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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

of each fraction was analyzed for total carbohydrate by the phenol-sulfuric acid method (Dubois et al., 1956).

2.6. Light microscopy observation during scleroglucan dissolution

A light microscope (Nikon Microscope ECLIPSE 80i) using polarizing filters and Nomarski differential interference contrast (DIC) optics was used. Images were captured with a microscope-coupled Nikon camera. Dry scleroglucan granules were placed between glass slide and cover slip and a small amount of water was added at the edges. To speed up the dissolution process, a small amount of 1 N NaOH was added at the edges of the cover slip (Cheeseman & Brown, 1995).

2.7. Transmission electron microscopy (TEM) of heat-alkali treated scleroglucan

Small amounts of scleroglucan (0.02–0.03 g) were dissolved in 10 mL of NaOH (0.1, 0.3 or 1 N). These solutions were then heated at constant temperature (65, 95 or 150 °C) for 30 min. Samples were prepared according to Cheeseman and Brown (1995), by dropping the gel solution onto a 300 mesh copper grid. The samples were first washed with water, then with Triton X-100 (0.25%, w/v) and again with water. Subsequently, the samples were negatively stained using uranyl acetate (2%, w/v), for TEM observation.

Microscopy was performed on a ZEISS EM 109 Electron Microscope (Oberkochen, Alemania) (Centro de Microscopía Electrónica – CCT – Tucumán) using an accelerating voltage of 80 kV, condenser and objective apertures of 400 and 30 μm respectively, and lens magnification between 50,000 and 85,000×.

3. Results and discussion

3.1. Properties and rheological characteristics of native and treated scleroglucans

Rheological behavior and pH of scleroglucan solutions were evaluated prior (control ≡ native) and after (treated) the different treatments performed. No significant differences were observed in the pH values of scleroglucan samples subjected to thermal or ultrasonic treatments (pH ~7) respect to the control, conversely to alkali-treated samples whose pH values oscillated between 12 and 13 (Table 1).

Rheological data were presented in the form of flow curves and fitted to the Ostwald–de Waele model (Figs. 1–3), where controls showed different levels of non-Newtonian pseudoplastic behavior. Larger pseudoplasticity involved higher consistency coefficient (K) and lower flow behavior index (n). The highest values of K (Table 1) were observed for EPSi and EPS I, followed by EPS II and LSCL, noting that EPS I was less sensitive to the presence of $NaNO_3$.

The addition of $NaNO_3$ as electrolyte to scleroglucan solutions, according to a previous protocol (Kulicke, Lettau, & Thielking, 1997), led to a decrease in η_{app} with respect to pure aqueous solutions (η_{app} % reduction in $NaNO_3$-added aqueous solution, EPSI: 0%, EPS II: 32%, EPSi and LSCL: 35%). The presence of $NaNO_3$, which would reduce intermolecular polymer interactions (Kulicke et al., 1997), could additionally have an osmotic effect at high concentration (0.1 M ≡ 8.5 g/L), compromising solvent molecules (H_2O), and thus reducing the macromolecule expansion ('coil contraction'), stiffness, and η_{app} (Fariña et al., 2001).

A similar response was previously noted particularly for EPS II when subjected to increasing concentrations of NaCl and different salts (Fariña et al., 2001). This may explain the herein observed lower pseudoplasticity of EPS II in the presence of $NaNO_3$ respect to EPS I (Table 1), reinforcing previous speculations on the conformational differences between EPS I and EPS II (Fariña et al., 2001; Viñarta et al., 2006, Viñarta, François, Daraio, Figueroa, & Fariña, 2007). Concerning EPSi, the effects of $NaNO_3$ on the final pseudoplasticity may have been attenuated because of the high viscosifying ability of this EPS (superior to EPS I and EPS II), a fact probably related to its better aqueous solubility after post-fermentation processing (Viñarta et al., 2013).

The performance of *S. rolfsii* ATCC 201126 native glucans (EPS I, EPS II and EPSi) was superior to that obtained for LSCL. In general terms, the more drastic assayed conditions (150 °C, 0.2 N NaOH and 10 min of ultrasonication), particularly showed a marked influence on the rheological behavior of scleroglucan solutions (Table 1), with an abrupt decline in η_{app} and loss of pseudoplastic behavior, with certain differences for EPSi and LSCL (see Sections 3.1.1, 3.1.2 and 3.1.3).

3.1.1. Thermal treatment

Rheological behavior of EPSi and LSCL solutions showed more similarity to EPS II when confronted to thermal treatment (Table 1, Fig. 1). For LSCL the initial η_{app} was quite low as compared with glucans from *S. rolfsii* ATCC 201126 (EPS I, EPS II and EPSi). This could be related to its lower M_w (4.5×10^5 Da), since it was similarly affected as EPS II and EPSi by $NaNO_3$ addition (see above).

Rheological parameters revealed no significant differences ($p > 0.05$) with respect to the control in all EPSs subjected to 65 and 95 °C. However, significant differences ($p < 0.001$) were found for samples subjected to 150 °C (from literature, $T_m \approx 135$ °C), with a loss of pseudoplastic behavior (Table 1, Fig. 1). That would allow inferring scleroglucan triplex denaturation due to destabilization induced by the thermal energy provided to the strands at high temperature (Sletmoen & Stokke, 2008).

Interestingly, increases in K at 65 and 95 °C could be associated with a minor increase in η_{app} as a consequence of the higher molecular order of certain polymers when treated at the annealing temperature (Falch, Elgsaeter, & Stokke, 1999). Aggregates formation and/or swelling of such aggregates as a result of higher intermolecular ordering (either triplets of triplex rods or supramolecular triple helical 'fasces-like' clusters) may be also suggested as a possible explanation (Gawronski, Park, Magee, & Conrad, 1999; Grassi, Lapasin, & Pricl, 1996; Hromádková et al., 2003).

The comparative study of EPSs against thermal degradation allowed identifying EPS I as the least sensitive polysaccharide, keeping a vestigial pseudoplastic behavior even when subjected to 150 °C. The coexistence of a certain proportion of triple helices may be speculated, but also, aggregates formation may be thought as a further explanation to the maintenance of η_{app} after high temperature treatment, followed by storage at room temperature (Gawronski et al., 1999; Grassi et al., 1996; Zentz, Verchère, & Muller, 1992). In a similar study, schizophyllan denaturation was incomplete and proceeded slowly at 130–140 °C with the coexistence of both EPS conformations for several hours, and was completed after 10 min at 161 °C (Kitamura & Kuge, 1989; Zentz et al., 1992). In the present work, the majority of evaluated EPSs could be denatured after 10 min at 150 °C.

3.1.2. Alkaline treatment

Although pseudoplasticity of all EPS solutions was abolished with 0.2 N NaOH, both LSCL and EPSi were particularly susceptible to the alkaline treatment, losing this character even at 0.05 and 0.1 N NaOH, respectively (Table 1, Fig. 2). Since the OH^- anion could fundamentally affect the interchain H-bonds (Bluhm et al., 1982; Bo et al., 1987; Shibata & Schurr, 1981; Sletmoen & Stokke, 2008; Tabata et al., 1981), the macromolecular conformation could be ascribed as the main cause of these differences. Alkali effects on rheological behavior were lower on EPS II and even less marked on EPS I (Table 1, Fig. 2). A more expanded macromolecule in LSCL and EPSi,

Table 1
Rheological parameters and pH of scleroglucan aqueous solutions subjected to thermal, alkaline and ultrasonic treatment *vs.* untreated controls.

Sample	Ostwald–de-Waele		pH	Sample	Ostwald–de-Waele		pH
	K (mPa s^n)	n			K (mPa s^n)	n	
EPS I control	1223.0	0.21	7.30	**EPS II control**	713.5	0.23	6.98
EPS I/65 °C	1238.0	0.21	7.28	EPS II/65 °C	705.5	0.23	7.00
EPS I/95 °C	1265.0	0.21	7.20	EPS II/95 °C	716.4	0.23	6.99
EPS I/150 °C	257.2	0.42	6.70	EPS II/150 °C	NA	NA	6.80
EPS I/0.01 N NaOH	1216.0	0.20	11.88	EPS II/0·01 N NaOH	901.2	0.22	11.77
EPS I/0.05 N NaOH	1104.0	0.24	12.60	EPS II/0·05 N NaOH	914.7	0.24	12.63
EPS I/0.1 N NaOH	774.1	0.30	12.88	EPS II/0·1 N NaOH	103.4	0.49	12.88
EPS I/0.2 N NaOH	NA	NA	13.06	EPS II/0·2 N NaOH	NA	NA	13.01
EPS I/1 min 20%[a]	1246.0	0.22	7.10	EPS II/1 min 20%[a]	712.0	0.22	7.00
EPS I/5 min 20%	695.3	0.25	7.16	EPS II/5 min 20%	222.2	0.21	7.08
EPS I/10 min 20%	128.5	0.30	7.33	EPS II/10 min 20%	NA	NA	7.00
EPSi control	1229.0	0.09	7.05	**LSCL control**	283.5	0.31	7.35
EPSi/65 °C	1275.0	0.09	7.15	LSCL/65 °C	337.0	0.19	7.18
EPSi/95 °C	1346.0	0.13	7.18	LSCL/95 °C	308.8	0.23	7.16
EPSi/150 °C	NA	NA	6.80	LSCL/150 °C	NA	NA	6.76
EPSi/0.01 N NaOH	193.4	0.40	11.59	LSCL/0·01 N NaOH	261.2	0.25	11.83
EPSi/0.05 N NaOH	NA	NA	12.54	LSCL/0·05 N NaOH	242.5	0.22	12.77
EPSi/0.1 N NaOH	NA	NA	12.84	LSCL/0·1 N NaOH	NA	NA	12.95
EPSi/0.2 N NaOH	NA	NA	12.98	LSCL/0·2 N NaOH	NA	NA	13.04
EPSi/1 min 20%[a]	1272.0	0.07	6.87	LSCL/1 min 20%[a]	294.8	0.24	7.16
EPSi/5 min 20%	1329.0	0.08	6.80	LSCL/5 min 20%	294.0	0.26	7.15
EPSi/10 min 20%	1231.0	0.10	6.80	LSCL/10 min 20%	308.9	0.27	7.22

Viscosity measurements were carried out at 25 °C and at shear rates between 0.396 and 79.21 1/s. Data were fitted to the Ostwald–de-Waele model (K = consistency coefficient; n = flow behavior index). Standard errors were all below 10%. For details on concentrations, see Section 2.
NA: not applicable to model.
[a] Ultrasonic treatment with amplitude = 20%.

as also suspected for EPS II to a lesser extent, could make them more susceptible to either salts (e.g. $NaNO_3$) or alkali. Macromolecule relaxation would then expose interchain linkages to the hydroxyl ion attack, promoting denaturation and loss of pseudoplasticity.

With respect to EPS II behavior, a slight increase in pseudoplasticity (>K) at the lowest NaOH concentrations (0.01 and 0.05 N, Table 1) could be associated to a controlled partial opening of the strands with a macromolecular reorganization which may favor polymer solubility and stiffness, thus increasing η_{app} as previously described (Young, Dong, & Jacobs, 2000). Alkalinization has been recommended to obtain 'molecularly true aqueous polysaccharide solutions' (Šoltés, Mislovičová, & Sébille, 1996). As reported for other β-1,3-glucans, beyond 0.1–0.2 N NaOH, trimer dissociation would be likely related to electrostatic repulsion between strands, due to the alkali-introduced high charge density (Sletmoen & Stokke, 2008).

Similar results were obtained for schizophyllan, where denaturation to single strands was also very quick and efficient at NaOH

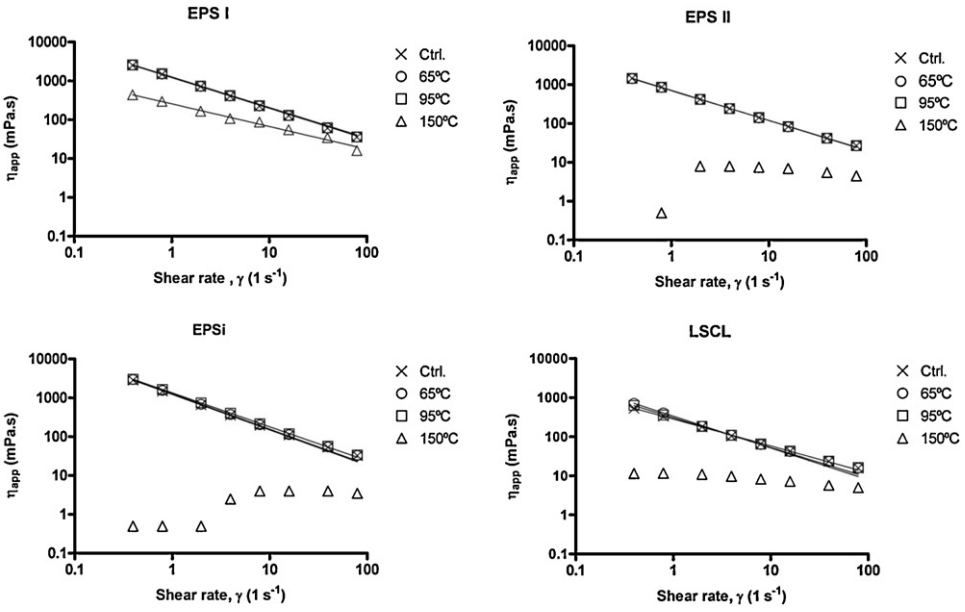

Fig. 1. Rheological behavior of scleroglucan aqueous solutions (0.2%, w/v) subjected to thermal treatment (65, 95 and 150 °C) *vs.* untreated control. For the sake of clarity, only critical fittings to the Ostwald–de-Waele model were displayed and standard errors were not plotted. For details, see Section 2.

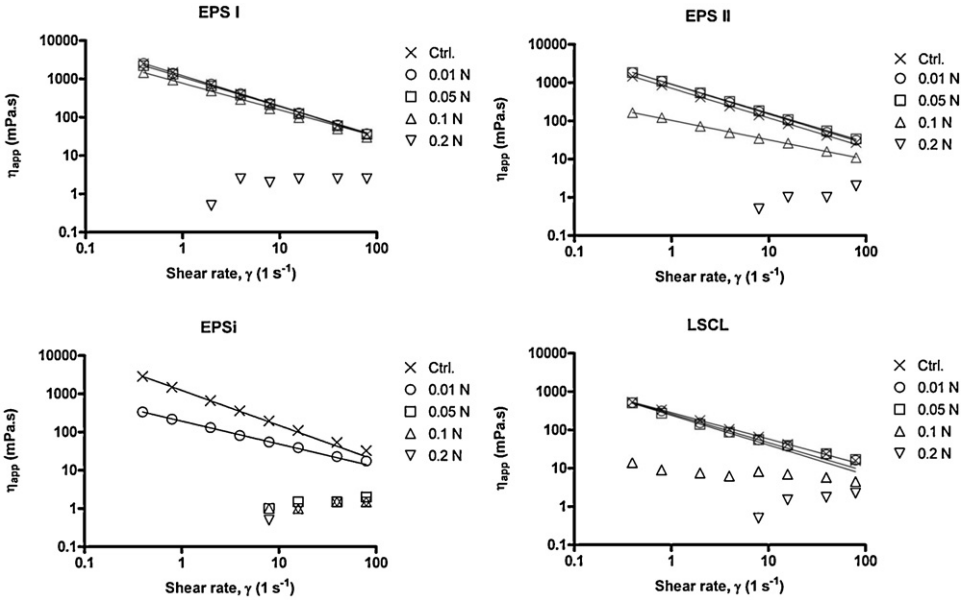

Fig. 2. Rheological behavior of scleroglucan aqueous solutions (0.2%, w/v) subjected to alkaline treatment (0.01; 0.05; 0.1 and 0.2 N NaOH) *vs.* untreated control. For the sake of clarity, only critical fittings to the Ostwald–de-Waele model were displayed and standard errors were not plotted. For details, see Section 2.

final concentrations superior to 0.05 M, but below this value the coexistence of triplex and single strands was revealed (Zentz et al., 1992).

3.1.3. Ultrasonic treatment

A significant decrease in η_{app} and K with the increase of ultrasonication time was observed, being EPS II more sensitive than EPS I, and showing a loss of pseudoplastic character after 10 min of treatment (Table 1, Fig. 3).

On the other hand, EPSi and LSCL experienced no significant differences in η_{app} after ultrasonic treatment under the assayed conditions (Fig. 3). Conversely to causing denaturation or fragmentation, this treatment seemed to induce higher solubility in these EPSs, which could be reflected in slight increases of K (Table 1). A treatment at higher amplitude (50%, 1 min), however, led to a marked decrease in η_{app} (data no shown).

These results would probably suggest that ultrasonication could have a predominant influence on polysaccharide solubility (Stahmann et al., 1995). As speculated above, a more expanded macromolecule in LSCL and EPSi, with a higher macromolecular relaxation, might turn the EPS more resistant to ultrasonication. It has been postulated that, during ultrasonication, polymer changes may be attributed to the stretching through elongational flow fields produced by imploding cavities (Kulicke, Otto, & Baar, 1993). Reversion between different transition states of the triplex and the delay in the appearance of lower M_w molecules as a consequence of bond breaking events could account for the apparent stability of these structures (Falch & Stokke, 2001).

Findings on the effects of ultrasonication on EPS II and to a lesser extent in EPS I are particularly valuable considering the potential biomedical application of scleroglucans. It has been already emphasized that the possibility to obtain a low-viscosity EPS

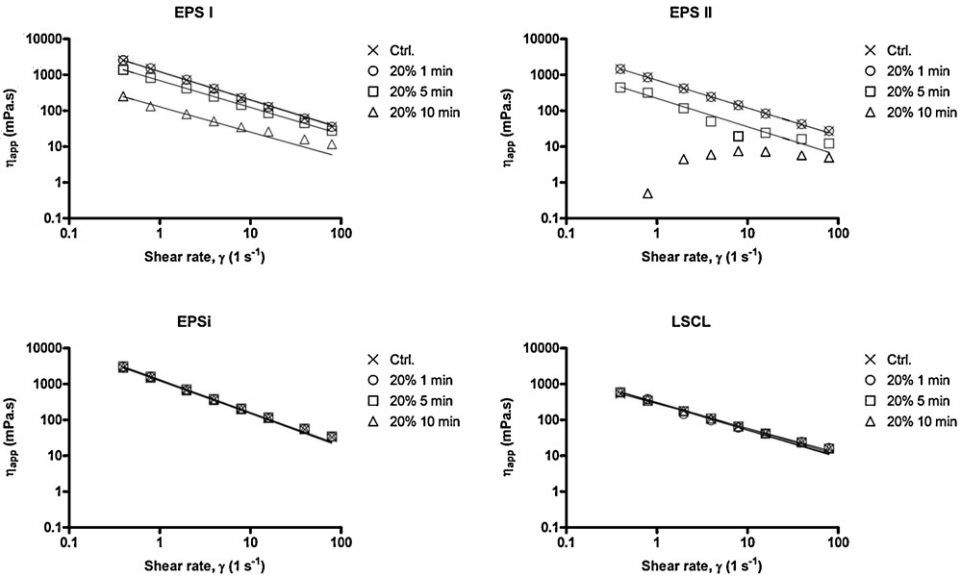

Fig. 3. Rheological behavior of scleroglucan aqueous solutions (0.2%, w/v) subjected to ultrasonic treatment (1, 5 and 10 min, amplitude: 20%) *vs.* untreated control. For the sake of clarity, only critical fittings to the Ostwald–de-Waele model were displayed and standard errors were not plotted. For details, see Section 2.

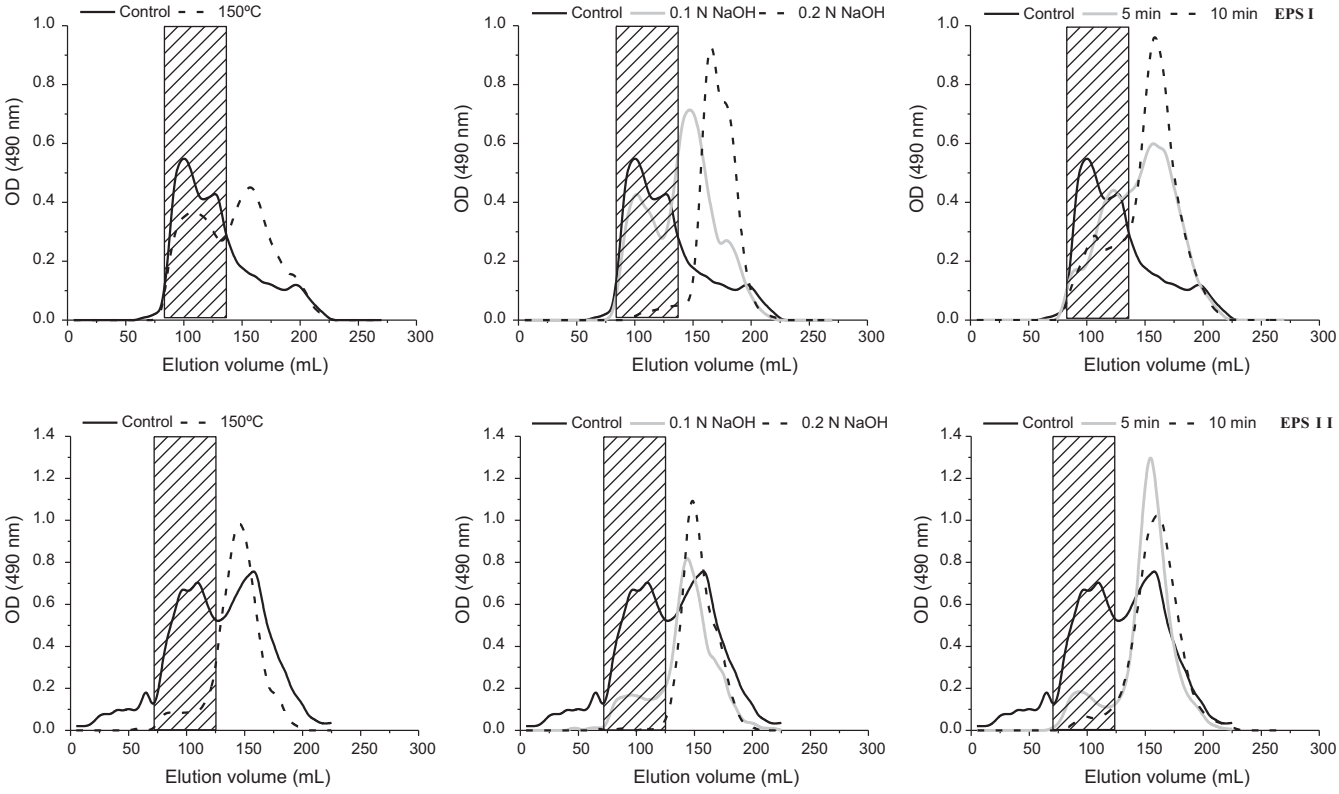

Fig. 4. Elution profiles obtained by SEC for aqueous solutions of EPS I, EPS II and LSCL after thermal (150 °C, 30 min), alkaline (0.1 and 0.2 N NaOH, 10 min, 25 °C) or ultrasonic (5 and 10 min, amplitude: 20%) treatment compared to untreated controls. Striped bars indicate elution zones presumed as single helix molecular aggregates. Chromatographic profiles for EPSi (not shown) were similar to EPS II.

solution but keeping the main macromolecular characteristics would be relevant at the time of testing biological activity, since β-glucans treated with DMSO or alkali may be antagonistic to cellular systems and biomacromolecules activity (Sletmoen & Stokke, 2008). Similarly, controlled ultrasonication was previously intended to overcome this problem when evaluating the potential use of high-viscosity native schizophyllan as a chemotherapeutic agent (Tabata et al., 1981).

Resuming, scleroglucan denaturation (triple helix → single brand transition) could be hypothesized as the underlying reason for the pronounced decline in η_{app} and K values (Table 1) for samples treated at 150 °C and with 0.2 N NaOH (Figs. 1 and 2). This speculation arises from considering the viscosity fall as a reliable evidence of the loss of triplex conformation and its semi-rigidity due to interchain H-bonds cleavage. In this sense, both temperature and alkali showed to be able to break such linkages, resulting in scleroglucan single strands.

With regard to sonication effects, and in accordance with observations for heating and alkaline treatments, EPS II showed higher susceptibility than EPS I (Table 1, Fig. 3). Conversely, EPSi and LSCL showed high stability against identical ultrasonication conditions, but were more sensitive to alkali, thus emphasizing the possibility for a different mechanistic rationale.

3.2. SEC profiling of treated scleroglucans

The use of SEC for studying M_w distribution in β-glucans has been already highlighted (Šoltés et al., 1996). Chromatographic profiles of EPSs (EPS I, EPS II and LSCL; EPSi ≈ EPS II, not shown) showed a common elution zone (between 75–80 and 125–140 mL) (Fig. 4), likely corresponding to single-stranded molecular aggregates or eventually, aggregates of remaining triplexes coexisting in a low proportion, which may be formed upon aging (Zentz et al., 1992). Although the presence of triple helices would be less probable under the elution conditions used (0.25 N NaOH), formation of molecular aggregates (e.g. dimers, trimers, etc.) (Picullel, 1998) likely bound by different and less alkali-susceptible stabilization forces may be suspected below a given temperature or alkali threshold upon aging (McIntire & Brant, 1998; Zentz et al., 1992). Such aggregates could present a greater hydrodynamic volume in relation to the single helix and therefore, a lower elution volume would be expectable (Grassi et al., 1996; Zentz et al., 1992).

As NaOH concentration increased, the proportion of these aggregates would have shown a gradual decrease, as well as with the rise in treatment temperature and ultrasonication time (Fig. 4). This fraction became minimal under the most drastic assayed conditions, which may have impeded molecular reassociation (e.g. 0.2 N NaOH). As more severe conditions were applied, a simultaneous increase of a higher elution volume peak was observed, a fact probably consistent with an increase in the single unaggregated strands proportion (Fig. 4).

The incidence of NaOH on aggregates formation in the higher molar mass region was recently revisited (Gidley & Nishinari, 2009), highlighting that this event would contribute (although less than triple helices) to the scleroglucan solution intrinsic viscosity. In agreement, laser light scattering (LLS) analysis in schizophyllan confirmed the single-helix aggregation hypothesis, showing an almost twofold increase in the scattered light intensity. Likewise, single-strands aggregation was previously associated to poor solution filterability (Zentz et al., 1992).

Many scleroglucan applications depend on its viscosifying capacity. However, it has been observed that scleroglucan samples can differ between lots according to production conditions and downstream processing (Viñarta et al., 2013). The latter generally involves alkaline and/or thermal treatments, which can promote denaturation or degradation (Zentz et al., 1992), but relatively few

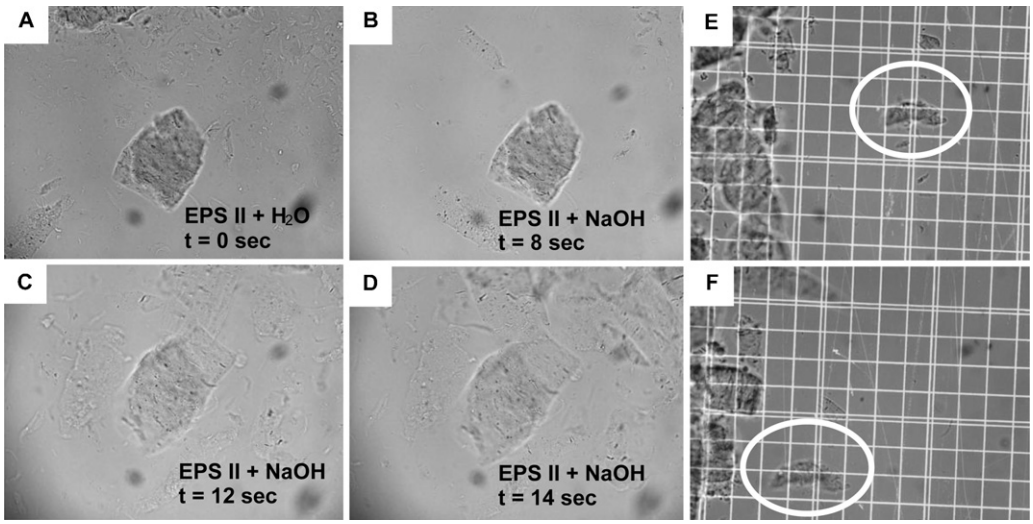

Fig. 5. Microphotographs obtained by DIC Nomarski (40×) during scleroglucan (EPS II) granule dissolution process. A–D: dissolution after adding 1 N NaOH. E and F: swelling measurements of scleroglucan granules in a Neubauer chamber (E: EPS II + H_2O, t = 0 s, granule length = 0.42 mm; F: EPS II + 1 N NaOH, t = 10 s, granule length = 0.62 mm).

works have deepened about downstream processing effects upon rheological characteristics of β-glucans (Viñarta et al., 2013). This processing could affect molecular, structural and functional features of EPS, and consequently their applicability and biological activity (Laroche & Michaud, 2007).

As herein evidenced, although the underlying mechanism could vary according to the applied treatment, the triple helix of EPSs from *S. rolfsii* would be denatured if subjected to drastic alkaline treatment (NaOH ≥ 0.2 N) or elevated temperatures (150 °C), accompanied by a rupture of interstrand H-bonds (Bo et al., 1987; Norisuye et al., 1980; Sletmoen & Stokke, 2008).

A more pronounced tendency to form molecular aggregates, as described for other β-glucans (Grassi et al., 1996; Picullel, 1998; Zentz et al., 1992), was detected in the control samples of EPSs from *S. rolfsii* ATCC 201126 (EPS I, EPS II and EPSi) than in commercial scleroglucan (LSCL) (Fig. 4). The persistence of these aggregates along with the coexistence of a minor proportion of triplexes might help interpreting the residual pseudoplasticity of some treated scleroglucans, particularly in EPS I (150 °C, 0.1 N NaOH and 10 min-20%-ultrasonication).

Concerning ultrasonication effects, previous works reported its use for high M_w polymer fragmentation (Falch et al., 1999; Falch & Stokke, 2001; Stahmann et al., 1995). Contrary to these observations, the herein tested scleroglucans revealed no signs of fragmentation under the applied conditions, although denaturation was suggested for EPS II after 10 min of ultrasonication (Table 1, Figs. 3 and 4).

3.3. Light microscopy observation through EPS dissolution

Scleroglucan dissolution process in alkaline environment (1 N NaOH) took about 8–14 s, but elapsed time may be dependent on the NaOH molarity. The process started with granule swelling (granule length: 0.42 mm at start point and 0.62 mm after 10 s, Fig. 5 E and F), followed by a loss of structure until becoming almost invisible (Fig. 5 A–D). The estimated dissolution mean rate of scleroglucan granule under alkaline conditions was ~0.02 mm/s, where NaOH may have promoted H-bond disruption due to ionization, as also described for the related unbranched β-glucan curdlan (Cheeseman & Brown, 1995).

3.4. Transmission electron microscopy (TEM) of treated EPS

Scleroglucan topologies shown by TEM were in accordance with denaturation at 150 °C and at NaOH concentrations above 0.1 N (Fig. 6). Microfibrils were observed in samples prepared at 65 and 95 °C using 0.1 N NaOH (Fig. 6 A and B), whereas microfibrils tended to disappear at 150 °C (Fig. 6 C) or at NaOH concentrations above 0.1 N (data not shown). β-(1,3)-glucan microfibrils have been described in the literature as associations of triple helices which held together (Cheeseman & Brown, 1995; Gawronski et al., 1999; McIntire & Brant, 1997; Vuppu, Garcia, & Vernia, 1997). As temperature increased under alkaline environment, disappearance of microfibrils could be related to the increase in single strands

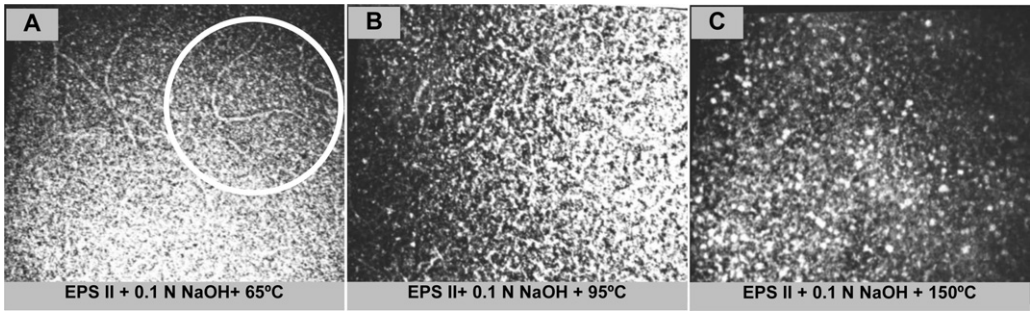

Fig. 6. Electron micrographs obtained by TEM (85,000×) for scleroglucan (EPS II) showing topologies obtained after treatment at different temperatures in the presence of moderate NaOH concentration (microfibrils are circled). For details, see Section 2.

proportion and the simultaneous decline or disappearance of triple helical conformation.

However it is worth to mention that scarce microfibrils were eventually observed in treated samples even under the most drastic conditions, which could not be totally surprising considering the speculation about the coexistence of different conformational states, a fact probably attributed to the lack of homogeneity of local pH when a concentrated base is added (Zentz et al., 1992). These observations were consistent with results on the scleroglucan rheological properties of thermally- or alkali-treated samples (see Sections 3.1.1 and 3.1.2).

As previously observed in curdlan, moderate temperature or NaOH concentration (e.g. at 95 °C) may induce association of microfibrils to form an organized structure of gel favored by hydrophobic forces, (Harada, Koreeda, Sato, & Kasai, 1979). A temperature increase able to induce triplex denaturation (herein 150 °C, see Section 3.1.1) would hamper microfibrils association probably leading to individual fiber swelling (Cheeseman & Brown, 1995; Harada et al., 1979).

4. Conclusions

Results supported that EPSs from *S. rolfsii* ATCC 201126 as well as the tested commercial scleroglucan resisted a wide range of temperatures and alkali concentrations, and were poorly affected by ultrasonication, but the most resistant EPSs could usually become denatured at 150 °C and beyond 0.2 N NaOH. These effects could be reflected on their rheological behavior and also by TEM.

The coexistence of native and denatured forms sometimes related to microheterogeneities during alkalinization or heating, and/or the presence of molecular aggregates may help to explain the observed residual pseudoplastic behavior after treatment.

In the light of the obtained results, the marked influence of downstream processing on biopolymer physicochemical behavior was once again confirmed, and therefore, it should be a stage within the biotechnological process to be particularly evaluated and standardized. The possibility to modify macromolecular characteristics or polysaccharide behavior depending on processing conditions, either during production or recovery/purification steps, was highlighted. Results would be also relevant for unraveling the influence of environmental conditions on the relationship between structural features and biological activity.

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