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Development of new ulvan-like polymer by regioselective oxidation of gellan exopolysaccharide using TEMPO reagent

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ABSTRACT

The produced, purified and deacylated gellan exopolysaccharide was subjected to regiospecific oxidation at C6 with NaOCl in the presence of 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) and NaBr in aqueous solution at pH 10. As with other polysaccharides, a high selectivity of oxidation was observed. The high performance anion-exchange chromatography (HPAEC) and ^{13}C NMR analysis of the TEMPO-oxidized products was used to confirm that the C6 primary hydroxyl groups of deacylated gellan were completely converted to carboxylate groups. Thus, after the addition of 7.5 mmol of NaClO per gram of polysaccharide, a new and original water-soluble rhamnoglucuronan polymer was obtained quantitatively with homogeneous chemical structures. This original carbohydrate might find use as surrogates of ulvan, a sulfated cell-wall polysaccharides extracted from green seaweeds (Ulva species), in pharmaceutical and medical areas.

1. Introduction

Polysaccharides represent a class of high-value polymers with many industrial applications in food, cosmetic, textile and pharmaceutical industries due to their rheological properties. They have been used as emulsifiers, as stabilizers and as texture enhances in the food industry. Traditionally, these polysaccharides have been obtained from plant or algae sources. However, in the last years, novel families of polysaccharides produced from microbial sources have received increase attention because of their novel functionality and reproducible chemical properties (Delattre, Laroche, & Michaud, 2009). From of them, Gellan is an exopolysaccharide produced in high yield by the non-pathogenic bacterium *Sphingomonas elodea* ATCC 31461 (Mazen, Milas, & Rinaud, 1999). It is well known as a multifunctional gelling agent able to form thermoreversible gels and highly viscous aqueous solutions (Dlamini & Peiris, 1997). In recent years, a wide range of gellan applications has been investigated in food and biomedical fields (Chandrasekaran & Radha, 1995; Fialho et al., 2008). As shown in Fig. 1, gellan is an anionic polysaccharide made up of a tetrasaccharide repeating unit composed by the backbone [→ 3)-β-D-Glcp-(1 → 4)-β-D-GlcpA-(1 → 4)-β-D-Glcp-(1 → 4)-α-L-Rhap-(1 →] (Jannson, Lindeberg, & Sandford, 1983; Jay et al., 1998). In modern biotechnologies, scientists are more and more looking for new processes to obtain gellan-like polymers with different properties than the gellan wild-type. Therefore, the producing strains can be either exposed to chemical mutagenesis or submitted to genetic engineering procedures (Jay et al., 1998). Despite the significant progresses that have been made in the genetic characterization of gellan biosynthesis, the success of genetic engineering approaches still requires a more detailed understanding of gellan's biosynthesis (Fialho et al., 2008; Jay et al., 1998). In order to improve the intrinsic biological properties and create new functional properties of polysaccharides, chemical modification has been commonly applied in the polysaccharide chemistry. For example, the sulfatation of hydroxyl groups is interesting since significant effects on the physiological functions of polysaccharides have been described such as anticoagulant, antitumor, and anti-HIV infection activities (Martinichen-Herreroa, Carboneroa, Gorina, & Iacominia, 2005; Wang, Zhang, Li, Hou, & Zeng, 2004). Moreover, carboxylation of polysaccharides provides a fast and useful way to improve the water solubility and increase biological activity of native polysaccharides (Delattre, Michaud, Elboutachfaiti, Courtois, & Courtois, 2006; Delattre et al., 2009; Fan, Saito, & Isogai, 2009; Fraschini & Vignon, 2000; Isogai & Kato, 1998; Muzzarellia, Muzzarellia, Cosanib, & Terbojevichb, 1999). These last years, a specific catalytic oxidation of C6 primary hydroxyl groups of polysaccharides by the stable nitroxyl radical 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) and NaOCl was abundantly described for its high regioselectivity and reaction rate

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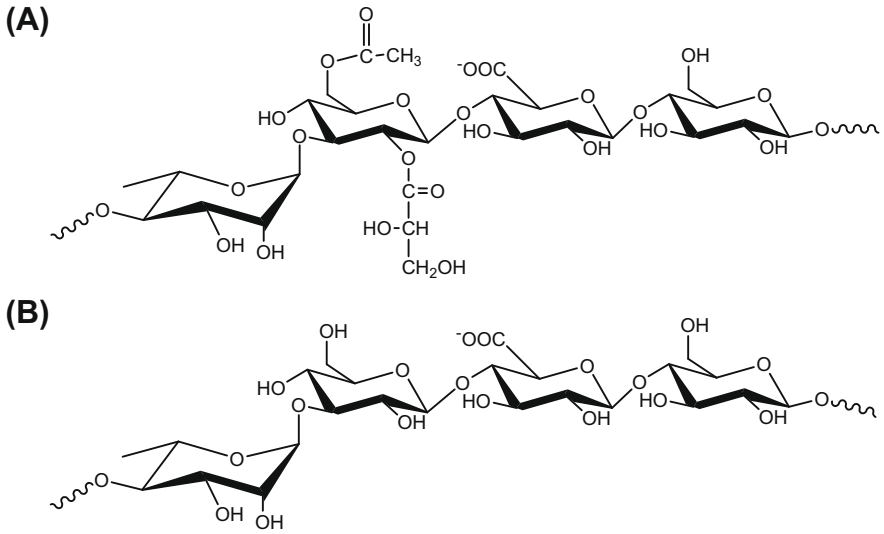

Fig. 1. Chemical structure of (A) gellan produced by *Sphingomonas elodea* sp. and (B) deacylated Gellan obtained by alkaline Treatment.

(Bragd, van Bekkum, & Besemer, 2004; De Nooy, Besemer, & Van Bekkum, 1995). This TEMPO-mediated oxidation is considered as a new method to improve the functional properties for many polysaccharides as for example to give their corresponding water-soluble derivatives (Delattre et al., 2006; Fan et al., 2009; Isogai & Kato, 1998). Consequently, the challenge was to perform this chemical modification on gellan in order to extend their range of putative biological applications. It is important to mention that the theoretical modified gellan structure, is an anionic polyelectrolyte having a structural similarity to ulvan which is made up of disaccharide formed by β-D-glucuronic acid (1 → 4)-L-rhamnose 3 sulfate as largely related in literature (Lahaye, Baumberger, Quemener, & Axelos, 1996; Robic, Sassi, & Lahaye, 2008). Ulvan have been reported to possess diverse biological activities and medicinal properties such as anticoagulant, antitumour and antioxidant activity (Lahaye & Kaeffer, 1997; Kaeffer, Bénard, Lahaye, Blottière, & Cherbut, 1999; Ivanova et al., 1994). Usually, Ulvan is extracted from green seaweeds *Ulva* sp. using hot water often containing a calcium chelating agent such as sodium oxalate (Elboutachfaiti et al., 2009; Lahaye & Robic, 2007). Nevertheless, under these extraction conditions, the chemical structure can varies according to (i) its period of collect, (ii) the seaweed species, and (iii) all the post-collect treatment (Lahaye & Robic, 2007). These problems are considered as one of the major drawbacks to really evaluate the biological activity of ulvan and the structure/activity relationships. In this study, the TEMPO-mediated oxidation was applied to gellan to prepare in good yield water-soluble rhamnoglucuronan polymer, which may be proposed as the first principal component for development of ulvan-like polymers for cosmetic and pharmaceutical applications. The chemical structures and molecular weights of the TEMPO-oxidized products thus obtained were analyzed in detail.

2. Materials and methods

2.1. Production and purification of gellan and its deacylated form

Gellan was produced by *S. elodea* sp. during incubation at 30 °C with orbital agitation (250 rpm) in basal media containing glucose as the carbon source (at 10 g/L). After 48 h of growth, cultures entered the stationary phase and gellan produced was recovered by the addition of 3 volumes of cold isopropanol (99%, v/v), followed by several washings of the precipitate with isopropanol (Dreveton, Monot, Lecourtier, Ballerini, & Choplin, 1996; Martins & Sk-Correia, 1994; Martins, Fialho, Rodrigues, & SCCorreia, 1996). The isopropanol precipitates isolated were redissolved in water, dialyzed against distilled water for 48 h. The amount of gellan produced was determined after lyophilization. The deacylated gellan was produced with alkaline treatment (NaOH, pH 12) and purified following the procedure previously by Milas, Shi, and Rinaudo (1990).

2.2. Deacylated gellan TEMPO/NaOCl/NaBr mediated oxidation

Deacylated gellan (1 g) was dissolved in water (100 ml) at pH 10 containing TEMPO (0.017 g) and sodium bromide (0.12 g), and the suspension was stirred at room temperature and 1000 rpm. The pH of this solution was adjusted to 10 with 1 M NaOH. Then, a desired amount of sodium hypochlorite solution from 1.7 to 7.5 mmol was added to the deacylated gellan solution to start the TEMPO-mediated oxidation. The pH was maintained at 10 by continuous addition of 1 M NaOH using a pH-stat until no NaOH consumption was observed. After neutralisation at pH 7 by addition of diluted HCl (1 M) the oxidized deacylated gellan was precipitated by addition of 3 volumes of cold isopropanol (99%, v/v). The isopropanol precipitates isolated were dissolved in water, dialyzed against distilled water for 48 h. The amount of oxidized deacylated gellan produced was determined after lyophilization. Yields of the oxidized products were about 90%.

2.3. Analysis of monosaccharides composition

The deacylated gellan and its different oxidized form (1 mg) dissolved in 4 M TFA (1 ml) were heated at 100 °C for 8 h. The acid was removed by flushing the sample with air and the hydrolysates was dried under vacuum. The hydrolysates (1 mg) were dissolved in pure water (1 mg/ml). Twenty-five microliters of these solutions was used for the ionic chromatography analysis by High performance anion-exchange chromatography (HPAEC) of Dionex ICS-2500 System. Analysis was performed on a Carbopac PA-1 column (4.5 × 25 mm) eluted with 16 mM NaOH at a flow rate of 1 ml/min for 40 min. Detection was performed using a pulsed amperometric detector (Dionex).

2.4. SEC-MALLS analysis

Average molecular weights and the molecular weight distributions were determined by high pressure size exclusion chromatog-

raphy (HPSEC) with on line multi-angle laser light scattering (MALLS) filled with a K5 cell (50 μl) and two detectors: a He–Ne laser (λ = 690 nm) and a differential refractive index (DRI). Columns [OHPAK SB-G guard column, OHPAK SB806, 804 and 803 HQ columns (Shodex)] were eluted with $NaNO_3$ 0.1 M at 0.7 ml/min. Solvent was filtered through 0.1 μm filter unit (Millipore), degassed and filtered through a 0.45 μm filter upstream column. The sample was injected through a 100 μl full loop. The collected data were analyzed using the Astra 4.50 software package.

2.5. NMR analysis

NMR analyses were performed at 80 °C with a Bruker Avance 300 spectrometer of 300 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 750 scans. The deacylated gellan and TEMPO-oxidized products were previously dissolved in deuterium (D_2O, 99.9% D) and lyophilized to replace exchangeable protons with deuterium. The lyophilized samples were then dissolved in D_2O at a 20–50 g/L concentration.

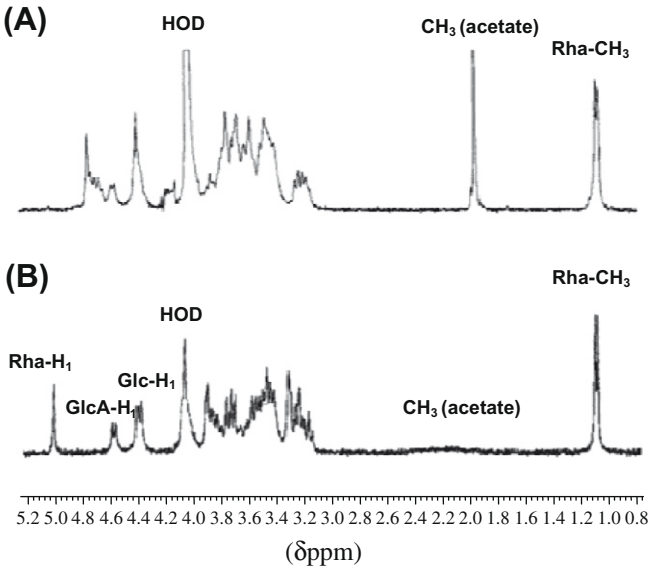

Fig. 2. ^{1}H NMR Analysis of (A) gellan produced by *Sphingomonas elodea* sp. and (B) deacylated gellan in D_2O at 80 °C.

3. Results and discussion

3.1. TEMPO-mediated oxidation of gellan

Gellan (Fig. 1A) was produced by *S. elodea* sp. in media containing glucose as the carbon source according to Jay et al. (1998). The total concentration of gellan produced was then estimated at 7 g/L. The ^{1}H NMR spectrum of the native polysaccharide was complex in anomeric region (4.2–5.2 ppm) due to partial acylation (Fig. 1A).

The deacylated gellan (Fig. 1B) was prepared and purified following the procedure previously described by Milas et al. (1990). Briefly, after alkaline treatment (NaOH 1 M, 90 °C), deacylated gellan was precipitated by using isopropanol (3 v) followed by dialysis (48 h). Note to mention that the deacylated gellan forms rigid and very clear gel which is comparable with agarose gel as related by Milas and Rinaudo (1996).

The total deacylation was confirmed by ^{1}H NMR analysis as shown in Fig. 2. The ^{1}H-1D NMR spectrum of the *O*-deacylated gellan (Fig. 1B) showed in the anomeric region, four proton signals: two at 4.39 and 5.05 ppm attributed to H-1 of the β-1,4-(Glc*p*) residues and α-1,4-(Rha*p*) residues, respectively and, two others closely overlapped at 4.56 ppm assigned to β-1,4-(Glc*p*A) residues and β-1,3-(Glc*p*) residues as already confirmed by Shi (1990).

Deacylated gellan was oxidized according to the TEMPO/NaOCl/NaBr oxidation mechanism as described in Fig. 3. Different designated amounts of NaOCl (used as the primary oxidant) and TEMPO (catalytic amounts) were added in deacylated gellan solutions (1%) as mentioned in Table 1. For each experiment, the end of oxidation was visualized once consumption of 1 M NaOH was stopped. We have shown that the amount of NaOCl to complete oxidation of the C6 primary hydroxyl groups of 1 g deacylated gellan was estimated at 7.5 mmol. During TEMPO-mediated oxidation of deacylated gellan, we found that its gelling power has visibly disappeared once the amount of NaOCl added was increased in the oxidation. Thus, active liquefaction phenomenon of gel-forming gellan is started once the amount of NaOCl reaches a value of surrounding 5.4 mmol/g. As against, when the NaOCl level reach about 1.7 mmol, the oxidized products still allows the ability to form a weak gel, slightly different from the starting materials. It seemed that this gel–sol phase transition accompanied with good water solubility is probably due to a change in the structure of deacylated gellan caused by the oxidation.

The comparison between the carboxylate groups formed, amounts of NaOCl added, and time reaction were established and listed in Table 1 for a better evaluation of different parameters involved in the deacylated gellan oxidation. The carboxylate groups formed by the oxidation, determined as the ratio of uronic acid monomer, were measured by the analytical anion-exchange HPLC and confirmed in carbazole colorimetric assay. A new carbohydrate family (Rhamnoglucuronan) called GO4 containing only rhamnose and glucuronic acid monomers was obtained quantitatively (89%) by the complete TEMPO-mediated oxidation of C6 primary hydroxyl groups of glucose using an amount of 7.5 mmol of NaClO per gram of deacylated gellan. If this rhamnoglucuronan polymer was obtained after a reaction-time of 40 min, the concomitant improvement of deacylated gellan solubility and decreasing of its apparent viscosity require only 20 min of reaction-time with NaOCl of about 3.6 mmol per gram of deacylated gellan.

3.2. Structural analyses of the TEMPO-oxidized products

In addition to the colorimetric assay and the analytical anion-exchange HPLC, NMR analysis was realized. As shown in Fig. 4, no signal around 60 ppm due to the C6 primary hydroxyls of the original deacylated gellan was present in the ^{13}C NMR spectrum of GO4 where the NaOCl addition level was 7.5 mmol/g. It is also important to point out that for GO4 there is the signal due to the C6 carboxylate groups at 175 ppm. Consequently, all C6 primary hydroxyls of deacylated gellan were converted to carboxylate groups by the TEMPO-mediated oxidation. On the other hand, if the NaOCl proportion is less than 7.5 mmol/g, the oxidized deacylated gellan had signals due to both the C6 primary hydroxyls and carboxylate groups in the NMR spectrum (data not shown). Moreover, according to the literature (Jay et al., 1998), the resonances in the region of 101–105 ppm in the ^{13}C NMR spectrum of deacylated Gellan and its oxidized form GO4 (Fig. 4A and B, respectively) were attributed to the anomeric carbon atoms of glucopyranoses (Glc*p*), glucuronopyranose (GlcA*p*) and rhamnopyranose (Rha*p*). In fact, the peaks at 103.71 ppm (a) , 103.35 ppm (b), 103.02 ppm (c), and 100.81 ppm (d) were attributed to C-1 of β-1,4-(Glc*p*) residues, C-1 of β-1,3-(Glc*p*) residues, C-1 of β-1,4-(Glc*p*A) residues, and C-1 of α-1,4-(Rha*p*) residues, respectively (Fig. 4A). Once again, we confirmed the total oxidation of glucopyranoses (Glc*p*) from deacy-

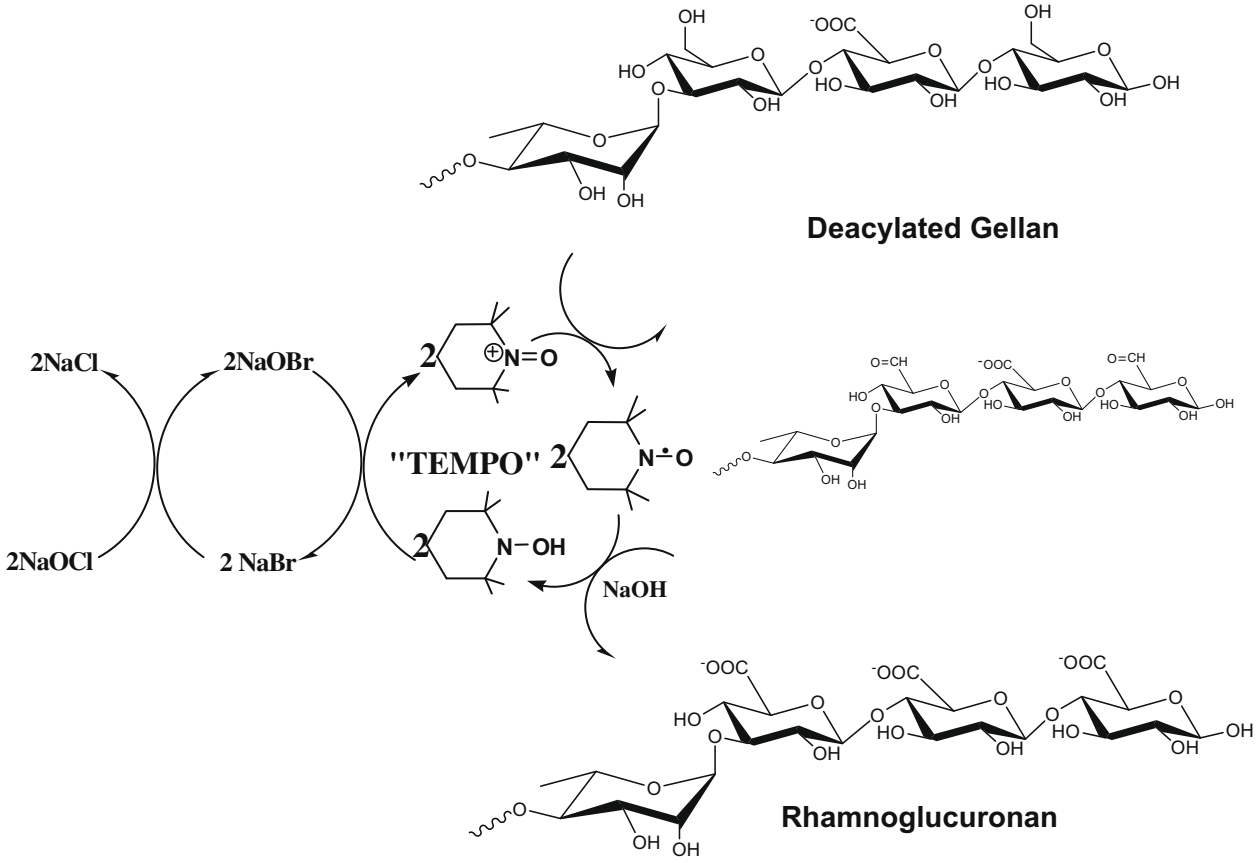

Fig. 3. Proposed Scheme of regioselective oxidation of deacylated Gellan using the TEMPO/NaOCl/NaBr system.

Table 1
Reaction conditions and chemicals structure of deacylated gellan and of their TEMPO-oxidized products.

Sample	NaClO added (mmol/g)	Reaction-time (min)[a]	Molar ratios Glc/GlcA/Rha	Oxidation rate (%)	Recovery ratio (%)
Gellan	–	–	2.10/0.98/1.00	–	–
GO1	1.7	10	1.73/1.24/0.98	22.5	95
GO2	3.6	20	1.02/1.98/0.96	52.0	90
GO3	5.4	30	0.56/2.43/0.97	76.2	93
GO4	7.5	40	0.03/3.10/0.90	100.0	89

[a] Time necessary for complete consumption of NaClO.

lated gellan into β-1,4-(Glc*p*A) and β-1,3-(Glc*p*A) since the signals (a) and (b) have disappeared when the NaOCl addition level was 7.5 mmol/g (Fig. 4B). Finally, the assignment of the carbon atoms signals are conformed to literature (Guentas et al., 2001; Jay et al., 1998) and were summarized in Table 2.

3.3. Molecular weights of the TEMPO-oxidized products

The TEMPO-oxidized products prepared from deacylated gellan with different NaOCl addition level (from 1.7 up to 7.5 mmol/g of polysaccharide) were analyzed by SEC-MALLS to determine their molecular weight parameters (Fig. 5). In Table 3 are mentioned the weight and number average DP values (DP_w and DP_n, respectively) of deacylated gellan and their TEMPO-oxidized products. The DP_w of deacylated gellan was estimated at 3084, whereas DP_w of its total oxidized form (GO4) decreased to 112, which corresponded to around 1/28 of that of deacylated gellan. As observed, more the amount of NaOCl increased more the DP_w decreased, certainly due to the high depolymerization occurred on the TEMPO-oxidized products. As it was largely described in lot of publications of TEMPO-oxidation, a remarkable depolymerization of polysaccharides was observed. In fact, during the last decade, the mechanism of this depolymerization of polyuronides such as cellouronic acid, amylouronic acid, chitouronic acid etc. during

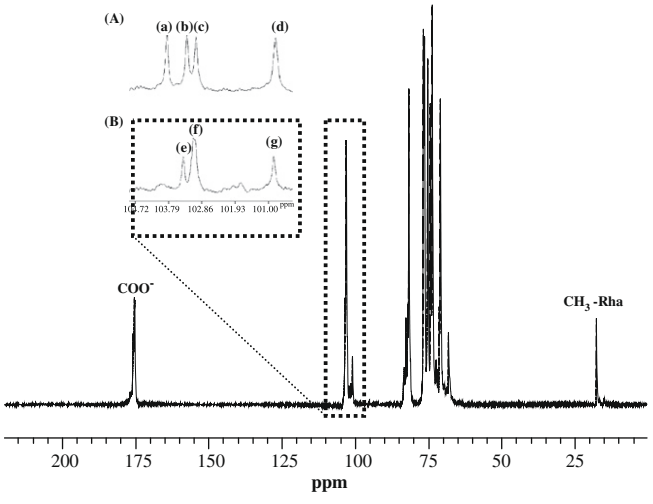

Fig. 4. ^{13}C NMR analysis of Oxidized gellan (GO4) and deacylated Gellan. The anomeric region (A) of Deacylated Gellan and (B) GO4. Signal assignment: (a) C-1-(β-1,4-Glc*p*) residues); (b) C-1-(β-1,3-Glc*p* residues); (c) C-1-(β-1,4-Glc*p*A residues), and (d) C-1-(α-1,4-Rha*p*) in deacylated Gellan. (e) C-1-(β-1,3-Glc*p*A residues); (f) C-1-(β-1,4-Glc*p*A residues), and (g) C-1-(α-1,4-Rha*p*) in GO4 in D_2O at 80 °C.

Table 2
Chemical shifts (ppm) of ^{13}C NMR signals for the deacylated gellan and total oxidized form (GO4) recorded in D_2O at 80 °C.

Residue	C-1	C-2	C-3	C-4	C-5	C-6
a → 4)-β-D-Glc*p*-(1 →	103.71	74.67	75.35	79.82	75.75	61.24
b → 3)-β-D-Glc*p*-(1 →	103.35	74.67	83.35	69.15	76.88	61.69
c → 4)-β-D-Glc*p*A-(1 →	103.02	73.70	75.35	81.57	76.35	175.1
d → 4)-α-L-Rha*p*-(1 →	100.81	71.34	71.14	82.16	68.28	17.68
e → 3)-β-D-Glc*p*A-(1 →	103.40	74.31	83.16	70.88	76.25	175.74
f → 4)-β-D-Glc*p*A-(1 →	103.05	73.65	75.19	81.64	76.65	175.17
g → 4)-α-L-Rha*p*-(1 →	100.86	71.75	71.25	82.03	68.12	17.60

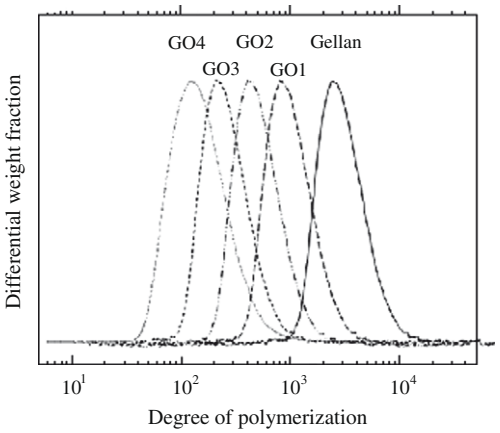

Fig. 5. DP distribution patterns of deacylated gellan and their TEMPO-oxidized products (GO1, GO2, GO3, GO4).

Table 3
Weight and number average molecular weights (M_w and M_n, respectively) and the corresponding DP_w and DP_n values of the deacylated gellan and of their TEMPO-oxidized products.

Sample	M_w	(DP_w)	M_n	(DP_n)	M_w/M_n
G	512,000	(3084)	345,900	(2083)	1.48
GO1	162,690	(989)	119,620	(727)	1.36
GO2	84,330	(502)	63,410	(376)	1.33
GO3	39,280	(232)	31,670	(187)	1.24
GO4	19,370	(112)	17,450	(101)	1.11

TEMPO-mediated oxidation was studied. This phenomenon was attributed to hydroxyl radicals formed such as super oxide anion radical and/or hydroperoxide radical ($HOO^{\cdot}$) from NaOBr and TEMPO at pH 10–11 (De Nooy, Besemer, Van Bekkum, Van Dijk, & Smit, 1996; Isogai & Kato, 1998; Kitaoka, Isogai, & Onabe, 1999; Miyazawa, Endo, & Okawara, 1985). Finally, it is well known that under alkaline conditions, the formation of hydroxyl radicals leads to remarkable decreases in viscosity of wood pulp fibres by oxidation of hydroxyl groups followed by β-elimination (Ishizu, 1973; Matsumoto, 2000).

4. Conclusions

This paper describes the oxidation of deacylated gellan mediated by TEMPO/NaBr/NaOCl to yield novel rhamnoglucuronan biopolymers soluble over an extended pH range. When the NaOCl addition level was 7.5 mmol/g of deacylated gellan, all the C6 primary hydroxyl groups were oxidized to carboxylate groups within 40 min. Thus, rhamnoglucuronan having almost homogeneous chemical structures can be obtained quantitatively by the TEMPO-mediated oxidation. The mechanism also implies an important kinetic process which causes variability in the properties of gel obtained depending on the NaOCl addition level. The preparation of the sulfated rhamnoglucuronan will be studied in more detail. It seems that it permits to increase the anionic character and to obtain modified gellan that might prove interesting applications as surrogates of ulvan in cosmetics, medical items and drug carriers.

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