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ORIGINAL ARTICLE

Structural stability of *Sclerotium rolfsii* ATCC 201126 *β*-glucan with fermentation time: a chemical, infrared spectroscopic and enzymatic approach

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enzymatic digestion, fermentation, FT-IR, scleroglucan, *Sclerotium rolfsii,* structure.

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Abstract

Aims: *Sclerotium rolfsii* ATCC 201126 exopolysaccharides (EPSs) recovered at 48 h (EPS I) and 72 h (EPS II) of fermentation, with differences in rheological parameters, hydrogel topography, salt tolerance, antisyneresis, emulsifying and suspending properties, were subjected to a polyphasic characterization in order to detect structural divergences.

Methods and Results: Fermenter-scale production led to productivity (P_r) and yield ($Y_{P/C}$) values higher at 48 h (P_r = 0·542 g l^{-1} h^{-1}; $Y_{P/C}$ = 0·74) than at 72 h (P_r = 0·336 g l^{-1} h^{-1}; $Y_{P/C}$ = 0·50). Both EPSs were neutral glucose-homopolysaccharides with a *β*-(1,3)-glycosidic backbone and single *β*-(1,6)-glucopyranosyl sidechains regularly attached every three residues in the main chain, as revealed by chemical analyses. The infra-red diagnostic peak at 890 cm^{-1} confirmed *β*-glycosidic linkages, while gentiobiose released by *β*-(1,3)-glucanases confirmed single *β*-1,6-glycosidic branching for both EPSs.

Conclusions: The true modular repeating unit of *S. rolfsii* ATCC 201126 scleroglucan could be resolved. Structural stability was corroborated and no structural differences could be detected as to account for the variations in EPSs behaviour.

Significance and Impact of the Study: Recovery of *S. rolfsii* ATCC 201126 scleroglucan at 48 h might be considered based on better fermentation kinetic parameters and no detrimental effects on EPS structural features.

Introduction

Exopolysaccharides (EPSs) from microbial origin have attracted worldwide attention because of their novel and unique physical properties and their variety of industrial applications (Crognale *et al.* 2007; Laroche and Michaud 2007), thus encouraging any progress in their economic competitiveness. Scleroglucan, a glucan produced by different *Sclerotium* species, has been considered as one of the EPSs with great potential for commercialization (Survase *et al.* 2007).

Early applications of scleroglucan in enhanced oil recovery proposed it as a promising competitor for xanthan, based on its viscosifying, suspending and gelling properties over a wide range of temperature, pH and salinity (Halleck 1967). Later investigations documented the antitumor, antiviral and antimicrobial activity of scleroglucan and its immune stimulatory effects, thus being classified as a biological response modifier (BRM) (Bohn and BeMiller 1995; Kogan 2000; Zhang *et al.* 2007). More recently, discoveries concerning the scleroglucan potential in controlled drug release (Viñarta *et al.* 2007), and based on the aforementioned immunopharmacological properties, the applicability in the medical market is becoming seriously taken into account (Survase *et al.* 2007).

Sclerotium rolfsii ATCC 201126 demonstrated ability to produce high amounts of scleroglucan under optimized

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fermentation conditions (Fariña *et al.* 1998) and advances on the physicochemical characterization (Fariña *et al.* 2001) led to reveal a promising potential for different biotechnological applications (Viñarta *et al.* 2006, 2007).

Despite responding to a general structure of β-1,3–β-1,6-glucan, scleroglucans may exhibit variable degrees of β-1,6-glycosidic branching, differences in molecular weight (M_W), number and length of side chains, degree of polymerization and rheological characteristics depending on the *Sclerotium* species, strains, culture conditions or even the downstream processing (Bluhm *et al.* 1982; Zentz and Muller 1992; Leung *et al.* 2006; Survase *et al.* 2007).

Samples of scleroglucan produced by *S. rolfsii* ATCC 201126 at fermenter scale and recovered at two different cultivation times (EPS I at 48 h, and EPS II at 72 h), though having quite similar M_W and intrinsic viscosities, showed differences concerning rheological parameters of aqueous solutions and tolerance to inorganic salts (Fariña *et al.* 2001), variations in hydrogel pore dimensions (Viñarta *et al.* 2007), and distinct anti-syneresis (Viñarta *et al.* 2006), emulsifying and suspending properties (Viñarta *et al.* 2007).

Some desirable properties of the polysaccharide are likely related to the M_W distribution, physicochemical and structural characteristics of the macromolecule, and these might be modified during the biosynthesis and even the recovery process (Zentz and Muller 1992; Survase *et al.* 2007). Although some of these parameters have been previously evaluated in *S. rolfsii* EPSs from different fermentation times (Fariña *et al.* 2001), no significant variations were detected as to explain the already mentioned different behaviour. Structural divergences were then thought as a possible rationale and accordingly assessed.

The scarcity concerning structural analysis of complex carbohydrates is still up to date emphasized (Leung *et al.* 2006; Zhang *et al.* 2007). Much of the difficulties to address this topic have been usually related to the diversification of naturally occurring glycans, which is obviously derived from the extensively varied building blocks. As a consequence, universal protocols for their analysis had to be frequently replaced by polyphasic studies.

The present study confirms the EPS structural stability and might be useful for determining an appropriate time for EPS recovery depending on the expected or required scleroglucan behaviour. For scleroglucan production competitiveness, biopolymer yields and productivity may still represent parameters susceptible to optimization in order to face the biopolymer potential demand (Fariña *et al.* 1998; Survase *et al.* 2007). Then, if the feasibility to reduce the fermenter time usage exists, provided that EPS properties of interest are unaffected, scleroglucan productivity may be significantly increased.

In this work, EPS I and EPS II were comparatively assessed by a polyphasic approach focusing on the following aspects:

1. study and resolution of the true modular repeating unit of the scleroglucan produced by *S. rolfsii* under optimized culture conditions; and

2. chemical and structural stability of EPSs recovered at two critical fermentation times.

Materials and methods

Seed cultures and scleroglucan production

Sclerotium rolfsii ATCC 201126 was preserved as previously described (Fariña *et al.* 1996). Strain activation, inocula preparation and scleroglucan production with optimized culture medium (MOPT) were performed as early reported (Fariña *et al.* 1998, 2001). EPSs were recovered and purified after either 48 or 72 h of fermentation for their subsequent characterization (see below).

Analytical determinations throughout fermentation

Periodic samples along fermentation were analysed for biomass, EPS, pH and residual sucrose according to Fariña *et al.* (1998). For extracellular β-glucanase activity, culture broth samples were fourfold diluted with distilled water and subsequently centrifuged (27 500 ***g***, 20 min, 5°C). Proteins from diluted supernatant were precipitated by adding $(NH_4)_2SO_4$ at 60% saturation, and neutrality was kept by adding 0·05 mol l^{-1} Na_2CO_3. After complete precipitation, samples were centrifuged (27 500 ***g***, 20 min, 5°C) and the pellet was resuspended in 100 mmol l^{-1} citrate buffer pH 4·6. Soluble proteins were quantified by the Folin-Lowry method with BSA as standard.

Extracellular β-glucanase (indistinctly, β-1,3- or β-1,6-) activity was determined by incubating 1 ml of 0·5% (w/v) EPS II in citrate buffer pH 4·6 plus 1 ml of crude enzyme solution at 55°C for 5 min. The mixture was then transferred to an ice bath and the reaction was stopped by adding 3 ml of 3,5-dinitro salicylic acid reagent (DNSA). Colorimetric reaction for determining released reducing sugars was performed by incubating the mixture in a boiling water bath for 12 min. One blank with no substrate (EPS) and another blank with no enzyme were included in order to discount for the reducing power contributed by the crude enzyme preparation or the EPS solution, respectively. Reducing sugars were determined by the colorimetric method of Miller (1959) with glucose as standard. One unit (U) of β-glucanase activity was defined as the amount of enzyme releasing 1 μmol of reducing sugars per min under the conditions assayed.

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Bulk polysaccharide recovery and purification

EPS from bioreactor was recovered and purified as previously described (Fariña *et al.* 2001). The EPSs corresponding to 48 and 72 h of cultivation were arbitrarily identified as EPS I and EPS II, respectively. The glucan powder grade of purity included analyses for: protein content (Folin-Lowry, with BSA as standard), reducing sugars (Somogyi-Nelson, with glucose as standard) and total carbohydrates (phenol-sulphuric acid method, with dextran as standard).

Precipitation with quaternary ammonium salts

EPS aqueous solutions (2 g l^{-1}) were treated with a 10% (w/v) aqueous solution of *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB) and precipitation characteristics were tested (Rapp *et al.* 1979).

Procedures for structural analysis of polysaccharides

The protocols described below were conducted for both EPS I and EPS II.

Total hydrolysis

The method of Saeman *et al.* for β-linked D-hexoglycans was applied (Adams 1965). Five millilitres of 72% (w/v) H_2SO_4 was added to 100 mg of EPS. The mixture was kept at 30°C in a water bath for 45 min (with stirring at 10-min intervals) and then, distilled water (140 ml) was added and the solution autoclaved (1 atm, 1 h). The hydrolysate was brought to a final volume of 250 ml, neutralized with $CaCO_3$ and centrifuged (27 500 ***g***, 15 min, 10–15°C). The hydrolytic products contained in supernatant were examined by HPLC with a Rezex Organic Acid column (Phenomenex) (300 × 7·8 mm), operated at a running temperature of 55°C. The mobile phase was 10 mmol l^{-1} H_2SO_4 in Milli-Q (Millipore) water at a flow rate of 0·6 ml min^{-1}. Chromatograph (Gilson, Villiers-le-Bel, France) was equipped with a differential refractive index detector (LKB 2142; Pharmacia, Bromma, Sweden).

Periodate oxidation

According to the procedure of Hay *et al.* (1965), 70 mg EPS were dissolved in 45 ml of acetate buffer pH 4 (0·2 mol l^{-1} acetic acid–0·2 mol l^{-1} sodium acetate) in a 100 ml-volumetric flask. To this, 50 ml of $28{\cdot}88 \times 10^{-3}$ mol l^{-1} *m*-KIO_4 (in acetate buffer) were added, and the solution was rapidly made up to 100 ml; the time of addition was annotated. Experiments were carried out in duplicate; a blank solution was made up omitting the glycol. Reaction mixture and blank aliquots were withdrawn at intervals for analysis of metaperiodate uptake and formic acid liberation until two consecutive determinations gave the same result.

Metaperiodate consumption was followed by the Fleury-Lange method (Guthrie 1962). An aliquot of oxidation sample was pipetted into a 100 ml-flask and treated with 10 ml of a saturated solution of $NaHCO_3$. Then, 20 ml of 0·01 mol l^{-1} $NaAsO_2$ and 2 ml of 20% (w/v) KI were immediately added. After setting aside for 15 min in the dark, the excess sodium metaarsenite was titrated against 0·005 mol l^{-1} I_2, using 1 ml of a 1% (w/v) sodium starch glycolate as indicator; the endpoint was the persistence of a faint blue-purple colour in solution. The molecular proportions of metaperiodate consumed per mole of glucose in the polysaccharide were as follows:

$$\frac{M}{G} \times \left[m - \frac{0 \cdot 01A(V - v)}{10\,000} \right]$$

where M = molecular weight of the monosaccharide component; G = grams of polysaccharide that consumed $[m - (0 \cdot 01A(V - v)/10\,000)]$ moles of *m*-KIO_4; m = moles of metaperiodate in the initial oxidant solution (blank-based calculation); A = ml of sample (5 ml in this case); V = ml of 0·005 mol l^{-1} I_2 ≡ 20 ml of 0·01 mol l^{-1} $NaAsO_2$; v = ml of 0·005 mol l^{-1} I_2 ≡ excess meta-arsenite in the titration; $(V - v)$ = ml of 0·005 mol l^{-1} I_2 ≡ metaarsenite consumed by 5 ml of sample.

The determination of formic acid was carried out by titration with alkali using indicators (Hay *et al.* 1965). Aliquots (10 ml) of the oxidation mixture were withdrawn at desired times and then treated with acid-free ethylene glycol (1 ml). After shaking, the mixture was held at room temperature for 10 min. Nitrogen was bubbled through the mixture for 10 min prior to titration with 0·01 mol l^{-1} NaOH using methyl red as indicator [0·1% (w/v) in ethanol]. A reagent blank was treated in the same way with ethylene glycol. The difference in acidity between the blank and the sample represented the formic acid liberated from the polysaccharide.

Mild Smith degradation

The metaperiodate oxidized polysaccharide was subjected to Smith degradation according to a modification of the procedure described by Goldstein *et al.* (1965). An aliquot of oxidation sample (10 ml) was treated with ethylene glycol to destroy the excess of metaperiodate and, after 30 min, the solution was dialysed (MWCO 12000) overnight against distilled water at 5°C. The dialysed sample containing the oxidized polysaccharide was brought to 25 ml with distilled water and then, 0·1 g of $NaBH_4$ were added all at once. After reduction at room

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temperature during 10 h, H_2SO_4 was added for a final concentration of 0·05 mol l^{-1} and degradation was allowed to proceed for 48 h at room temperature (mild Smith degradation).

The reaction mixture was centrifuged (27 500 ***g***, 20 min, 5°C) and supernatant was analysed to determine the liberation of glycerol per mol of glucose in the polysaccharide. Glycerol was determined by HPLC with a Rezex Organic Acid column (Phenomenex, 300 × 7·8 mm) under the same conditions as above described for EPS hydrolytic products.

Fourier-transformed infra-red spectroscopy

Infra-red spectra of purified EPSs (I and II) were obtained using a Fourier transform infrared spectrophotometer (Perkin-Elmer FTIR 1600 IR). The sample was ground with spectroscopic grade KBr powder and then pressed into 1 mm pellets for Fourier-transformed infra-red (FT-IR) measurement in the frequency range of 4000–400 cm^{-1} (Mid infrared region). Approximately 16 scans per sample were collected.

End-point enzymatic digestions of EPSs

Solutions of EPS were digested with *β*-1,3-glucanase (endo- and/or exo-) enzymes: cellulase from *Aspergillus niger* (Sigma), Novozym 234 (Novo Biolabs, Bagsvaerd, Denmark), zymolyase (Seikagaku Kogyo, Co. Ltd, Tokyo, Japan) and lysing enzymes from *Trichoderma harzianum* (Sigma). Since treatment with alkali makes *β*-glucans more susceptible to be attacked by *β*-(1,3)-D-glucanases (Schmid *et al.* 2001), 1 ml of EPS solution (5 g l^{-1}) was treated with 500 *μ*l of 1 mol l^{-1} NaOH prior to enzyme addition. After incubation of the mixture during 20 min at room temperature, 2·5 ml of 0·2 mol l^{-1} phosphate-citrate (McIlvaine) buffer pH 5·8 was added. The appropriate amount of enzyme was incorporated for a final concentration of 5 nkat in a final reaction volume of 5 ml, and digestion proceeded at 37°C for 5 h. Enzymes were inactivated by heating (100°C, 10 min).

Digestion products were evidenced by descending paper chromatography (PC) on Whatman paper no.3, using n-propanol : water:ethyl acetate (7 : 2 : 1, v/v) as solvent system. After two runs at 30°C, sugar spots were detected by alkaline $AgNO_3$ reagent (Smith 1958). The presence of glucose and/or gentiobiose (*β*-(1,6)-D-glucopyranosil glucose) was investigated by comparing the R_f values with the corresponding standards, and quantification of these sugars was performed by HPLC as above described.

Controlled enzymatic digestions of EPSs

EPS structure was also analysed by controlled digestion with lysing enzymes from *Rhizoctonia solani* (Sigma), an enzyme preparation with both endo- and exo-*β*-1,3-D-glucanase activity, minimal *β*-1,4-glucanase and no *β*-1,6-glucanase activities (Totsuka and Usui 1986). Polysaccharide (0·26 g l^{-1}, either *S. rolfsii* EPS, laminarin, barley- or baker's yeast glucan) was dissolved in phosphate-citrate buffer pH 4·8 and, after enzyme addition (0·13 g l^{-1}), digestion proceeded at 37°C for 72 h. Periodic aliquots were taken and enzymes were inactivated by heating in a boiling water bath for 15 min. Digestion supernatants were centrifuged at 15 000 ***g***, stored at −20°C if necessary, and subsequently analysed for reducing power (Somogyi-Nelson) and TLC profile.

From 2-ml aliquots at 72 h of incubation with *R. solani* enzymes, a further digestion (60 h at 37°C) was performed with *T. harzianum* enzymes (1 g l^{-1}) (Sigma). Enzyme inactivation, centrifugation and storage were as above. Digestion products were fractionated by TLC and glucose/gentiobiose quantification was performed by HPLC as above described.

Aliquots were spotted onto Silica gel $60F_{254}$ TLC aluminium sheets (Merck) and the mobile phase consisted in acetonitrile : ethyl acetate : *n*-propanol : H_2O (60 : 60 : 60 : 20, v/v) (four ascents). The spots were made visible by immersing the plates in 0·5% (w/v) *α*-naphtol and 5% (w/v) H_2SO_4, followed by heating (105°C, 10 min). Under these conditions, glucose, laminaribiose and gentiobiose (89% *β*- and 7%-*α*-anomers) standards migrated with different R_f values whilst polysaccharides remained at the loading point.

Chemicals

Standards for chromatography [D-(+)-glucose, *β*-gentiobiose mixed anomers, laminaribiose], and substrates for enzymatic digestions (laminarin from *Laminaria digitata*, *β*-glucan from barley, *β*-glucan from Baker's Yeast) were all purchased from Sigma and prepared as 2 g l^{-1} standard solutions.

Statistical analysis

All results are expressed as mean values of at least triplicate determinations. Statistical significance was assessed using the ANOVA and Tukey–Kramer multiple comparisons tests (Miller and Miller 1988).

Results

Scleroglucan production at fermenter scale

The corresponding fermentation kinetic parameters for scleroglucan production at fermenter scale were: at 48 h (EPS I), volumetric productivity (P_r) 0·542 g l^{-1} h^{-1} and yield on consumed C-source ($Y_{P/C}$) 0·74; at 72 h (EPS II), 0·336 g l^{-1} h^{-1} and 0·50.

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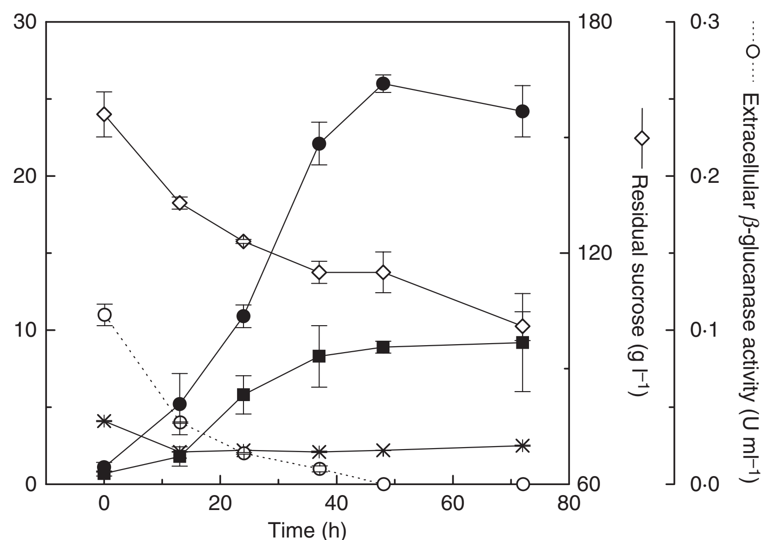


Figure 1 Scleroglucan production at fermenter scale by *S. rolfsii* ATCC 201126 cultured in MOPT culture medium. Data points ± SEM are means of triplicate determinations from two independent fermentations. For cultivation conditions see 'Materials and methods'. –■–, Biomass; –●–, EPS (gl^{-1}); –×–, pH.

EPS values at 48 and 72 h of cultivation were 26·0 ± 0·6 and 24·2 ± 1·7 g l^{-1}, respectively. Despite the apparent decrease in EPS at 72 h, the differences were not statistically significant. Low levels of extracellular β-glucanase activity were detected at the first stages of cultivation but, after 24 h, it became almost negligible (Fig. 1).

Grade of purity, chemical composition and electrical charge of EPSs

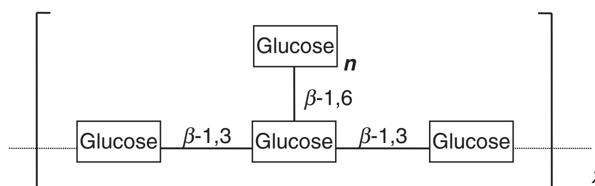
The purified polysaccharides (EPS I and II) consisted in a whitish fibrous solid with a total carbohydrate content of 98% (w/w) and 1·6–1·9% (w/w) of protein impurities; no reducing sugars were detected. They were largely soluble in water and after total hydrolysis they yielded glucose as the sole monosaccharide component, as shown by HPLC. The reducing power of the hydrolysate was equivalent to 87–88% (w/w) anhydroglucose. Confirming their electroneutrality, both scleroglucan samples exhibited no precipitation after addition of 10% (w/v) CTAB.

Structural analysis of EPSs

Periodate oxidation and mild Smith degradation

First analysis consisted in the EPS oxidation with metaperiodate. For αβγ-triols such as β-(1,3)-β-(1,6)-D-glucan-like polysaccharides, a double cleavage of the carbon chain by two molecular proportions of metaperiodate with the concomitant formation of two aldehydic groups and the liberation of one molecular proportion of formic acid was expected (Scheme 1).

With a hypothetical repeating unit represented as follows:



where n represents the number of glucose units involved in branching and, x the degree of polymerization (DP), the following expressions could be applied:

$$\text{moles } m\text{-KIO}_4/\text{mol glucose (in EPS)} = 2n/(n+3)$$

$$\text{moles HCOOH/mol glucose (in EPS)} = n/(n+3)$$

The oxidation kinetics of both EPSs showed 0·56 mol of metaperiodate consumption and 0·23 moles of formic acid production per glucose residue in the macromolecule. These values, when introduced in the above formulae, led to $n \sim 1$ for both EPSs. That value would correspond to single D-glucopyranose side chains and a structure with one (1,6) linkage at every third unit of the main chain (Scheme 1, I).

The reduction of the metaperiodate-oxidiz'ed glucan (II) with sodium borohydride, followed by treatment of the resulting polyalcohol (III) with 0·05 mol l^{-1} H_2SO_4 at room temperature, led to the liberation of 0·25 moles of glycerol per glucose residue in the polysaccharide and, to the formation of a whitish precipitate (IV) (Scheme 1). Since for this reaction a similar equation to that of formic acid production can be applied, glycerol production gave further evidence of the repetitive unit previously proposed ($n = 1$). The Smith-degraded glucan (IV) was shown to be insoluble in water and alkali, which might be related to the

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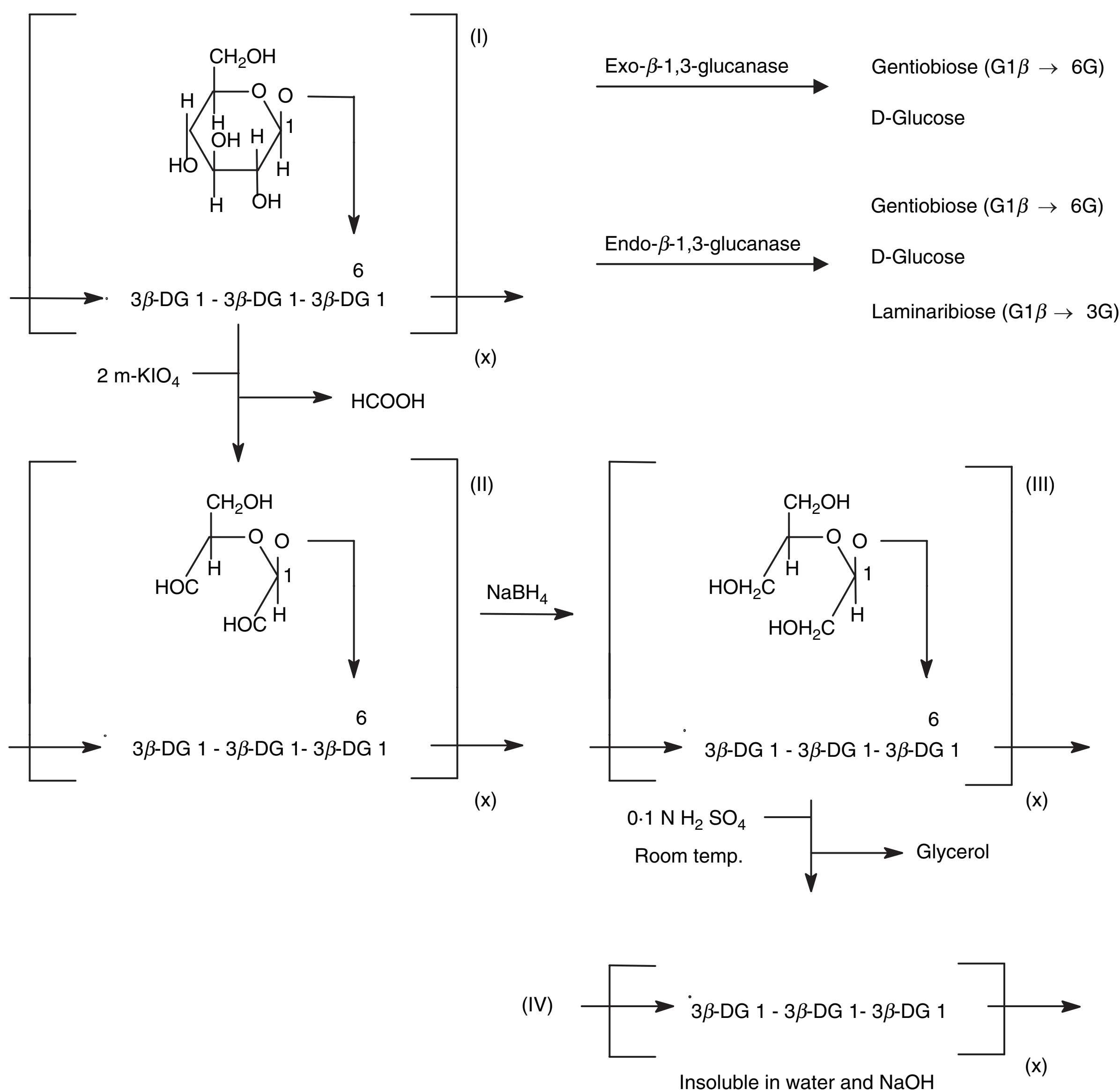

Scheme 1. Chemical events during periodate oxidation, borohydride reduction and mild Smith degradation of *β*-(1,3)-*β*-(1,6)-D-glucans. On the right side, expected products of *β*-glucanases action on structure (I) are also indicated.

formation of an unbranched curdlan type *β*-(1,3)-D-glucan. Identical reactions were found for both EPSs.

FT-IR spectroscopy

The FT-IR spectra of the purified EPSs were very similar to each other (Fig. 2a,b), showing a diagnostic peak at 891 ± 7 cm^{-1} (2b type bending C_1-H_{axial} vibrational mode, for *β*-glycosidic linkages) and no peak at 844 ± 8 cm^{-1} (2a type bending C_1-$H_{equatorial}$ vibrational mode, for *α*-glycosidic linkages). The absence of *α*-linked molecule bands (920 ± 5 cm^{-1}, type 1 vibration, and 774 ± 9 cm^{-1}, type 3 vibration) was also observed. Spectra also included a large absorption band at ~3400 cm^{-1}, with additional bands at 2937 cm^{-1}, in the range of 1475–1250 cm^{-1} and in the 1000–1200 cm^{-1} region (Fig. 2a,b).

Enzymatic digestions

For both EPSs, end-point enzymatic digestions with cellulase, Novozym 234, zymolyase and lysing enzymes from *T. harzianum* all yielded two spots in paper chromatograms with R_f = 63 and R_f = 39, respectively coincident with the standards of glucose (G) and gentiobiose (G1*β* → 6G).

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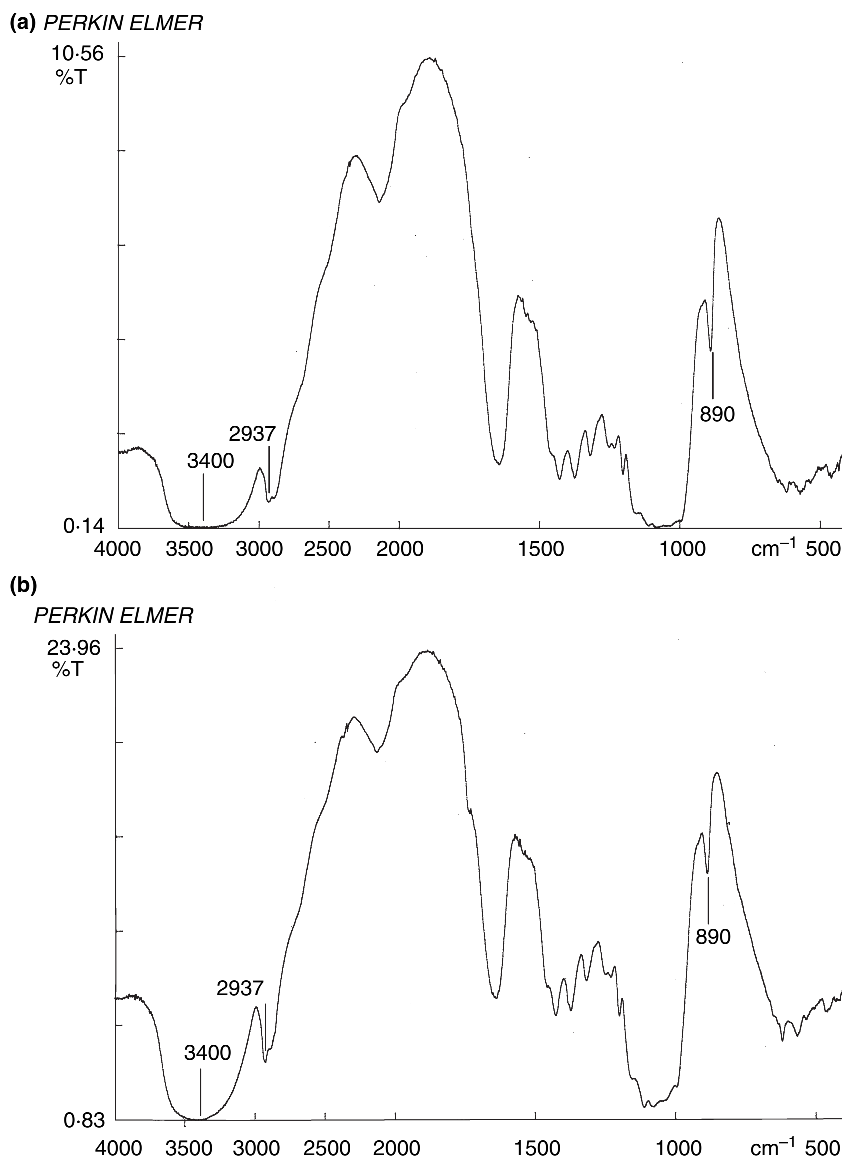

Figure 2 Infra-red spectra of scleroglucan. (a) EPS I (produced by *S. rolfsii* ATCC 201126 after 48-h fermentation). (b) EPS II (produced by *S. rolfsii* ATCC 201126 after 72-h fermentation). Critical absorption peaks are annotated.

The reducing power liberated along the controlled enzymatic hydrolysis increased continuously as digestion progressed (0–72 h), both for EPS I and EPS II (Fig. 3). Comparatively, EPS digestions proceeded slower than for the rest of β-glucans.

Controlled enzymolysis followed by TLC (Fig. 4a) led to quite identical findings as above, with D-glucose and gentiobiose as the main hydrolytic products, with an estimated final glucose to gentiobiose molar ratio of 2 : 1. In comparison to the migration rates of standards, the spots obtained in digestion samples appeared somewhat delayed (Fig. 4a). It could be confirmed that some components in the digestion mixture (e.g. oligosaccharide complexes, etc.) may have retarded the migration of standards (data not shown).

For all compared β-glucans, glucose showed a progressive increment as digestion proceeded (Fig. 4a). Retarded gentiobiose also tended to increase towards the end of EPSs digestion. Laminaribiose was mainly present at the beginnings of hydrolysis for the less branched glucans (laminarin, Baker's Yeast and barley glucans). Other laminari-oligosaccharides were presumed in digestion samples from laminarin and Baker's Yeast glucan, as well as cellobiose from barley β-glucan (data not shown). Finally, when *R. solani*-digested glucans (72 h) were further treated (60 h) with *T. harzianum* enzymes, only glucose could be revealed as the exclusive hydrolytic product (Fig. 4b).

Based on the molecular mass of the repeating unit herein confirmed (Scheme 1, I), and taking into account the previously determined M_W ($1{\cdot}6 \times 10^6$ Da and

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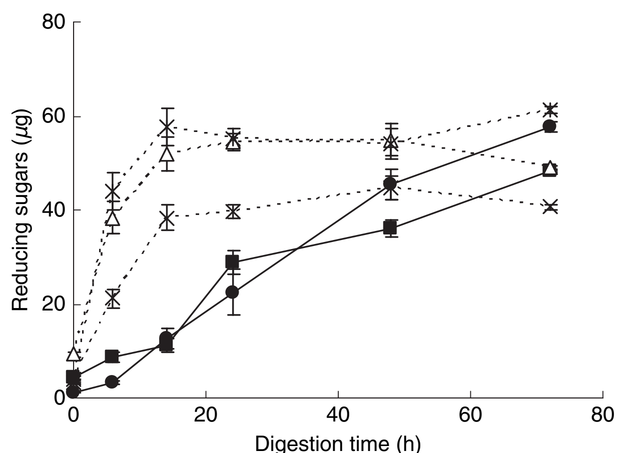

Figure 3 Kinetics of reducing sugars release during controlled enzymatic digestion of β-glucans with *Rhizoctonia solani* enzymes. For details, see 'Materials and methods'. (–•–, EPSI; –▪–, EPS II; ··△··, Laminarin; ··×··, Barley β-glucan; ··✳··, Baker's Yeast β-glucan).

$1{\cdot}7 \times 10^6$ Da in DMSO, for EPS I and EPS II, respectively), the degree of polymerization (DP) estimated for a single coil was around 2400 (EPS I) and 2500 (EPS II). Likewise, an approximate degree of branching (DB) of 0·33 could also be confirmed.

Discussion

Differences observed in rheological behaviour, hydrogel topography, antisyneresis, suspending and emulsifying properties between EPSs produced by *S. rolfsii* ATCC 201126 and recovered at 48 or 72 h of fermentation (i.e. EPS I or EPS II), could not be earlier explained in the light of M_W or other physicochemical characteristics (Fariña *et al.* 2001; Viñarta *et al.* 2006, 2007). Consequently, structural divergences were thought as a possible rationale for these observations.

Variations in branching length have been previously reported among scleroglucans, but the incidence of cultivation parameters on this feature is still poorly understood and optimizations usually follow an empirical basis (Lecacheux *et al.* 1986; Morin 1998; Laroche and Michaud 2007). Despite that no significant changes in EPS concentration were found (Fig. 1), it was suspected that scleroglucan structure may have been the target for modifications, for instance, by the action of β-glucanases (Rau 2004).

Extracellular β-glucanase activity could be detected at the beginning of fermentation (Fig. 1), but after transferring the inoculum from PM_{20} (with 20 g l^{-1} sucrose) to MOPT (with 150 g l^{-1} at fermenter scale), glucanases

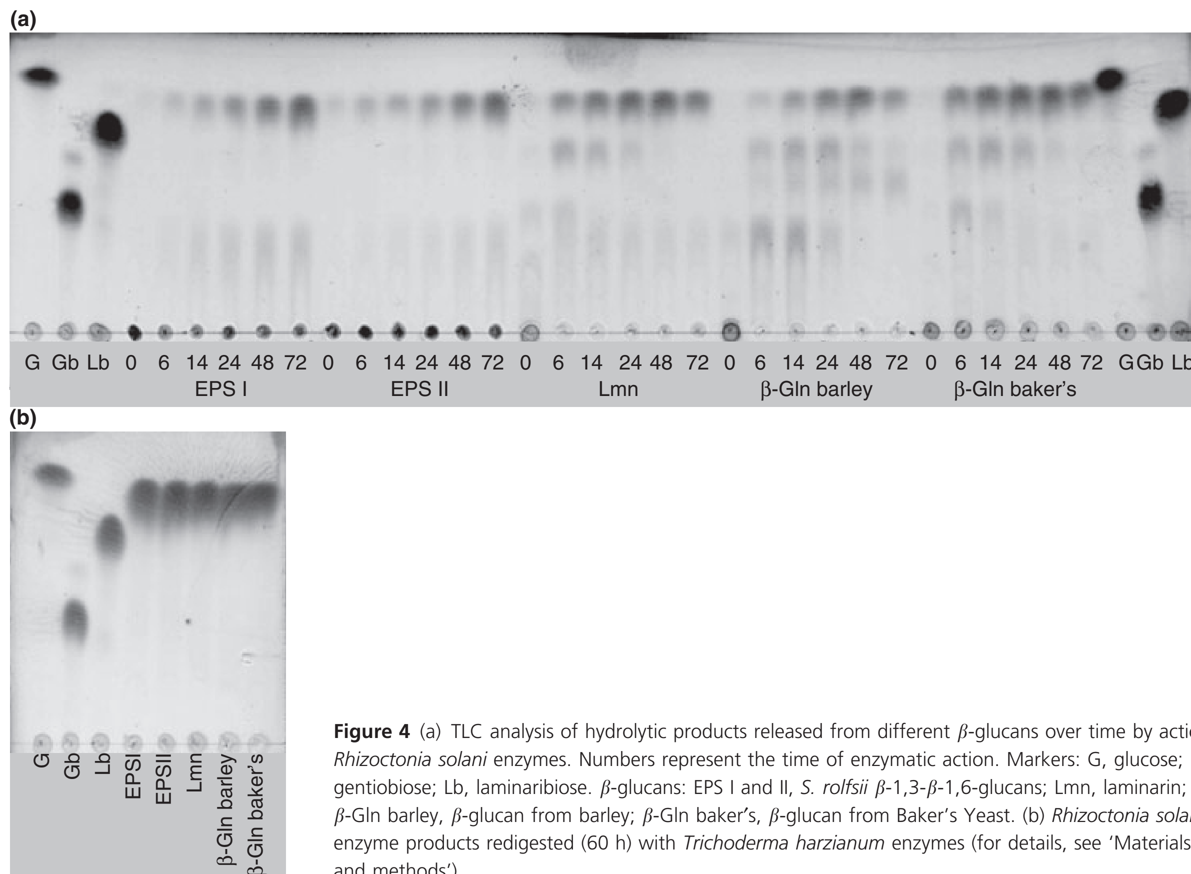

Figure 4 (a) TLC analysis of hydrolytic products released from different β-glucans over time by action of *Rhizoctonia solani* enzymes. Numbers represent the time of enzymatic action. Markers: G, glucose; Gb, gentiobiose; Lb, laminaribiose. β-glucans: EPS I and II, *S. rolfsii* β-1,3-β-1,6-glucans; Lmn, laminarin; β-Gln barley, β-glucan from barley; β-Gln baker's, β-glucan from Baker's Yeast. (b) *Rhizoctonia solani*-enzyme products redigested (60 h) with *Trichoderma harzianum* enzymes (for details, see 'Materials and methods').

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levels started to decrease until being undetectable at 48 h of cultivation. In principle, we hypothesized that high residual sucrose concentrations (*c.* 100 g l^{-1}) might have repressed β-glucanases (Rau 2004). Nevertheless, regulation mechanisms different from catabolic repression, like β-glucan enhancement action, might also be implied (Jayus *et al.* 2005; Crognale *et al.* 2007). Additionally, when scaling up from shake-flask to fermenter scale, the shear stress impossed by Rushton turbine impellers at high stirring speed (400 rev min^{-1}) may have also affected β-glucanases production (Jayus *et al.* 2005).

Considering the undetectable levels of extracellular glucanases, and despite that mycelium-bound glucanases should not be neglected, the eventual action of these polysaccharases on extracellular scleroglucan seemed not very likely. Previous research on *Botryosphaeria rhodina* β-glucan-producing cultures concluded that, even at high glucan-hydrolytic activity levels, EPS decrease could not be attributed to the effect of these enzymes. In turn, the complexity of biomass-sticked biopolymer recovery was argumented (Crognale *et al.* 2007).

On the other hand, the measurement of mycelium-bound glucanases when mycelium was subjected to complete disruption (Crognale *et al.* 2007) might be well attributed to the enzymes responsible for apical hyphal extension (McIntyre *et al.* 2001; Adams 2004; Jayus *et al.* 2005). Furthermore, in spite of scleroglucan may induce glucanases when supplemented as the sole C-source (Jayus *et al.* 2005; Crognale *et al.* 2007), it appears difficult to assume that the fungus would use a complex polysaccharide if a readily assimilable sugar as sucrose was still remaining at high concentration.

Accordingly, changes on scleroglucan characteristics were not expected as a consequence of extracellular hydrolytic activities but those arising from either EPS biosynthetic mechanism (Schmid *et al.* 2001), cultivation conditions or mycelium-bound glucanases could not be discarded.

With respect to the EPSs grade of purity, the applied downstream processing rendered clear advantages regarding appearance, protein and carbohydrate contents, as previously reported for other purified polysaccharides (Wu *et al.* 2007).

The combined use of periodate oxidation/borohydride reduction/Smith degradation and enzymatic hydrolysis has been already successfully applied to provide structural information from a variety of β-glucans (Kishida *et al.* 1989; Aouadi *et al.* 1991; Kiho *et al.* 1991; Schmid *et al.* 2006). For the herein described EPSs, chemical analyses led to the confirmation of a β-1,3-β-1,6-glycosidic structure (Scheme 1, I), where β-1,6-glycosidic linkages were esential for solubility. The lack of branching in the Smith-degraded glucan (Scheme 1, IV) would have enabled the molecules to close to each other in such an extent that extensive H-bonding took place and aggregated forms started to precipitate (Kath *et al.* 1999). Similar values of metaperiodate consumption and formic acid production were also reported for another scleroglucan after first Smith degradation (Schmid *et al.* 2006).

Among the different procedures for assigning configuration to polysaccharides, infra-red absorption can provide definitive evidence about the type of glycosidic linkages (Zhang *et al.* 2007). FT-IR spectra of EPS I and EPS II (Fig. 2a,b) were very similar to each other and also to already published scleroglucan spectrum (Halleck 1967). The presence of a diagnostic peak corresponding to β-glycosidic linkages along with the absence of α-linked molecule bands was consistent with a β-type glucan, as noted for other fungal polysaccharides (Kiho *et al.* 1991; Šandula *et al.* 1999; Schmid *et al.* 2001).

The large absorption band at $\sim$3400 cm^{-1} (Fig. 2a,b) would be directly related to the stretching and bending modes of the large –OH groups fraction in the macromolecule. The incidence of absorbed water in this assignment might be neglected since the polysaccharide was hardly hygroscopic. Absorbance at 2937 cm^{-1} might be attributed to the C–H stretching vibrations (Wu *et al.* 2007). Additional bands between 1475 and 1250 cm^{-1} might be assigned to C–H bending modes (i.e. 1383 cm^{-1}), whilst bands appeared in the region of 1000–1200 cm^{-1} would correspond to C–O–C glycosidic frequencies and C–OH stretching vibrations overlapped with ring vibrations.

Further support for the structural evidence given by periodate oxidation and FT-IR spectroscopy was obtained through enzymolysis with β-glucanases of known specificity and mode of action, as suggested for β-glucan structure elucidation (Bielecki and Galas 1991). End-point enzymatic digestions giving glucose and gentiobiose as hydrolytic products served to confirm the single unit branches linked by β-1,6 linkages (Scheme 1, I). On the other hand, along the concerted action of exo- and endo-β-D-(1,3)-glucanase activity from *R. solani* lysing enzymes, the reducing power of anomeric groups released from EPSs could be compared to other β-glucans (Fig. 3).

Taking into account the initial amount of β-glucans, only half percent could be hydrolysed by *R. solani* enzymes. That may be related to the reduced hydrolytic activity consequent on enzyme affinity modifications as substrates with different degree of polymerization appeared. A diminished activity of *R. solani* β-glucanases as β-1,6-linkages increase could also be speculated, highlighting the comparatively higher EPSs degree of branching. Eventually, the decrease in reducing power at the end of digestion might be associated to the transglycosylase activity of *R. solani* endo-β-(1,3)-D-glucanases (Totsuka and Usui 1986).

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At the end of the *R. solani*-enzymes digestion, the final glucose to gentiobiose molar ratio (2 : 1) was in agreement with the suggested structure (Scheme 1, I) (Aouadi *et al.* 1991; Kiho *et al.* 1991). Glucose could be again confirmed as the exclusive hydrolytic product by further treatment with *T. harzianum* enzymes (Fig. 4b), a lytic preparation with β-1,6-glucanase activity (de la Cruz *et al.* 1995). The slowered migration of digestion products (Fig. 4a,b) showed similarities with previous observations in epiglucan digests (Schmid *et al.* 2001).

Differences in the TLC-monitored hydrolytic profiles (Fig. 4a) might be likely related to the β-glucan structures: β-glucan from baker's yeast, mainly a β-1,3-glucan with highly spaced β-1,6-linkages (Bielecki and Galas 1991; http://www.beta-glucan-13d.com, 2007); laminarin, with 85–98% β-1,3 and 2–15% β-1,6-linkages (Klarzynski *et al.* 2000; Schmid *et al.* 2001); EPSs with 66% β-1,3 and 33% β-1,6-linkages (this work); and barley β-glucan with 25–30%, β-1,3 and 70–75%, β-1,4-linkages (Totsuka and Usui 1986).

The similarities found between the results from enzymatic and chemical analyses were quite essential in order to confirm the structural homogeneity of scleroglucan EPSs. Discrepancies between these results may have denoted heterogeneities with regard to the DB, as already described of other fungal β-1,3-D-glucans (Kishida *et al.* 1989).

With reference to DP, values in literature seem to be extremely variable: ~110 for *S. glucanicum* scleroglucan (Bielecki and Galas 1991), ~800 for commercial scleroglucan (Bluhm *et al.* 1982), 500–1600 for related glucans (Sandford 1979), and up to ~5600 for another reported scleroglucan (Rice *et al.* 2004). The DP values found for *S. rolfsii* ATCC 201126 scleroglucan (2400–2500) would be among the highest reported, a fact that might account for the outstanding rheological behaviour and the successful applications reported earlier (Fariña *et al.* 2001; Viñarta *et al.* 2006, 2007). Likewise, considering the estimated DB for both EPSs (~0·33), they would be well positioned among the most biologically active β-(1,3)-glucans (Bohn and BeMiller 1995).

Mimicking the similarities between intrinsic viscosities of EPS I and EPS II ([η] = 9610 and 9510 ml g^{-1} in water, respectively) and weight-average molecular weights (M_W = 1·6 and 1·7 × 10^6 Da in 0·25 mol l^{-1} NaOH or DMSO for the random coil, respectively) (Fariña *et al.* 2001), all the herein described structural properties indicated no differences between the EPSs recovered and purified at 48 and 72 h of fermentation.

In literature, scleroglucan productivities lower than those herein presented have been frequently reported (Taurhesia and McNeil 1994; Survase *et al.* 2006, 2007), either because of the lower EPS concentrations produced or the longer cultivation times required. In this context, the possibility to increase productivities by reducing fermentation time would represent a fact undoubtedly favourable not only from the production point of view but also in economical costs terms (Survase *et al.* 2007), and may be validly considered in *S. rolfsii* ATCC 201126.

Molecular weight and chemical structure of β-glucans are features under permanent investigation as key parameters for biological activity (Bohn and BeMiller 1995). Therefore, it seems relevant to emphasize the identical structures between EPS I and EPS II. Accordingly, for applications such as controlled drug delivery (Viñarta *et al.* 2007), where either EPS I or EPS II were equally efficient only exhibiting a polysaccharide-concentration dependence, or when M_W as well as β-1,6-branching seem to be basis for activity, for example, immunopharmacological effects (Leung *et al.* 2006; Survase *et al.* 2007), these results would be particularly valuable. Nevertheless, conformational differences in EPSs may play a significant role (Leung *et al.* 2006; Zhang *et al.* 2007) and will be the focus of future work.

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References

Adams, G.A. (1965) Complete acid hydrolysis. In *Methods in Carbohydrate Chemistry*, Vol. V ed. Whistler, R.L., BeMiller, J.N. and Wolfrom, M.L. pp. 269–276 New York/London: Academic Press.

Adams, D.J. (2004) Fungal cell wall chitinases and glucanases. *Microbiology* **150**, 2029–2035.

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

Bielecki, S. and Galas, E. (1991) Microbial β-glucanases different from cellulases. *Crit Rev Biotechnol* **10**, 275–304.

Bluhm, T.L., Deslandes, Y., Marchessault, R.H., Pérez, S. and Rinaudo, M. (1982) Solid-state and solution conformation of scleroglucan. *Carbohydr Res* **100**, 117–130.

Bohn, J. and BeMiller, J.N. (1995) (1 → 3)-β-D-glucans as biological response modifiers: a review of structure-functional activity relationships. *Carbohydr Polym* **28**, 3–14.

Crognale, S., Bruno, M., Fidaleo, M., Moresi, M. and Petruccioli, M. (2007) Production of β-glucan and related

© 2008 The Authors

glucan-hydrolases by *Botryosphaeria rhodina*. *J Appl Microbiol* **102**, 860–871.

de la Cruz, J., Pintor-Toro, J.A., Benítez, T. and Llobell, A. (1995) Purification and characterization of an endo-β-1,6-glucanase from *Trichoderma harzianum* that is related to its mycoparasitism. *J Bacteriol* **177**, 1864–1871.

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

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

Fariña, J.I., Siñeriz, F., Molina, O.E. and Perotti, N.I. (2001) Isolation and physicochemical characterization of soluble scleroglucan from *Sclerotium rolfsii.* Rheological properties, molecular weight and conformational characteristics. *Carbohydr Polym* **44**, 41–50.

Goldstein, I.J., Hay, G.W., Lewis, B.A. and Smith, F. (1965) Controlled degradation of polysaccharides by periodate oxidation, reduction, and hydrolysis. In *Methods in Carbohydrate Chemistry*, Vol. V ed. Whistler, R.L., BeMiller, J.N. and Wolfrom, M.L. pp. 361–370. New York/London: Academic Press.

Guthrie, R.D. (1962) Periodate oxidation. In *Methods in Carbohydrate Chemistry*, Vol. I ed. Whistler, R.L., Wolfrom, M.L., BeMiller, J.N. and Shafizadeh, F. pp. 435–441. New York/London: Academic Press.

Halleck, F.E. (1967) Polysaccharides and methods for production thereof. (to the Pillsbury Company). Application: United States Patents Office: 3,301,848, 31 January 1967.

Hay, G.W., Lewis, B.A. and Smith, F. (1965) Periodate oxidation of polysaccharides: general procedures. In *Methods in Carbohydrate Chemistry*, Vol. V ed. Whistler, R.L., BeMiller, J.N. and Wolfrom, M.L. pp. 357–361. New York/London: Academic Press.

Jayus, O.J., McDougall, B.M. and Seviour, R.J. (2005) Synthesis of extracellular $(1 \rightarrow 3)$- and $(1 \rightarrow 6)$-β-glucanase activities in carbon limiting chemostat culture by the fungus *Acremonium* sp. IMI 383068. *Enzyme Microb Technol* **36**, 680–686.

Kath, F., Lange, S. and Kulicke, W.-M. (1999) Influence of the glycosidic linkage on the solution conformation of glucans. *Die Angewandte Makromolekulare Chemie* **271**, 28–36.

Kiho, T., Sakushima, M., Wang, S., Nagai, K. and Ukai, S. (1991) Polysaccharides in fungi. XXVI. Two branched $(1 \rightarrow 3)$-β-D-glucans from hot water extract of Yū ěr. *Chem Pharm Bull* **39**, 798–800.

Kishida, E., Sone, Y. and Misaki, A. (1989) Purification of an antitumor-active, branched $(1 \rightarrow 3)$-β-D-glucan from *Volvariella volvacea*, and elucidation of its fine structure. *Carbohydr Res* **193**, 227–239.

Klarzynski, O., Plesse, B., Joubert, J.-M., Yvin, J.-C., Kopp, M., Kloareg, B. and Fritig, B. (2000) Linear β-1,3 glucans are elicitors of defense responses in tobacco. *Plant Physiol* **124**, 1027–1037.

Kogan, G. (2000) $(1 \rightarrow 3, 1 \rightarrow 6)$-$\beta$-D-glucans of yeasts and fungi and their biological activity. In *Studies in Natural Products Chemistry*, Vol. 23: Bioactive Natural Products, Part D. ed. Atta-Ur-Rahman, H.E.J. pp. 107–152. Netherlands: Elsevier Science B.V.

Laroche, C. and Michaud, P. (2007) New developments and prospective applications for β (1,3) glucans. *Recent Patents Biotechnol* **1**, 59–73.

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

Leung, M.Y.K., Liu, C., Koon, J.C.M. and Fung, K.P. (2006) Polysaccharide biological response modifiers. *Immunol Lett* **105**, 101–114.

McIntyre, M., Müller, C., Dynesen, J. and Nielsen, J. (2001) Metabolic engineering of the morphology of *Aspergillus*. *Adv Biochem Eng Biotechnol* **73**, 103–128.

Miller, G.L. (1959) Use of the dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* **31**, 426–428.

Miller, J.C. and Miller, J.N. (1988) Significance tests. In *Statistics for Analytical Chemistry* ed. Chalmers, R.A. and Masson, M. pp. 53–80 Chichester: Ellis Horwood Ltd.

Morin, A. (1998) Screening of polysaccharide-producing microorganisms, factors influencing the production, and recovery of microbial polysaccharides. In *Polysaccharides: Structural Diversity and Functional Versatility* ed. Dumitriu, S. pp. 275–296. New York: Marcel Dekker Inc.

Rapp, P., Beck, Ch.H. and Wagner, F. (1979) Formation of exopolysaccharide by *Rhodococcus erythropolis* and partial characterization of a heteropolysaccharide of high molecular weight. *Eur J Appl Microbiol Biotechnol* **7**, 67–78.

Rau, U. (2004) Glucans secreted by fungi. *Turkish Electr J Biotechnol* **2**, 30–36.

Rice, P.J., Lockhart, B.E., Barker, L.A., Adams, E.L., Ensley, H.E. and Williams, D.L. (2004) Pharmacokinetics of fungal (1-3)-β-D-glucans following intravenous administration in rats. *Int Immunopharmacol* **4**, 1209–1215.

Sandford, P.A. (1979) Exocellular, microbial polysaccharides. *Adv Carbohydr Chem Biochem* **36**, 265–313.

Šandula, J., Kogan, G., Kačuráková, M. and Machová, E. (1999) Microbial $(1 \rightarrow 3)$-β-D-glucans, their preparation, physico-chemical characterization and immunomodulatory activity. *Carbohydr Polym* **38**, 247–253.

Schmid, F., Stone, B.A., McDougall, B.M., Bacic, A., Martin, K.L., Brownlee, R.T.C., Chai, E. and Seviour, R.J. (2001) Structure of epiglucan, a highly side-chain/branched $(1 \rightarrow 3;1 \rightarrow 6)$-$\beta$-glucan from the micro fungus *Epicoccum nigrum* Ehrenb. ex Schlecht. *Carbohydr Res* **331**, 163–171.

Schmid, F., Stone, B.A., Brownlee, R.T.C., McDougall, B.M. and Seviour, R.J. (2006) Structure and assembly of

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epiglucan, the extracellular (1 → 3;1 → 6)-*β*-glucan produced by the fungus *Epicoccum nigrum* strain F19. *Carbohydr Res* **341**, 365–373.

Smith, I. (1958) Sugars. In *Chromatographic Techniques – Clinical and Biochemical Applications* ed. Heinemann, W. pp. 164–170 UK: Pitman Press.

Survase, S.A., Saudagar, P.S. and Singhal, R.S. (2006) Production of scleroglucan from *Sclerotium rolfsii* MTCC 2156. *Biores Technol* **97**, 989–993.

Survase, S.A., Saudagar, P.S. and Singhal, R.S. (2007) Use of complex media for the production of scleroglucan by *Sclerotium rolfsii* MTCC 2156. *Biores Technol* **98**, 1509–1512.

Taurhesia, S. and McNeil, B. (1994) Production of scleroglucan by *Sclerotium glucanicum* in batch and supplemented batch cultures. *Enzyme Microb Technol* **16**, 223–228.

Totsuka, A. and Usui, T. (1986) Separation and characterization of the endo-*β*-(1 → 3)-D-glucanase from *Rhizoctonia solani*. *Agric Biol Chem* **50**, 543–550.

Viñarta, S.C., Molina, O.E., Figueroa, L.I.C. and Fariña, J.I. (2006) A further insight into the practical applications of exopolysaccharides from *Sclerotium rolfsii*. *Food Hydrocol* **20**, 619–629.

Viñarta, S.C., François, N.J., Daraio, M.E., Figueroa, L.I.C. and Fariña, J.I. (2007) *Sclerotium rolfsii* scleroglucan: the promising behavior of a natural polysaccharide as a drug delivery vehicle, suspension stabilizer and emulsifier. *Int J Biol Macromol* **41**, 314–323.

Wu, Y., Cui, S.W., Tang, J., Wang, Q. and Gu, X. (2007) Preparation, partial characterization and bioactivity of water-soluble polysaccharides from boat-fruited sterculia seeds. *Carbohydr Polym* **70**, 437–443.

Zentz, F. and Muller, G. (1992) Post-fermentation processing conditions and solution properties of an extracellular fungal polysaccharide isolated from the culture filtrate of *Schizophyllum commune*. *Carbohydr Polym* **19**, 75–81.

Zhang, M., Cui, S.W., Cheung, P.C.K. and Wang, Q. (2007) Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. *Trends Food Sci Technol* **18**, 4–19.

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