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Production and characterization of new families of polyglucuronic acids from TEMPO–NaOCl oxidation of curdlan

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ABSTRACT

Curdlan from *Agrobacterium* sp. was oxidized using 2,2,6,6,-tetramethylpiperidine-1-oxyl radical (TEMPO)–NaBr–NaClO systems at pH 11. The effects of oxidation conditions on degrees of oxidation and polymerization of the products obtained were studied using SEC–MALLS, NMR and IR analyses. Different families of water-soluble β-(1,3)-polyglucuronic and β-(1,3)-polyglucoglucuronic acid sodium salts were quantitatively generated with a yield of 80% and without significant loss of their molecular weights.

Given that β-(1,3)-polyglucuronic acids prepared from the regioselective oxidation of curdlan by the TEMPO–NaBr–NaClO systems regularly consist of the glucuronic acid repeating unit; they may open new biotechnological fields for the utilizations of water soluble forms of curdlan.

1. Introduction

Curdlan is a bacterial neutral linear β-D-glucan essentially produced by *Agrobacterium* biovar 1 (*Alcaligenes faecalis* var. *myxogenes* strain 10C3) [1–3]. This polysaccharide is made up of around 12,000 D-glucose monomers linked by β-glucosidic bonds between C-1 and C-3. It is totally insoluble in most organic solvents and in water at ambient temperature but it can form two types of heat-induced gels, called "high-set gels" and "low-set gels" by heating process of aqueous solution [1–4]. In contrast, it is important to note that curdlan is totally soluble in the alkali solution without heating. This feature, not commonly found with other types of hydrocolloids or gelling agents has been largely exploited from long time by food industries in Korea, Taiwan, and Japan [5]. Effectively, curdlan can be applied as texturing processing aid, stabilizer, thickener and texture modifier in a wide range of food preparations [6]. Furthermore, curdlan can offer lots of health benefits [7]. In fact, as many of other β-glucan polysaccharides, it has been used (functionalized or not) for its biological properties in pharmacology as anticoagulant, antithrombotic, and anti-HIV agent [1,8,9]. Since curdlan is totally insoluble in water, its biological applications and properties are extremely limited. Its insolubility in water is generally attributed to the existence of extensive inter/intramolecular hydrogen bonds. Consequently, there is a strong need to increase the solubility of curdlan to improve its biological activities. In the past years, lots of publications have investigated the chemical modification of this β-(1,3)-D-glucan to produce water soluble derivatives as for example aminated [10], carboxymethylated [11], sulphated and phosphorylated polymers [8,12].

Other methodologies can be applied to produce large amount of anionic curdlan. In the past years a specific catalytic oxidation of primary hydroxyl groups by the stable nitroxyl radical 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) was abundantly described for its high regioselectivity and reaction rate [13,14]. This TEMPO mediated oxidation is considered as a new method to improve the functional properties for many polysaccharides as the water-soluble polyglucuronic acid derivatives generated by this reaction have a great potential in regard to their numerous biological and rheological properties. Moreover, contrary to carboxymethylated polysaccharides, those generated by regioselective oxidation of primary hydroxyl groups are real polyuronides.

In this paper, a native insoluble β-(1,3)-glucan from *Agrobacterium* sp. bacteria has been modified using TEMPO–NaBr–NaOCl system in order to produce a soluble β-(1,3)-polyglucuronic acid which may be proposed as new surrogate of glycosaminoglycans for cosmetic and pharmaceutical applications.

2. Experimental

2.1. Production of curdlan

To produce curdlan, the *Agrobacterium* sp. strain (ATCC 31750) was cultivated in Yeast Broth media at 30 °C, 600 rpm and 20 SLPM in a 7.5 L bioreactor (Bioflo® 110 Newbrunswick Scientific) accord-

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ing to Lee et al. [15] in which the culture pH was changed at the end of the growth phase from pH 7.0 to 5.5 by the addition of HCl.

Curdlan was recovered from cultures after 96 h of cultivation by dissolution in 0.85 M NaOH and removal of bacteria by centrifugation (15,000 × *g* for 20 min at 4 °C). Curdlan in supernatant was then precipitated by neutralization with HCl (3 M), collected by centrifugation (15,000 × *g* for 30 min at 4 °C), washed with distilled water to remove salts, and finally freeze-dried.

2.2. Production of β-(1,3)-polyglucuronic acid

β-(1,3)-Polyglucuronic acid was prepared according to the cellulose oxidation procedure described by Delattre et al. [16]. Curdlan (10 g) was dissolved at room temperature in distilled water (1 L) during 60 min at pH 11.5 by adding NaOH (4 M). The solution was kept below 4 °C in an ice bath. TEMPO (86.6 mg), NaBr (1.92 g) and NaOCl (100 mL at 9.6%) were added to start oxidation. The pH was kept at 11 by the addition of NaOH (1 M). The reaction was quenched by adding methanol (50 mL) after 1 h and neutralized with HCl (1 M). After concentration (1/3) by evaporation, β-(1,3)-polyglucuronic acid was precipitated with cold isopropanol (3 volumes). The precipitate was washed with isopropanol, dissolved in distilled water, dialyzed against water during 24 h at 10 kDa cut-off and freeze-dried.

2.3. Production of β-(1,3)-polyglucoglucuronic acid

β-(1,3)-Polyglucoglucuronic acid was prepared according to the oxidation procedure mentioned above. After 15, 30 and 45 min, the oxidation was stopped and the samples collected were neutralized with HCl (1 M) before to be precipitated with cold isopropanol (3 volumes). So, the three β-(1,3)-polyglucoglucuronic acids were dialyzed and freeze-dried as described above.

2.4. SEC–MALLS analysis of β-(1,3)-polyglucuronic acids

Average molecular weights and molecular weight distributions were determined by high pressure size exclusion chromatography (HPSEC) with on-line multi-angle laser light scattering (MALLS) and differential refractive index (DRI) detectors. The MALLS apparatus is the EOS from Wyatt Technology (Ca, USA) filled with a K5 cell and a Ga–As laser (λ = 690 nm). The DRI detector is the ERC7515A from Erma Cr., Inc. (Japan). Columns [OHPAK SB-G guard column, OHPAK SB804 and 806 HQ columns (Shodex)] were eluted with $LiNO_3$ 0.1 M at 0.6 mL min^{-1}. Solvent was filtered through 0.1 μm filter unit (Millipore), degassed (ERC-413) and filtered through a 0.45 μm filter upstream column. The sample, filtered on 0.45 μm unit filter (Millipore) was injected through a 100 μL full loop. The used dn/dC value is 0.15 mL g^{-1}. The collected data were analysed using the Astra V-4-81-05 software package from Wyatt Technology (Ca, USA).

2.5. Sugar assays

Glucuronic acid and glucose contents of β-(1,3)-polyglucuronic acid and β-(1,3)-polyglucoglucuronic acid extracts were assayed with *meta*-hydroxyldiphenyl [17] and resorcinol [18] in order to quantify the oxidation level. D-Glucose (Sigma) and D-glucuronic acid (Sigma) were used as standards. Quantification of neutral sugars was done according to the corrective formula described by Montreuil and Spick [19]. The uronic acid ratio (UA %) of polysaccharides obtained was expressed as the amount of glucuronic acid compared with this of all sugars (glucose and glucuronic acid).

2.6. Determination of carboxylate content by conductimetry titration

In order to quantify the degrees of oxidation of β-(1,3)-polyglucuronic and β-(1,3)-polyglucoglucuronic acids, conductimetry titration method was used according to the literature [20]. Dried polysaccharides (50 mg) were dissolved in 0.01 M HCl (15 mL) to set the pH value in the range of 2.5–3.0. The titration was started by the addition of a 0.01 M NaOH solution at the rate of 0.1 mL min^{-1} up to pH 11 and the conductivity was measured using CDM 210 Meterlab conductimeter. The degree of oxidation was given by the following equation:

$$\mathrm{DO} = \frac{162\,(V_2 - V_1)\,\mathrm{M_{NaOH}}}{m - (36\,(V_2 - V_1)\,\mathrm{M_{NaOH}})} \quad (1)$$

V_1 and V_2 are respectively the volumes (mL) of NaOH solution necessary to neutralize HCl, the strong acid and to neutralize the polyglucuronic acid (weak acid).

2.7. NMR analysis

NMR analyses were performed at 30 °C with a Bruker Avance 400 spectrometer of 400 MHz equipped with $^{13}C/^{1}H$ dual probe. The NMR experiments were recorded with a spectral width of 3000 Hz, an acquisition time of 1.36 s, a pulse width of 7 μs, a relaxation time of 1 s and a number of 1500 scans. Oxidized polysaccharides were dissolved in D_2O at a 50 g L^{-1} concentration. Curdlan was dissolved in NaOD (1 M) at a 50 g L^{-1} concentration.

2.8. IR analysis

Dried polysaccharides samples (1 mg) were dispersed in 100 mg of anhydrous KBr and pressed. The IR spectra were recorded at room temperature in the wavenumber range of 500–2000 cm^{-1} and referenced against air with a Nicolet 380 FT-IR instrument (Thermoelectron Corporation).

3. Results and discussion

3.1. TEMPO–NaBr–NaOCl mediated oxidation of curdlan

A new generation of polyuronides composed of β-(1,3)-polyglucuronic acid has been developed using the TEMPO chemistry. The regioselective TEMPO oxidation of polysaccharides is a complex reaction. In fact, when TEMPO is added as catalyser; the oxidation process involves different reaction steps as mentioned in Fig. 1. Oxidation of 1 mol of primary alcohol to the carboxylic acid requires 2 mol of NaOCl and 2 mol of TEMPO radical.

Curdlan was oxidized with TEMPO–NaOCl–NaBr system in order to investigate the production of β-(1,3)-polyglucuronic and/or β-(1,3)-polyglucoglucuronic acids. Therefore, as resumed in Table 1, an oxidation kinetic of curdlan (10 g) allowed to generate four families of soluble anionic polysaccharides in good yields except for this obtained after 15 min of oxidation. As we can observe after sugar

Table 1
Characterization of oxidized products prepared from curdlan (10 g) by TEMPO-mediated oxidation process.

Oxidation time	Carbohydrate (g)	Soluble fraction (%)	UA (%)[a]	DO (%)[b]
T-15 min	9.5	10	16	25
T-30 min	9.0	54	41	40
T-45 min	8.5	85	60	60
T-60 min	8.0	95	98	100

[a] Uronic acid ratios of the soluble fraction.
[b] Degree of oxidation of the soluble fraction.