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Implications of recovery procedures on structural and rheological properties of schizophyllan produced from date syrup

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ABSTRACT

This study investigates the effects of different recovery procedures on high molar mass schizophyllan produced by *Schizophyllum commune* using low value agricultural residues. Recovered extracellular polysaccharides (EPSs) were compared in terms of purity, sugar composition, degree of branching, molecular weight, and rheological properties. Performing different recovery methods, such as re-dissolving in water and re-precipitation with ethanol on produced EPS, provided schizophyllan with purity similar to the commercial grade. Besides, Freeze-thawing cycles allowed the fractionation of schizophyllan based on branching degree and solubility. The EPSs with higher purity and lower degree of branching (less conformational flexibility) showed higher viscosity. This study evidences the possibility of producing EPSs with excellent rheological properties using low value agricultural side products. Furthermore, our results demonstrate the importance of recovery methods for tailoring the purity, molecular structure and macroscopic properties of the produced polysaccharides for specific applications.

1. Introduction

Schizophyllan is an exopolysaccharide (EPS) produced by *Schizophyllum commune*, a white-rot fungus and ubiquitous mushroom. It is a non-ionic, water-soluble homoglucan, which consists of a backbone of β-1 → 3-glucose residues with β-1 → 6-linked glucose side chains at every third residue on average [1]. Schizophyllan is known to be a biological response modifier and to act as a non-specific stimulator of the immune system. Because of its immunotherapeutic properties, it has been used as an immune effect enhancer in anticancer, vaccines and also as a bioactive ingredient in cosmetics [2]. Simultaneously, due to the high molecular weight and the very stiff triple helical conformation in water, schizophyllan aqueous solutions exhibit well recognized rheological properties, which suggest its potential application as thickener in enhanced oil recovery [3] and as biodegradable bioflocculant [4]. The biological activity and rheological properties of aqueous schizophyllan solutions are mainly under the influence of purity, molecular structure, molecular weight, and the macromolecular conformation in solution [5–9]. There is evidence that extracellular β-glucans produced from the same strain and under the same conditions but yet subjected to different post-fermentation treatments (downstream processing), may show different solution properties and macromolecular features [8,10]. In spite of the importance of post fermentation treatments and their influence on the properties and the macromolecular features of recovered schizophyllan, limited investigations have mainly focused on this subject. Rau et al. [11] investigated the effect of cross-flow filtration, an effective procedure for washing out low molecular substances (salts, mono- and oligosaccharides, peptides) and concentrating the cell free schizophyllan solution, on molecular weight and shear viscosity of schizophyllan solutions and showed that molecular weight and shear viscosity of the β-glucan solution decreased after this procedure. Zentz and Muller [8] found that impurities from the fermentation broth can unfavorably affect the solution properties of schizophyllan and that aggregation of the exopolysaccharide molecules result from the thermal treatment of the unpurified fermentation broth (higher molecular weight). These observations indicate that downstream processing greatly influences the macromolecular features of schizophyllan, which as a consequence can affect its macroscopic properties and potential applications.

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We recently demonstrated a simplified process at laboratory scale level for the recovery of schizophyllan produced from date syrup and corn steep liquor by *S. commune* ATCC 38548 [12]. The efficient fermentation of the low-cost residues for schizophyllan production along with the importance of post-fermentation processing on schizophyllan properties encouraged us to further investigate the influence of the recovery and purification processes on the molecular features and rheological properties of schizophyllan. In this study, the effect of recovery procedures on the produced schizophyllan was investigated by comparing two parallel processes: (i) a two-step redissolution and precipitation in ethanol and (ii) three cycles of freeze-thawing of the crude extracellular polysaccharide. The purity, monosaccharide composition, degree of β-(1 → 3)(1 → 6)-glycosidic branching, molecular weight, and rheological properties were compared, in order to elucidate the structure-property relationships of the recovered and purified exopolysaccharides.

2. Materials and methods

2.1. Preparation of downstream-processed schizophyllan samples

For the production of crude schizophyllan, *Schizophyllum commune* ATCC 38548 was grown on PDA plates at 28 °C for 7 days. For the first subculture, an approximately 5 × 5 mm square of *S. commune* covered agar was used to inoculate 50 ml of sterile seed culture medium in 250 ml shake flask with 2 baffles, which was then incubated at 28 °C, 180 rpm for 2 days on a rotary shaker. The second seed culture with 100 ml medium in 250 ml shake flasks was inoculated with 10 ml of homogenized culture suspension prepared using a Potter-type homogenizer and cultivated for 2 days as described. The seed culture medium consisted of glucose (30 g l^{-1}), yeast extract (3 g l^{-1}), KH_2PO_4 (1 g l^{-1}), and $MgSO_4 \cdot 7H_2O$ (0.5 g l^{-1}) [1]. The second seed culture was added at a 7.7% (v/v) inoculum size to the optimized medium for schizophyllan production containing 7% (w/v) date syrup and 0.1% (w/v) corn steep liquor. The culture was incubated for 180 h at agitation rate of 181 rpm at 28 °C [12]. The sugar composition of date syrup was 29.5 (%w/w) glucose, 33.2 (%w/w) fructose and 0.1 (%w/w) sucrose.

Downstream processing started with a 3-fold dilution of culture broth with distilled water and homogenization (Power Gen 700, Fisher Scientific) for 40 s, and then centrifuging at 15,000 × *g* for 20 min at 4 °C. In order to isolate the extracellular polysaccharides (mainly schizophyllan), an equivalent volume of 95% (v/v) ethanol was added to precipitate the schizophyllan from the supernatant. After 1 h at 4 °C, the precipitate was collected by centrifugation at 15,000 × *g* for 20 min at 4 °C [12].

The crude schizophyllan was further treated with two different methods (Fig. 1). In the first method, the crude schizophyllan was re-dissolved in distilled water and then purified by re-precipitation in 95% ethanol (two times). The polysaccharide precipitate was freeze-dried, milled (by means of two 10 s-pulses in a domestic coffee grinder), and stored as EPS-r (Fig. 1). In the second method, after freeze drying of the crude schizophyllan, the method involved 3 consecutive freeze-thawing cycles of the crude schizophyllan (−80 °C for 30 min, 65 °C for 3 h), and separation of the precipitate fraction (EPS-pre) from the supernatant (EPS-sup) fraction by centrifugation (6000 × *g*, 15 min, 4 °C) (Fig. 1). Finally, each fraction was freeze-dried and milled as above (Fig. 1). Commercial scleroglucans (grade CS11and CS6) were kindly provided by Cargill (France) and used without further modification or purification for comparison with the recovered schizophyllan samples. The samples were analyzed for protein content by the Bradford method [13] using BSA as standard, and total carbohydrates were determined by the phenol–sulfuric acid method [14] with glucose as standard.

2.2. Sugar composition analysis of schizophyllan

The sugar composition of the recovered schizophyllan and the commercial scleroglucans (CS 11 and CS6) was determined by acid hydrolysis using trifluoroacetic acid (TFA) and subsequent analysis by high-pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Approximately 1 mg of dry sample was suspended in 1 ml of 2 M TFA and incubated for 3 h at 120 °C. After centrifuging, 100 μl of the supernatant were transferred to new Eppendorf tubes and then air dried to completion. The hydrolysates were redissolved in 1 ml of deionized water and filtered through 0.2-μm filters prior to analysis. The released neutral sugars and uronic acids were separated and quantified by HPAEC-PAD on an ICS3000 system (Dionex, Sunnyvale, CA) using a Dionex Carbopac PA1 column maintained at 30 °C at a flow rate of 1 ml/min. The samples were quantified by comparing the peak areas of the analytes with those of monosaccharide standards at known concentrations for calibration. The experiments were performed in triplicate and the results are reported as the average with the corresponding standard deviations.

2.3. Glycosidic linkage analysis of the produced schizophyllan

Glycosidic linkage analysis was performed following the methodology reported by Pettolino et al. [15]. Briefly, 1 mg of the EPS was fully methylated with methyl iodide under alkaline conditions (with excess of NaOH) in dimethyl sulfoxide (DMSO) [16]. The partially methylated polysaccharides were hydrolyzed with 2 M TFA at 121 °C for 1.5 h, further reduced with $NaBH_4$ and acetylated with acetic anhydride and pyridine. The obtained permethylated alditol acetates (PMAAs) were identified and quantified by gas chromatography–mass spectrometry (GC–MS) using an HP-6890 GC coupled to an HP-5973 electron-impact mass spectrometer (EI-MS) (Agilent Technologies, United States). Separation was performed on an SP-2380 capillary column (30 m × 0.25 mm ID, Agilent Technologies) from 160 °C to 210 °C at 1 °C min^{-1}. The samples were analyzed in triplicate. The retention times and the fragmentation spectra by EI-MS could be assigned to the different glycosidic linkages by comparison with those of commercial polysaccharide standards.

2.4. Molecular weight determinations

The molar mass distribution of the EPSs were determined by size exclusion chromatography (SECcurity 1260, Polymer Standard Services, Mainz, Germany) coupled to a multiple-angle laser light scattering detector (MALLS; BIC-MwA7000, Brookhaven Instrument Corp., New York), and a refractive index detector (SECcurity 1260, Polymer Standard Services, Mainz, Germany) thermostatted at 45 °C. The lyophilized EPS was dissolved in the separation solvent consisting of dimethyl sulfoxide (DMSO) with 0.5% (w/w) LiBr at concentrations ranging between 0.5–3 mg ml^{-1}. The sample solution was applied to a column set consisting of a GRAM PreColumn, 100 and 10,000 analytical columns (Polymer Standards Services, Mainz, Germany) and eluted with the SEC eluent consisting of DMSO (HPLC grade, Scharlab, Sweden) with 0.5% w/w LiBr (ReagentPlus) at a flow rate of 0.5 ml/min and 60 °C. Calibration of the SEC set-up was performed using the Mark-Houwink relation by the injection of pullulan standards of known molar masses provided by Polymer Standards Services (PSS, Mainz, Germany) (Supplementary Material, Fig. S1). The Mark-Houwink parameters for pullulan in DMSO/LiBr (0.5 wt%) at 80 °C are $K = 2.427 \times 10{-}4$ dL g^{-1} and a = 0.6804. Data treatment and collection was performed using WinGPC software (Polymer Standards Services, Mainz, Germany) and further analyzed by additional mathematical procedures presented elsewhere (insert citation: