a greater insight into the rheological characteristics of scleroglucan, viscosity values of aqueous solutions were fitted to the Power Law (Ostwald–de-Waele) relationship (1), applicable to fluids which exhibit pseudoplastic behaviour (Bongenaar, Kossen, Metz & Meijboom, 1973). Consequently, the values of the parameters K (consistency index) and n (flow behaviour index) have been estimated (Table 1) and they were in agreement with the high pseudoplasticity of scleroglucan.

3.2.2. Ostwald–de-Waele model

$$\eta = K\dot{\gamma}^{(n-1)} \quad (1)$$

where $\eta = \tau\dot{\gamma}^{-1}$, with η being the apparent viscosity, τ the shear stress and $\dot{\gamma}$ the shear rate.

3.2.3. Effects of heating on viscosity

The change on viscosity of EPS I and II aqueous solutions after different periods of heating (15, 30 and 60 min) at temperatures ranging from 50 to 100°C was investigated (Table 2). The viscosity values of heat-treated polysaccharide solutions were statistically compared (one-way ANOVA test) revealing no significant differences even after heating at 100°C for 1 h ($P = 0.4952$, $F = 1.004$).

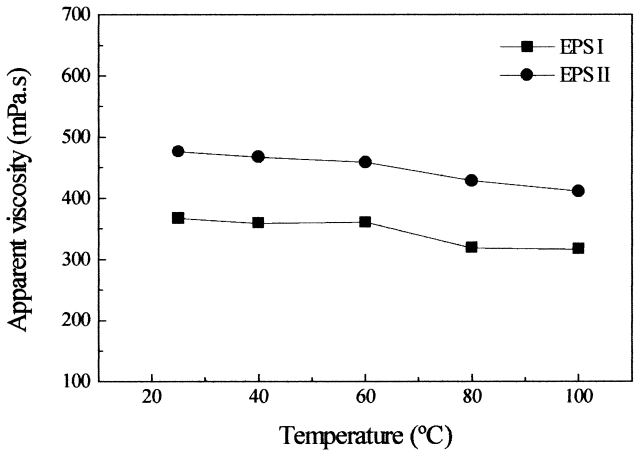

Fig. 3. Effects of measurement temperature T_M on viscosity. The viscosity of EPS aqueous solutions (2 g/l) was measured at the indicated temperatures with a TL-5 spindle at $\gamma = 3.96$ l/s.

Resistance to high temperatures has been described as one of the remarkable rheological properties of scleroglucan. Very slight variations in viscosity were detected when scleroglucan was subjected to temperatures ranging from 15 to 90°C, both in concentrated and semi-dilute solutions. Moreover, when tested in oil reservoirs, this polysaccharide retained more than 90% of its viscosity after 500 days at 90°C in sea water (Brigand, 1993; El Ouriaghli, François, Sarazin & Dinh, 1992; Lecacheux et al., 1986; Sandford, 1979; Stokke et al., 1992; Wang & McNeil, 1996; Zentz et al., 1992). This resulted in striking contrast with the marked heat-sensitivity exhibited by other biopolymers such as seed and *Bacillus circulans* polysaccharides, even at high concentrations (Isobe et al., 1992).

3.2.4. Effects of measurement temperature (T_M) on viscosity

The viscosity of scleroglucan aqueous solutions was determined at different temperatures (T_M) ranging from 25 to 100°C and results are displayed in Fig. 3. As it can be seen, viscosity was hardly affected by temperature it remaining practically constant until the temperature reached 100°C. Even at the latter temperature, scleroglucan retained almost 90% of its viscosity in both cases (EPS I and II). The polysaccharides studied in this work seem to have similar rheological properties to other *Sclerotium* biopolymers reported so far (Brigand, 1993; Noïk & Lecourtier, 1993; Stokke et al., 1992).

3.2.5. Effects of pH on viscosity

The viscosity of scleroglucan solutions remained extremely stable on shifting the pH either to highly acidic or moderately alkaline values. Whereas low pH values did not affect viscosity, a pH above 13 (reached at very high concentrations of alkali) brought about a steep decrease in apparent viscosity (Fig. 4). This is likely to be related to the loss of the triple-helical arrangement caused by the transition to the single-stranded conformation (denaturation), a

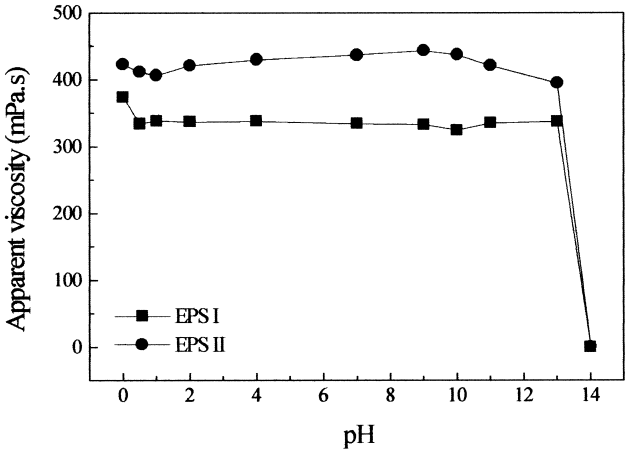

Fig. 4. Effects of pH on viscosity. The pH of 2 g/l EPS aqueous solutions was adjusted to the indicated pH values with HCl or NaOH as necessary. Initial pH values were as follows: EPS I, 6.48 and EPS II, 6.57. $T_M = 25°C$, TL-5 spindle, $\gamma = 3.96$ l/s.

fact which is usually favoured in very alkaline environments (Stipanovic & Giammatteo, 1989; Zentz et al., 1992).

The pH-stability may be because scleroglucan is not a polyelectrolyte. It gives it an advantage over other polysaccharides which are polyelectrolytes and exhibit a pH dependence because of changes in the polymer architecture as a consequence of variations in the net electric charge on the molecule (Isobe et al., 1992; Rau et al., 1990).

Although the above results themselves have such a critical importance regarding the performance of scleroglucan under extreme conditions, heat- and pH-stability assessment is also crucial in order to prevent polysaccharide deterioration consequent on unsuitable post-biosynthesis operations

Table 3
Relative viscosity of 2 g/l scleroglucan aqueous solutions (EPS I and II) after addition of different inorganic salts for a final concentration of 1% w/v. Measurements were carried out after complete dissolution of the salt and pH determination. $T_M = 25°C$, TL5 spindle, $\gamma = 3.96$ l/s

Polymer	Salt (1% w/v)	Final pH	Relative value of viscosity ± S.E.M. (%)[a]
EPS I	NaCl	6.14	96.8 ± 0.6
	KCl	6.26	96.4 ± 0.4
	$CaCl_2$	5.99	96.9 ± 1.7
	$MgCl_2$	6.16	97.4 ± 0.8
	$MnCl_2$	5.91	98.1 ± 1.2
	$FeCl_3$	1.34	102.2 ± 0.6
EPS II	NaCl	5.98	83.7 ± 3.5
	KCl	6.00	81.8 ± 1.2
	$CaCl_2$	5.66	83.4 ± 2.8
	$MgCl_2$	5.84	82.0 ± 1.4
	$MnCl_2$	5.81	83.2 ± 1.7
	$FeCl_3$	1.44	111.0 ± 0.6

[a] Viscosity values are relative to the controls of pure polymer aqueous solutions (EPS I: 100 ± 0.2%, pH = 6.31; EPS II: 100 ± 0.1%, pH = 6.13).

Table 4
Relative viscosity of 2 g/l scleroglucan aqueous solutions (EPS I and II) after addition of different NaCl concentrations. Measurements were carried out after complete dissolution of the salt and pH determination. $T_M = 25°C$, TL5 spindle, $\gamma = 3.96$ l/s

Polymer	NaCl (% w/v)	Final pH	Relative value of viscosity ± S.E.M. (%)[a]
EPS I	1	5.73	95.2 ± 2.6
	5	5.50	96.4 ± 2.2
	10	5.42	95.7 ± 1.9
	20	5.39	91.7 ± 1.9
EPS II	1	6.04	85.2 ± 1.6
	5	5.67	80.4 ± 3.8
	10	5.68	78.8 ± 2.8
	20	5.58	79.8 ± 2.4

[a] Viscosity values are relative to the controls of pure polymer aqueous solutions (EPS I: 100 ± 1.0%, pH = 6.12; EPS II: 100 ± 2.7%, pH = 6.37).

(Rau et al., 1990; Wang & McNeil, 1996; Zentz et al., 1992).

3.2.6. Effects of addition of inorganic salts on viscosity

As it can be seen in Table 3, a slight decrease on viscosity values of EPS aqueous solutions was detected after the addition of NaCl, KCl, $CaCl_2$, $MgCl_2$ and $MnCl_2$, while the addition of $FeCl_3$ brought about a small increase. Although the same tendency was observed for both EPS, according to the ANOVA test, no significant differences were detected when adding different salts at 1% w/v to the EPS I aqueous solutions ($P > 0.05$). However, for EPS II, the addition of salts did cause a significant viscosity change ($P < 0.05$).

The decrease in viscosity after salt addition has been previously reported for other polysaccharides. It is possible that the reduction in the solvent quality might increase interactions within the multi-stranded structure resulting in coil contraction and hence a reduction in viscosity (Rau et al., 1990).

The pH of polysaccharide solutions was determined after complete dissolution of the added salts. It was not so different from the initial pH of pure scleroglucan solutions, except in the case of $FeCl_3$ when a large decrease to pH 1.3–1.5 was observed. It has been speculated that under acidic conditions some breakage of glycosidic linkages at the insertion point of the side chains could occur resulting in a less highly branched molecule which enables aggregation and gelation phenomena (Brigand, 1993).

The influence of NaCl concentration on viscosity is displayed in Table 4. Again, though the general trends after adding NaCl to both EPS were similar, no significant changes on viscosity were detected in the case of EPS I, even at 20% w/v NaCl ($P > 0.05$). In contrast, for EPS II solutions a slight decrease on viscosity was evidenced ($P < 0.05$), though the increase in salt concentration caused no significant differences. In both cases the pH after NaCl addition was almost unaffected.

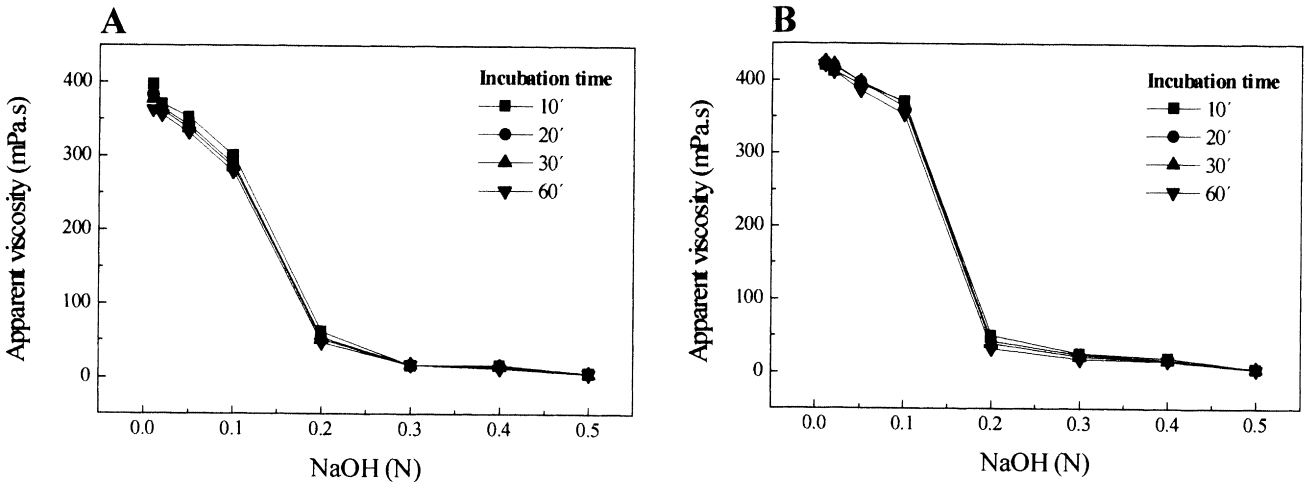

Fig. 5. Alkaline denaturation of scleroglucan (EPS I: A; EPS II: B). EPS concentration = 2 g/l, $T_M = 25°C$, TL-5 spindle, $\gamma = 3.96$ l/s.

As previously reported, a polysaccharide having a polyelectrolyte character is affected by the addition of salts. The binding between charged moieties of the polymer and the surrounding ions usually determines variations on the cross-linking degree of the macromolecule and, consequently, on viscosities (Isobe et al., 1992). In the present case, the incidence of salts on viscosity of EPS II solutions is difficult to explain according to the previous considerations because of the neutral characteristics of scleroglucan, and it is more likely related to the solvent quality as discussed above (Cesàro, Delben, Flaibani & Paoletti, 1987).

It is not clear why the effect of salt on EPS I and EPS II differs. A possible explanation is that subtle conformational differences between EPS II and EPS I such as variations in the degree of intramolecular interactions or coiling might exist. This could change the accessibility of surrounding molecules or the chain stiffness. In this sense, further information about the scleroglucan conformation based on such direct observation methods as X-ray diffraction measurements could be important (Ogawa, Watanabe, Tsurugi & Ono, 1972).

3.2.7. Effects of addition of NaOH on viscosity

Beta-glucans with a tendency to adopt a triple-helical conformation and semi-rigid structure in neutral aqueous solution, in association with high molecular weight (about 5×10^6 Da), exhibit marked viscosifying ability (Yanaki, Kojima & Norisuye, 1981). Nevertheless, when these biopolymers are subjected to high alkali concentrations, a steep decrease in viscosity is usually observed because of their denaturation. In the present work, the EPS aqueous solutions were subjected to different concentrations of alkali to determine the critical point at which the polymer denaturation takes place.

According to the results obtained (Fig. 5) we can assume that scleroglucan adopts an ordered triple-helical conformation at low concentrations of alkali, below 0.15 M NaOH. At higher NaOH concentrations, where drastic changes in viscosity are observed, the triple-strand helices of scleroglucan probably undergo the ionization of hydroxyl groups which results in the destruction of hydrogen bonds and the subsequent denaturation of the polysaccharide (Hara et al., 1982).

3.2.8. Weight-average molecular weight (M_w) determination according to intrinsic viscosity measurements

Intrinsic viscosity is related to the conformation in solution and the molecular weight of the macromolecule. The application of the well-known Mark–Houwink–Staudinger equation (2) was used to calculate the scleroglucan M_w:

$$[\eta] = K_S M_w^a \tag{2}$$

where $[\eta]$ is the intrinsic viscosity, K_S the prefactor traditionally deduced from measurements of M_w and $[\eta]$ for a series of homologous fractions of the polymer and a the Mark–Houwink exponent, relates the power law dependence of the molecular weight (M_w) of the intrinsic viscosity. It is related to the shape of the macromolecule and the nature of the solvent.

For linear chain molecules the Mark–Houwink exponent has a theoretical value of 0.5. The better the solvent and the more rigid the molecular backbone are, the more open the structure of the coil becomes, and the Mark–Houwink exponent increases up to a value of 1.8 for ideal rods. In the case of scleroglucan from *S. rolfsii* a solution structure of an expanded coil has been previously reported (Kulicke et al., 1997).

Yanaki established a same relationship between intrinsic viscosity and molecular weight for two polysaccharides, schizophyllan and scleroglucan, at 25°C in aqueous solution (Noïk & Lecourtier, 1993; Yanaki et al., 1981). In water, where the polymer exhibits a triple-helical conformation, the power law relation is:

$$[\eta] \propto M_w^{1.8} \qquad \text{for } M_w < 5 \times 10^5 \text{ Da}$$

$$[\eta] \propto M_w^{1.1} \qquad \text{for } M_w > 10^6 \text{ Da}$$

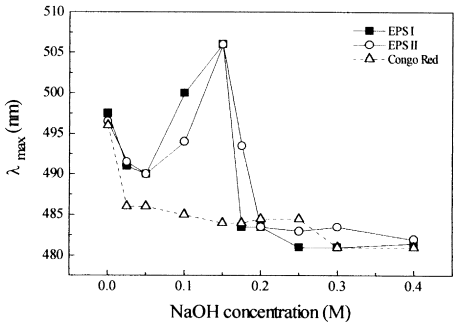

Fig. 6. Helix–coil transition analysis of scleroglucan according to the change in the absorption maximum of the Congo Red–polysaccharide complex at various concentrations of NaOH. Congo Red in NaOH served as the negative control. For more details, see Materials and Methods.

An exponent value a of 1.8 is the theoretical prediction for a rigid-rod conformation. In the case of high molecular weight polymers, which behave more like a semi-rigid chain, Yanaki suggested the lower value of 1.1 (Yanaki et al., 1981). Thus, in the case of scleroglucan aqueous solutions a value of $a = 1.1$ was applied. The value of K_S was calculated according to previous results for the intrinsic viscosity and the molecular weight, determined by light scattering for scleroglucan (Noïk & Lecourtier, 1993). This value was 3.93×10^{-4} ([η] in ml/g) and it was applied in the following calculations, both for water and DMSO.

Contrary to aqueous solutions, the polysaccharide showed Newtonian behaviour in DMSO, which was verified up to a polymer concentration of 1 g/l. It means that viscosity does not depend on the shear rate, which was varied from 0.396 to 79.2 l/s (data not shown). In DMSO scleroglucan exhibits a random coil structure as a consequence of the destruction of the triplex, so a different a value (0.94) was used. It was estimated from intrinsic viscosity and molecular weight data previously reported for DMSO solutions of scleroglucan N-2 (Yanaki et al., 1981).

Intrinsic viscosities [η] of scleroglucan in water and DMSO at 25°C were determined according to Huggins and Kraemer plots. The calculated intrinsic viscosities were:

EPS *I* : 9,610 ml/g (in water) and 277 ml/g (in DMSO)

EPS *II* : 9,510 ml/g (in water) and 282 ml/g (in DMSO)

The found values of [η] for scleroglucan in water were very similar to that reported by Aouadi, Heyraud, Seigle-Murandi, Steiman, Kraus and Franz (1991) for SCL scleroglucan, which corresponded to a M_w about 4.9×10^6. Using the Mark–Houwink–Staudinger equation and the observed [η] values, it was possible to estimate the approximate molecular weight of scleroglucan using reported correlations between [η] and M_w (see parameters above):

EPS *I* : 5.2×10^6 Da (in water) and 1.6 $\times 10^6$ Da (in DMSO)

EPS *II* : 5.2×10^6 Da (in water) and 1.7 $\times 10^6$ Da (in DMSO)

The ratio of the M_w of the polymer in water to the M_w in DMSO was roughly 3, which was in accordance with the assumed triple-helical structure.

Although the weight-average molecular weight of scleroglucan samples were similar, some slight differences were found with respect to the steady shear viscosity and the interactions with salts when comparing EPS I and EPS II behaviour. This might be due to the variations in the level of cross-linking and the consequent binding energy (ΔH) of the macromolecule (Na & Lee, 1997). It has been mentioned that a clear relationship between M_w and viscosity of polymer solutions does not always exist, since the latter could be affected by the EPS solubility (Lecacheux et al., 1986).

3.3. Helix–coil transition analysis

Scleroglucan from *S. rolfsii* exhibited a triple-helical conformation as shown by the shift in the absorption maxima (λ_{max}) between 0.15 and 0.25 M NaOH (Fig. 6). The same behaviour was observed for both EPS I and II. Congo Red in NaOH served as the negative control. These results are consistent with an order–disorder transition which may be attributable to the breakage of intermolecular hydrogen bonds (Hara et al., 1982; Williams et al., 1991).

The macromolecular conformation adopted by the polysaccharide in solution underpins two widely different applications, EOR and pharmaceutics (Stokke et al., 1993).

3.4. Gel filtration chromatography on Sepharose CL-4B

In order to obtain a second value that enables to estimate the accuracy of molecular weight determination according to the intrinsic viscosity, an independent measurement based on the scleroglucan exclusion profile by gel filtration chromatography was carried out. The polysaccharide showed a narrow molecular weight distribution and the weight-average molecular weight was calculated to be 1.6×10^6 and 1.7×10^6 Da (corresponding to random coils eluted with 0.25 M NaOH) for EPS I and EPS II respectively, according to the calibration curve with standard dextrans (Fig. 7A and B).

The estimated M_w of scleroglucan from *S. rolfsii* ATCC 201126 was in agreement with results of intrinsic viscosity determinations and also with previous data reported so far (El Ouriaghli et al., 1992; Lecacheux et al., 1986; Stokke et al., 1992; Wang & McNeil, 1996).

4. Conclusions

The work reported here represents a contribution to the knowledge of the physicochemical and rheological

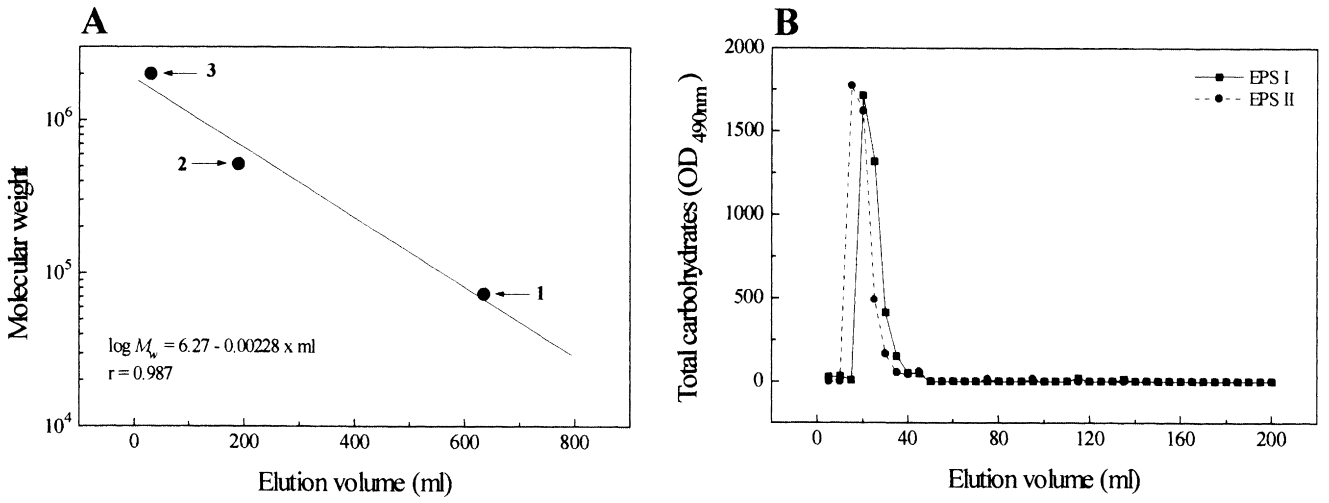

Fig. 7. Weight-average molecular weight determination by gel filtration chromatography on Sepharose CL-4B. (A) Calibration curve with standard dextrans. The elution volume is plotted against the logarithm of the molecular weight of dextrans (1—$M_w \sim 73,000$; 2—$M_w \sim 515,000$ and 3—$M_w \sim 2,000,000$. from *L. mesenteroides* B-512, Sigma). (B) Chromatogram of scleroglucan (EPS I and II) eluted with 0.25 M NaOH. Total carbohydrates determined according to the phenol–sulphuric acid method (results referred as optical density at 490 nm).

properties of the fungal polysaccharide produced by *S. rolfsii* ATCC 201126.

To understand this is important for specialized applications such as BRM, since the determination of physicochemical, conformational and structural properties of scleroglucan is required for the subsequent correlation with the biological activity observed.

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References

Aouadi, S., Heyraud, A., Seigle-Murandi, F., Steiman, R., Kraus, J., & Franz, G. (1991). Structure and properties of an extracellular polysaccharide from *Laetisaria arvalis*. Evaluation of its antitumour activity. *Carbohydrate Polymers*, *16*, 155–165.

Bongenaar, J. J. T. M., Kossen, N. W. F., Metz, B., & Meijboom, F. W. (1973). A method for characterizing the rheological properties of viscous fermentation broths. *Biotechnology and Bioengineering*, *15*, 201–206.

Brigand, G. (1993). Scleroglucan. In R. L. Whistler & J. N. BeMiller, *Industrial gums* (pp. 461–474). New York: Academic Press.

Cesàro, A., Delben, F., Flaibani, A., & Paoletti, S. (1987). Polyelectrolytic effects in carboxylic derivatives of natural polysaccharides. *Carbohydrate Research*, *160*, 355–368.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

El Ouriaghli, T., François, J., Sarazin, D., & Dinh, N. T. (1992). Influence of nonionic surfactant on aggregation state of scleroglucan in aqueous solution. *Carbohydrate Polymers*, *17*, 305–312.

Fariña, J. I., Siñeriz, F., Molina, O. E., & Perotti, N. I. (1996). Low-cost method for the preservation of *Sclerotium rolfsii* PROIMI F-6656: Inoculum standardization and its use in scleroglucan production. *Biotechnology Techniques*, *10*, 705–708.

Fariña, J. I., Siñeriz, F., Molina, O. E., & Perotti, N. I. (1998). High scleroglucan production by *Sclerotium rolfsii*: influence of medium composition. *Biotechnology Letters*, *20*, 825–831.

Fariña, J.I. (1997). Producción de escleroglucano por *Sclerotium rolfsii*. Doctoral Thesis, Universidad de Tucumán, Argentina.

Gómez, C., Navarro, A., Manzanares, P., Horta, A., & Carbonell, J. V. (1997). Physical and structural properties of barley (1→3),(1→4)-β-D-glucan. Part II: viscosity, chain stiffness and macromolecular dimensions. *Carbohydrate Polymers*, *32*, 17–22.

Gorin, P. A. J., & Spencer, J. F. T. (1968). Structural chemistry of fungal polysaccharides. In M. L. Wolfrom & R. S. Tipson, *Advances in carbohydrate chemistry* (pp. 367–417). New York: Academic Press.

Hara, C., Kiho, T., Tanaka, Y., & Ukai, S. (1982). Anti-inflammatory activity and conformational behavior of a branched (1→3)-β-D-glucan from an alkaline extract of *Dictyophora indusiata Fisch*. *Carbohydrate Research*, *110*, 77–87.

Hodge, J. E., & Hofreiter, B. T. (1962). Determination of reducing sugars and carbohydrates. In R. L. Whistler, M. L. Wolfrom, J. N. BeMiller & F. Shafizadeh, *Methods in carbohydrate chemistry* (pp. 380–394). vol. I. New York: Academic Press.

Isobe, Y., Endo, K., & Kawai, H. (1992). Properties of a highly viscous polysaccharide produced by a *Bacillus* strain isolated from soil. *Bioscience, Biotechnology and Biochemistry*, *56*, 636–639.

Kulicke, W. -M., Lettau, A. I., & Thielking, H. (1997). Correlation between immunological activity, molar mass, and molecular structure of different (1→3)-β-D-glucans. *Carbohydrate Research*, *297*, 135–143.

Lecacheux, D., Mustiere, Y., Panaras, R., & Brigand, G. (1986). Molecular weight of scleroglucan and other extracellular microbial polysaccharides by size-exclusion chromatography and low angle laser light scattering. *Carbohydrate Polymers*, *6*, 477–492.

Na, K., & Lee, K.-Y. (1997). Characteristics of the lactan gum produced from various carbon sources by *Rahnella aquatilis*. *Biotechnology Letters*, *19*, 1193–1195.

Noïk, C., & Lecourtier, J. (1993). Studies on scleroglucan conformation by rheological measurements versus temperature up to 150°C. *Polymer*, *34*, 150–157.

Ogawa, K., & Hatano, M. (1978). Circular dichroism of the complex of a (1→3)-β-D-glucan with congo red. *Carbohydrate Research*, *67*, 527–535.

Ogawa, K., Watanabe, T., Tsurugi, J., & Ono, S. (1972). Conformational behavior of a gel-forming (1→3)-β-D-glucan in alkaline solution. *Carbohydrate Research*, *23*, 399–405.

Pretus, H. A., Ensley, H. A., McNamee, R. B., Jones, E. L., Browder, W., & Williams, D. L. (1991). Isolation, physicochemical characterization and preclinical efficacy evaluation of soluble scleroglucan. *Journal of Pharmacology and Experimental Therapeutics*, *257*, 500–510.

Rau, U., Muller, R.-J., Cordes, K., & Klein, J. (1990). Process and molecular data of branched 1,3-β-D-glucans in comparison with xanthan. *Bioprocess Engineering*, *5*, 89–93.

Sandford, P. A. (1979). Exocellular, microbial polysaccharides. *Advances in Carbohydrate Chemistry and Biochemistry*, *36*, 265–312.

Sarkar, J. M., Hennebert, G. L., & Mayaudon, J. (1985). Optimization and characterization of an extracellular polysaccharide produced by *Glomerella cingulata*. *Biotechnology Letters*, *7*, 631–636.

Singh, P. P., Whistler, R. L., Tokuzen, R., & Nakahara, W. (1974). Scleroglucan, an antitumor polysaccharide from *Sclerotium glucanicum*. *Carbohydrate Research*, *37*, 245–247.

Steiner, E., Divjak, H., Steiner, W., Lafferty, R. M., & Esterbauer, H. (1988). Rheological properties of solutions of a colloid-disperse homoglucan from *Schizophyllum commune*. *Progress in Colloids and Polymer Sciences*, *77*, 217–220.

Stipanovic, A. J., & Giammatteo, P. J. (1989). Curdlan and scleroglucan. NMR characterization of solution and gel properties. In J. E. Glass, *Polymers in aqueous media: performance through association*, *194th National Meeting of the ACS: Advanced Chemical Series No. 223* (pp. 73–87). Washington, DC: American Chemical Society.

Stokke, B. T., Elgsaeter, A., Bjørnestad, E. Ø., & Lund, T. (1992). Rheology of xanthan and scleroglucan in synthetic seawater. *Carbohydrate Polymers*, *17*, 209–220.

Stokke, B. T., Elgsaeter, A., Hara, C., Kitamura, S., & Takeo, K. (1993). Physicochemical properties of (1→6)-branched (1→3)-β-D-glucans. 1. Physical dimensions estimated from hydrodynamic and electron microscopic data. *Biopolymers*, *33*, 561–573.

Wang, Y., & McNeil, B. (1995). Production of the fungal exopolysaccharide scleroglucan by cultivation of *Sclerotium glucanicum* in an airlift reactor with an external loop. *Journal of Chemical Technology and Biotechnology*, *63*, 215–222.

Wang, Y., & McNeil, B. (1996). Scleroglucan. *Critical Reviews in Biotechnology*, *16*, 185–215.

Williams, D. L., McNamee, R. B., Jones, E. L., Pretus, H. A., Ensley, H. E., Browder, I. W., & Di Luzio, N. R. (1991). A method for the solubilization of a (1→3)-β-D-glucan isolated from *Saccharomyces cerevisiae*. *Carbohydrate Research*, *219*, 203–213.

Yanaki, T., Kojima, T., & Norisuye, T. (1981). Triple helix of scleroglucan in dilute aqueous sodium hydroxide. *Polymer Journal*, *13*, 1135–1143.

Zentz, F., Verchère, J. -F., & Muller, G. (1992). Thermal denaturation and degradation of schizophyllan. *Carbohydrate Polymers*, *17*, 289–297.