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Partial and total C-6 oxidation of gelling carrageenans. Modulation of the antiviral activity with the anionic character

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ABSTRACT

The optimal conditions for the full C-6 oxidation of κ- and ι-carrageenans using (2,2,6,6-tetramethylpiperidinyl)oxy (TEMPO) in the presence of sodium hypochlorite and sodium bromide were assessed. The fully oxidized products were characterized by NMR spectroscopy. Partially oxidized products were also obtained and analyzed by chemical and spectroscopical methods. The antiviral activity of carrageenans against herpes simplex virus HSV-1 and HSV-2 determined by plaque reduction assay, was not largely affected by full oxidation of the polysaccharides, but an increase in activity was detected by partial oxidation. A specific overoxidation on C-2 of the 3,6-anhydrogalactose moiety of κ-carrageenan was identified, solved experimentally and rationalized through the application of molecular modeling.

1. Introduction

Many natural polysaccharides have been used in the food industry, as well as in other applications (Stephen & Churms, 2006). Polysaccharides from marine sources (alginates, agarans and carrageenans) are especially chosen for industrial applications due to their availability, and their (usually) regular structure (Stortz & Cerezo, 2000). Carrageenans have structures based on linear chains of alternating 3-linked β-D-galactopyranosyl residues and 4-linked α-D-galactopyranosyl or 3,6-anhydrogalactopyranosyl residues, substituted with sulfate esters in different positions. The polyanionic characteristics of carrageenans allow them to carry many proven biological activities, such as antitumor (Bondu, Deslandes, Fabre, Berthou, & Guangli, 2010; Haijin, Xiaolu, & Huashi, 2003;), anticoagulant (Carlucci et al., 1997; Wijesekara, Pangestuti, & Kim, 2011), and especially antiviral (Carlucci et al., 1997; Damonte, Matulewicz, & Cerezo, 2004; Talarico et al., 2004; Tischer et al., 2006).

Carrageenans are classified according to their idealized structure, and named by specific Greek letters. The most important gelling carrageenans (κ- and ι-) are 4-sulfated on the galactose moiety, have a 4-linked 3,6-anhydrogalactose moiety and differ only by the sulfation pattern of O-2 of this anhydro residue (sulfated in ι-, not sulfated in κ-).

A number of different chemical modifications of carrageenans have been carried out. The simplest and best known, even at an industrial level, is the alkaline treatment which converts by an intramolecular nucleophilic attack 6-sulfated α-D-galactose units into 3,6-anhydro-α-D-galactose moieties (Ciancia, Noseda, Matulewicz, & Cerezo, 1993; Navarro & Stortz, 2005). Other modifications included oversulfation (Yuan et al., 2005), phosphorylation (Yuan et al., 2005), replacement of sulfate groups by seleniate groups (Campos, Kawano, da Silva Jr., & Carvalho, 2009), *O*-maleolylation (Jiang & Guo, 2005), and *O*-succinylation (Jiang, Guo, & Chen, 2007), used to increase and/or modify the anionic properties, and thus their interactions with biological receptors.

Oversulfation, phosphorylation, and introduction of spacers terminating in carboxyl groups were thus the most common ways to increase the anionic charge of carrageenans, and then improve their biological activities. A simpler way might be the oxidation of the primary hydroxyl group of the galactose units to generate a C-6 carboxyl group (galacturonic acid). The most successful reagent for this reaction has been (2,2,6,6-tetramethylpiperidinyl)oxy or TEMPO, a stable water-soluble nitroxyl radical which can be used

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in catalytic amounts by the addition of another oxidant (usually NaOCl or commercial bleach) which regenerates the nitroxyl oxidant (Bragd, van Bekkum, & Besemer, 2004). With an adequate pH regulation to avoid oxidation by the hypochlorite, selective C-6 oxidation was achieved for different polysaccharides like cellulose (Follain, Montanari, Jeacomine, Gambarelli, & Vignon, 2008; Saito & Isogai, 2005; Xu, Dai, Sun, Wang, & Wu, 2012) and starch (Bragd, Besemer, & van Bekkum, 2001; de Nooy, Besemer, & van Bekkum, 1995; Kato, Matsuo, & Isogai, 2003; ter Haar et al., 2010; Thaburet, Merbouh, Ibert, Marsais, & Queguiner, 2001), among many others. Recently, the C-6 oxidation of agarose (Su et al., 2013), a polysaccharide related to carrageenan, was reported. The modified polysaccharides showed improved solubility properties (Chang & Robyt, 1996), metal chelating abilities (Muzzarelli, Muzzarelli, Cosani, & Terbojevich, 1999; Saito & Isogai, 2005) and the possibility of introducing new functional groups through amidation (Follain et al., 2008; Su et al., 2013) or esterification (Muzzarelli et al., 1999). Besides, the biological activity was modified by the introduction of new anionic groups (Bae, Kim, Lee, & Lee, 2011; Delattre et al., 2015; Elboutachfaiti et al., 2011; Wang et al., 2011).

Herpes simplex virus (HSV) types 1 and 2 may cause a broad range of human diseases, including oral and genital infections, keratoconjunctivitis and encephalitis, with different degrees of severity (Whitley & Roizman, 2009). As prolonged therapies with acyclovir, the most successful antiherpetic drug, have resulted in the emergence of drug-resistant variants (Piret & Boivin, 2011), the development of new compounds with different targets is required. In particular, sulfated polysaccharides like carrageenans have shown a potent anti-HSV activity (Damonte et al., 2004). Thus, the improvement of their antiviral effectiveness becomes of considerable interest.

With the aim of introducing modified materials with enhanced biological or functional properties, we have carried out a detailed study of the optimal conditions for achieving different degrees of C-6 oxidation of the κ-carrageenan from *Hypnea musciformis* (Cosenza, Navarro, Fissore, Rojas, & Stortz, 2014). We also report the study of a side reaction and its rationalization through molecular modeling, the extension of the reaction to ι-carrageenan, the chemical and spectroscopical characterization of all the products, and the assessment of the anti-herpes simplex virus (HSV) activity of fully and partly oxidized carrageenans.

2. Materials and methods

2.1. Materials

The κ-carrageenan from *Hypnea musciformis* was obtained as reported elsewhere (Cosenza et al., 2014). It corresponds to the fraction isolated after extraction of the seaweed with hot water, precipitation with 0.125 M KCl, dialysis and freeze-drying. ι-Carrageenan and TEMPO were purchased from Sigma–Aldrich. Dialysis was carried out using cellulose membranes of molecular weight cut-off 3500 against distilled water. All chemical reagents and solvents were of analytical grade.

2.2. Optimization of the reaction of κ-carrageenan with TEMPO

Oxidation experiments were performed at different pHs, reaction times and quenching methods. The general method was as follows: 100 mg of κ-carrageenan (containing *ca.* 0.25 mmol of primary alcohol), 1.6 mg of TEMPO and 20 mg of NaBr were dissolved in 15 mL of water and cooled to 0 °C in an ice bath. The solution was adjusted to the expected pH by adding 0.1 M aq NaOH, or else, when a $NaHCO_3/Na_2CO_3$ buffer solution was used, the reagents were dissolved directly in 15 mL of the buffer solution. The external oxidant was prepared by diluting commercial bleach (0.77 M NaClO) with an equal volume of water and adjusting to the desired pH by the addition of 1 M aq HCl. The bleach solution was titrated by a $Na_2S_2O_3$ solution using iodine/soluble starch as end point indicator. The oxidant solution was added dropwise to the carrageenan solution during 20 min. The volume of bleach added was calculated as to keep a ratio of 1.25 eq of NaClO per eq of primary alcohol. The appropriate pH was kept constant by adding 0.1 M NaOH. Both the pH adjustment of the bleach solution and the addition of 0.1 M NaOH were not necessary when buffer solutions were used. The reaction was left for the desired length of time. At this moment, the reaction volume was divided in two halves. One was quenched by addition of 2 mL of ethanol, neutralized with 0.1 M HCl and dialyzed. The other half was quenched by the addition of 50 mg of $NaBH_4$ and left 2 h at 0 °C before dialysis. Finally, the product was isolated by freeze-drying. The set of different conditions used, together with the acronyms of the treated polysaccharides are shown in Table 1.

Table 1
Nomenclature of the polysaccharides and conditions assayed for the oxidation with TEMPO.

Acronym	Treated polysaccharide	Base	pH	Time (h)	Eq NaClO	Quencher
K-Ox$_E$-N$_{10.5}$	κ-carrageenan	NaOH	10.5	2	1.25	EtOH
K-Ox$_R$-N$_{10.5}$	κ-carrageenan	NaOH	10.5	2	1.25	$NaBH_4$
K-Ox$_R$-B$_{10.4}$	κ-carrageenan	Buffer	10.4	2	1.25	$NaBH_4$
K-Ox$_R$-B$_{10}$	κ-carrageenan	Buffer	10	2	1.25	$NaBH_4$
K-Ox$_R$-B$_{9.4}$	κ-carrageenan	Buffer	9.4	2	1.25	$NaBH_4$
K-Ox$_R$	κ-carrageenan	NaOH	10	2	1.25	$NaBH_4$
K-Ox$_R$-1h	κ-carrageenan	NaOH	10	1	1.25	$NaBH_4$
K-Ox$_R$-3h	κ-carrageenan	NaOH	10	3	1.25	$NaBH_4$
K-1/10-Ox$_R$	κ-carrageenan	NaOH	10	2	0.125	$NaBH_4$
K-1/4-Ox$_R$	κ-carrageenan	NaOH	10	2	0.313	$NaBH_4$
K-1/2-Ox$_R$	κ-carrageenan	NaOH	10	2	0.625	$NaBH_4$
S-Ox$_E$	Starch	NaOH	10.5	2	1.25	EtOH
I-Ox$_E$	ι-carrageenan	NaOH	10.5	2	1.25	EtOH
I-Ox$_E$-D	ι-carrageenan	NaOH	10.5	2	1.25	EtOH
I-Ox$_R$	ι-carrageenan	NaOH	10	2	1.25	$NaBH_4$
I-1/4-Ox$_R$	ι-carrageenan	NaOH	10	2	0.313	$NaBH_4$
I-1/2-Ox$_R$	ι-carrageenan	NaOH	10	2	0.625	$NaBH_4$

2.3. Optimal conditions for the oxidation of carrageenans with TEMPO

The optimal conditions found for κ-carrageenan were used for the total oxidation of this and other polysaccharides. Briefly, an amount of polysaccharide containing 0.25 mmol of primary alcohol, 1.6 mg of TEMPO and 20 mg NaBr were dissolved in 15 mL of water and cooled to 0 °C in an ice bath. The solution was adjusted to the pH 10 by adding 0.1 M aq NaOH. The oxidant solution, prepared as indicated above containing 1.25 eq NaClO per eq of primary alcohol was added dropwise in 20 min. The reaction mixture was left for 1–2 h, keeping the pH at 10 with 0.1 M aq NaOH. After that period, $NaBH_4$ was added and left for 2 h at 0 °C before dialysis.

2.4. Partial oxidation of polysaccharides with TEMPO

The partial oxidation was carried out using the optimal conditions (see above), but reducing the amount of oxidant added: partially oxidized κ-carrageenan was obtained by addition of 0.625, 0.313 and 0.125 eq NaClO per eq primary alcohol. For ι-carrageenan the reaction was carried out after addition of 0.625 and 0.313 eq NaClO (Table 1).