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Effects of thermal, alkaline and ultrasonic treatments on scleroglucan stability and flow behavior

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ABSTRACT

Aqueous solutions (0.2%, w/v) of scleroglucans from *Sclerotium rolfsii* ATCC 201126 from different cultivation time or purification protocol (EPS I, EPS II, EPSi) as well as a commercial scleroglucan (LSCL) exhibited different sensitivity against thermal (65, 95 and 150 °C), ultrasonic (1, 5 and 10 min; 20% amplitude) or alkaline (0.01–0.2 N NaOH) treatments. Scleroglucan triple helix usually showed signs of denaturation at 150 °C or with 0.2 NaOH with a pronounced decrease in apparent viscosity and loss of pseudoplastic behavior. Differences in sensitivity could be noted depending on the scleroglucan sample, which may be likely related to polysaccharide conformational features, and these latter to production and/or downstream processing conditions. Transmission electron microscopy showed scleroglucan topologies in accordance with thermal and alkaline denaturation. Size exclusion chromatography of control scleroglucans revealed elution profiles compatible with macromolecular aggregates which tended to diminish or disappear as thermal, alkali or sonication treatments progressed. Scleroglucan granule dissolution process took ~8–14 s, according to DIC-light microscopy, and showed to be facilitated by addition of NaOH.

1. Introduction

Scleroglucan is a neutral β-1,3-β-1,6-glucan produced by different *Sclerotium* species. Oriented fiber X-ray diffraction revealed its triple helical conformation in the solid state (Bluhm, Deslandes, Marchessault, Pérez, & Rinaudo, 1982). In water, scleroglucan molecules exist in a stiff, triple stranded helical structure, where side chains are exposed toward the exterior (Gawronski, Aguirre, Conrad, Springer, & Stahmann, 1996; Norisuye, Yanaki, & Fujita, 1980; Sletmoen & Stokke, 2008; Yanaki, Kojima, & Norisuye, 1981). The driving force for triple helix construction would be based in interstrand H-bonds inside the helical core (Atkins & Parker, 1968; Bluhm et al., 1982), receiving a contribution from hydrophobic forces (McIntire & Brant, 1998).

When the strength of these bonds is decreased below a critical limit, scleroglucan triplex dissociates into random coils. This helix-coil transition, which is a cooperative process, is commonly referred as 'denaturation' (Cantor & Schimmel, 1980; Sletmoen & Stokke, 2008). Denaturation of β-1,3-D-glucans has been reported to occur in alkaline solutions (>0.25 M NaOH) (Bo, Milas, & Rinaudo, 1987; Kitamura et al., 1996; Tabata, Ito, Kojima, Kawabata, & Misaki, 1981), in dimethylsulfoxide (DMSO; water weight fraction $W_H < 0.13$) (Kitamura & Kuge, 1989; Norisuye et al., 1980; Sato, Norisuye, & Fujita, 1981; Yanaki, Norisuye, & Fujita, 1980), or when increasing the temperature above the triplex melting temperature, $Tm = 135$ °C (Norisuye et al., 1980; Yanaki, Tabata, & Kojima, 1985).

The underlying mechanism inducing helix dissociation would differ in each case. Based on the present knowledge, in high pH environments, the triplex likely dissociates due to the high charge density introduced along the strands which leads to electrostatic repulsions in between. In DMSO, H-bonds are destabilized due to the chaotropic solvent properties, whilst when exposed to high temperatures the thermal energy supplied to the strands leads to helix destabilization (Sletmoen & Stokke, 2008).

Under optimized conditions, *Sclerotium rolfsii* ATCC 201126 is able to secrete into the culture medium important amounts of scleroglucan (Fariña, Siñeriz, Molina, & Perotti, 2001). Scleroglucan aqueous solutions use to exhibit interesting and very well recognized rheological properties, mainly due to the high molecular weight and the macromolecular spatial conformation corresponding to a very stiff helical structure (Yanaki et al., 1981). Unique properties make it especially attractive for diverse industrial applications. Neutral aqueous solutions, where scleroglucan adopts a triple helical conformation, typically yield high viscosity solutions

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with pseudoplastic behavior. *S. rolfsii* ATCC 201126 scleroglucan aqueous solutions showed stable viscosities over a wide range of temperature (up to 100 °C/60 min) and pH (0–13) (Fariña et al., 2001). This β-glucan, as other closely related glucans, would be however susceptible to denaturation into single coiled chains when subjected to high pH, high temperature and/or in DMSO, with the consequent viscosity loss due to intermolecular H-bonds disruption (Bo et al., 1987; Norisuye et al., 1980).

In this work, we report on the triplex stability of scleroglucans (EPS I, EPS II and EPSi) produced by *S. rolfsii* ATCC 201126 at fermenter scale, recovered and subsequently purified. The stability of aqueous solutions of these scleroglucans and a commercial scleroglucan (LSCL) after undergoing heating, alkaline or ultrasonic treatments was comparatively assessed in between and against the untreated controls, as witnessed by their rheological behavior and size exclusion chromatography (SEC) profiles. Events occurred through alkali-facilitated scleroglucan granule dissolution were also monitored by polarized light microscopy and topological changes under heat-alkali combined treatment were recorded by transmission electron microscopy (TEM).

2. Materials and methods

2.1. Scleroglucans

EPS I and EPS II (M_w ~5.2×10^6 Da) from *S. rolfsii* ATCC 201126 were produced under batch culture mode with optimized culture medium (MOPT) and selected operative conditions, recovered at two different fermentation times (48 and 72 h, respectively), and subsequently purified with ethanol as previously described (Fariña et al., 2001). EPSi from *S. rolfsii* ATCC 201126 was produced under the same conditions, recovered at 72 h and purified with isopropanol (Viñarta, 2009; Viñarta, Yossen, Vega, Figueroa, & Fariña, 2013).

All EPS productions were started with inocula from PM20 liquid cultures (Fariña, Siñeriz, Molina, & Perotti, 1998) which were used for inoculation at 10% (v/v) of stirred-tank bioreactors fitted with baffles and six-flat bladed Rushton turbine impellers. The MOPT medium for EPS 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·H_2O, 0.7 (initial pH adjusted to 4.5). Operative conditions were set as follows: air flow rate, 0.5 vvm; stirrer speed, 400 rpm; temperature, 30 °C, pH uncontrolled.

At the end of fermentations, EPS-containing broths were harvested at the above specified times and were homogenized with the aid of a hand blender (medium output, 30 s-pulses), three-fold diluted with distilled water, neutralized, and heated at 80 °C for 30 min. Treated samples were then centrifuged (27,500 × *g*, 20 min, 10–15 °C) and EPS from clear supernatant was cooled at 5 °C and subsequently precipitated by adding an equivalent volume of either ethanol 96° (EPS I and EPS II) or isopropanol (EPSi). Mixtures were allowed to stand overnight at 5 °C to complete EPS precipitation. The precipitate was then recovered with a fine sieve (Macotest A.S.T.M. No. 60) and redissolved in distilled water. Crude EPSs were further purified by alcohol-reprecipitation (two times) (Fariña et al., 2001). Finally, EPSs were freeze-dried (~1 day) and milled (by means of two 10 s-pulses in a domestic coffee grinder) to a whitish glucan powder, showing a high purity grade (~98% for EPS I and EPS II and ~88% for EPSi) according to the phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) with dextran as standard.

Commercial scleroglucan, LSCL (M_w = 4.5×10^5 Da), from CarboMer Inc. (USA) was used without modification or subsequent purification.

2.2. Scleroglucan solutions

Polymer solutions (0.2% or 0.4%, w/v) were prepared according to the following protocols: EPS I, EPS II and LSCL were added to the appropriate volume of distilled water and hydrated overnight under magnetic stirring at 400 rpm and 25 °C. Thereafter, magnetic stirring at 60 °C was continued until polymer became completely dissolved (~48–72 h for EPS I and EPS II; ~72–96 h for LSCL). EPSi was hydrated under the same conditions for 3 h and complete dissolution required 3 h under magnetic stirring. If necessary, at the end of solution preparation, volume was corrected by addition of distilled water, and blend was left aside to reach room temperature (25 °C). In all cases, NaN_3 (0.02%, w/v) was added as preservative, in the presence of $NaNO_3$ (0.1 M) as electrolyte (Kath, Lange, & Kulicke, 1999).

2.3. Polymer treatments

Polymer aqueous solutions (0.2%, w/v) were subjected to either 65, 95 and 150 °C during 30 min.

Alkaline treatment with 0.01, 0.05, 0.1 and 0.2 N NaOH was performed in polymer aqueous solutions at 0.2% (w/v) final concentration by addition of an adequate volume of 0.4 N NaOH under magnetic stirring for 10 min at room temperature.

Different ultrasonication times (1, 5 and 10 min) in a sonicator (Sonics Vibra Cell, VCX 130) with a 20% amplitude was applied to polymer aqueous solutions (0.2%, w/v). Higher amplitudes were eventually tested for EPSs not susceptible to standard treatments.

2.4. Rheological measurements

Thermal, alkaline and ultrasonic treatments were followed by apparent viscosity determinations; pH values were also registered. Apparent viscosity (η_{app}) measurements were carried out according to already reported methodology (Viñarta, Molina, Figueroa, & Fariña, 2006), with a rotational viscometer (Cannon® LV 2000, Cannon® Instrument Co., State College, PA, USA) with narrow gap concentric cylinders or spindles equipped with a Temperature Controlled Unit TCU1000 and a Small Sample Adapter. Measurements were carried out with a TL-5 spindle at 25 °C and at shear rates between 0.396 and 79.2 1/s. Presented data are average of at least three measurements. Rheological parameters were estimated by fitting η_{app} data to the Ostwald–de-Waele model:

$$\eta_{app} = K\gamma^{(n-1)}$$

where γ is the shear rate, K the consistency coefficient and n the flow behavior index.

Assays were run in triplicate from independent assays and the statistical significance was assessed according to one-way ANOVA and Tukey–Kramer multiple comparisons tests (GraphPad Instat Biostatistics package, version 3.0).

2.5. Size exclusion chromatography (SEC) of EPSs

Scleroglucan solutions (treated and controls) were analyzed by SEC using a XK 16/100 column (General Electric Healthcare, Sweden) (1.6 cm × 100 cm, working volume: 183 cm^3) packed with Sephacryl S-1000 resin (General Electric, Healthcare, Sweden). Dextrans from *Leuconostoc mesenteroides* B-512 (Sigma) (M_w = 7.30×10^4, 5.15×10^5 and 2.00×10^6 Da) dissolved in 0.25 N NaOH were used as standards for calibration. The column was eluted with 0.25 N NaOH at a flow rate of 0.18 mL/min. The sample was injected (250 μL) and fractions were collected in a fraction collector Frac-920 (General Electric Healthcare, Sweden). An aliquot