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Effects of sulfate group in red seaweed polysaccharides on anticoagulant activity and cytotoxicity

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ABSTRACT

In this paper, the structural effects of two main red seaweed polysaccharides (agarose and carrageenan) and their sulfated derivatives on the anticoagulant activity and cytotoxicity were investigated. The substitution position rather than the substitution degree of sulfate groups shows the biggest impact on both the anticoagulant activity and the cell proliferation. Among them, C-2 of 3,6-anhydro-α-D-Gal*p* is the most favorable position for substitution, whereas C-6 of β-D-Gal*p* is the most disadvantageous. Moreover, the secondary structures of glycans also play a key role in biological activities. These demonstrations warrant that the red seaweed polysaccharides should be seriously considered in biomedical applications after carefully tailoring the sulfate groups.

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1. Introduction

As an important portion of polysaccharides, red seaweed polysaccharides have attracted much attentions because of their biological activities and abundant marine storage. Among them, agarose and carrageenan are two easily available ones which have been widely used as gelling and thickening agents (Jiao, Yu, Zhang, & Ewart, 2011). Their structures are both linear, with alternating α-(l-3) and β-(l-4) linkages situated between galactose or glactose analogs. The structural differences appear in the stereo configuration of 4-linked α-galactose residues (D in carrageenan and L in agarose) (Rochas and Lahaye, 1989). Their sulfated derivatives have also been found to possess unique anticoagulant, antioxidative, antitumor and antiviral functions (Chen, Wu, & Wen, 2008; Jiao et al., 2011; Mayer, Rodriguez, Berlinck, & Hamann, 2009; Pomin & Mourao, 2008; Yoshida et al., 1988). Sulfate groups can be located on C-2, C-4, and/or C-6 of D-galactose, for instance, natural sulfated agar was found to be substituted on C-6 and C-2 of β-D-galactopyranose (Gal*p*, symbolized as G) unit (Goncalves et al., 2005; Jiao et al., 2011). The most commonly used carrageenans are normally classified as κ, ι and λ forms according to their sulfation patterns and the existence of 3, 6-anhydro-α-D-galactopyranose (3,6-AG, symbolized as DA) on D-units (Shanmugam & Mody, 2000). As shown in Fig. 1, ideally, in the three forms of carrageenans, the number of sulfate groups in every disaccharide unit is 1, 2, and 3, respectively, and in agarose, no sulfate group exists. Compared with the κ- form, the ι-carrageenan has an additional sulfate group on C-2 of the DA residue, resulting in two sulfates per disaccharide unit. Meanwhile, each disaccharide unit of λ-carrageenan has three sulfate groups on C-2 of G residue, C-2 and C-6 of the 4-linked residue (α-1, 4-G), and with little or no 3, 6-anhydride bridge (Jiao et al., 2011). But commercially, the sulfate content of κ-, ι-, and λ-carrageenan is only 15–20%, 28–30% and 32–39%; while the 3,6-AG content is 28–35%, 25–30%, and 0–10%, respectively (Usov, 2011).

Many researches have been published concerning the relationship between the structures and the biological activities of sulfated red algal galactans (Toida, Amornrut, & Linhardt, 2003; Usov, 1992). But the results were mainly based on data from oligosaccharides. In most cases, sulfated polysaccharides with high-molecular-weight from natural source or after chemical modification were degraded by acid hydrolysis, methanolysis, partial reductive hydrolysis, or enzymolysis, and their structures were analyzed primarily relying on 1D or 2D ^{1}H and ^{13}C NMR spectroscopy, as well as electrospray ionization-mass spectrometry (ESIMS) (Gonçalves, Ducatti, Duarte, & Noseda, 2002; Goncalves et al., 2005; Pavlenko, Belogortseva, Kalinovskii, & Ovodov, 1976; Yu et al., 2002). Considering that the hydrolytic treatment might bring about changes in the secondary structure, and at the same time, the conformations of the whole polymer chains might also have synergistic effect on biological activities, a clear analysis on the chemical structure of pristine polysaccharides with high molecular weight is necessary. However, there have been some but still few reports on this issue owing to the difficulties in structural analysis of complex conformations (Opoku, Qiu, & Doctor, 2006; Yuan et al., 2005, Araújo, Noseda,

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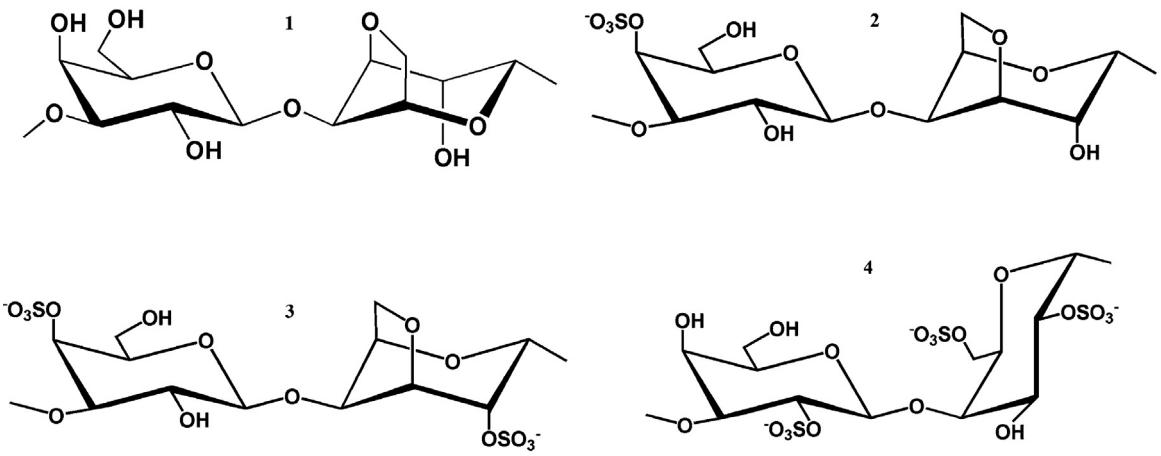

Fig. 1. Chemical structure of (1) agarose, (2) κ-, (3) ι- and (4) λ- carrageenan.

Cipriani, & Goncalves, 2013). In these researches, additionally, the sulfation pattern and the molecular weight of glycans have been revealed as two important factors on their biological activities (Bao, Duan, Fang, & Fang, 2001; Haroun-Bouhedja, Ellouali, Sinquin, & Boisson-Vidal, 2000; Nishino & Nagumo, 1991, 1992). Meanwhile, many recent evidences indicated that besides charge density arose from sulfate groups, some structural effects which were stereospecific could also be important to biological activity, for example, the anticoagulant activity could be influenced by the glycosidic linkage (either (1 → 3) or (1 → 4)) and the neighboring sulfate groups (Chaidedgumjorn et al., 2002; Pereira, Melo, & Mourão, 2002). Nevertheless, the existing researches on various factors were discrete, so comprehensive explorations combining these factors together remain to be investigated. Consequently, to carry out analyses based on intact structures and multifactor influences, we here prepared sulfated agarose, sulfated κ-carrageenan, desulfated κ-carrageenan, and κ-carrageenan oligosaccharide, respectively, and evaluated their anticoagulant activities and cytotoxicities, so that hopefully find the relationship between D-galactose structures and their bioactivities.

2. Materials and methods

2.1. Materials

κ-Carrageenan (B.R.) was purchased from Weijia (Guangzhou, China). ι-carrageenan (commercial grade, C1138), λ-carrageenan (low substitution degree, commercial grade, 22049, Lot No. 1408463), and 1,2,4,5-pyromellitic acid (A.R.) were purchased from Sigma–Aldrich (USA). Agarose (B.R.) was purchased from Genebase (Shanghai, China). Chlorosulfonic acid (A.R.) was purchased from Xihua (Tianjing, China). Heparin sodium standard (197 IU/mg) was purchased from Hengyuan Qitian (Beijing, China). 1640 culture medium, fetal bovine serum (FBS), and double resistant were purchased from Gibico (USA). All other chemical reagents used, including KCl, 30% H_2O_2, absolute EtOH (without dehydration), $NaBH_4$, phenol, $BaCl_2$, D-galactose, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and isopropanol, were of analytical grade. Some anhydrous organic solvents, including pyridine, formamide, dimethyl formamide (DMF), and dimethylsulfoxide (DMSO) were also of analytical grade and dehydrated through routine approaches before use.

2.2. Purification, degradation and classification of κ-carrageenan

The κ-carrageenan was purified by KCl precipitation method (S.F-Tischer et al., 2006). κ-Carrageenan was firstly dissolved in H_2O at 75 °C to form 0.5% aqueous solution. The solution was quickly filtered before cooling down. Then equal volume of 8% KCl solution was added by stirring for 3 h. The resulting mixture was kept to rest overnight at room temperature. After discarding the supernatant by filtration, the insoluble part was dialyzed (cut-off 3500) for 3d, and freeze-dried.

The purified κ-carrageenan was degraded through ultrasonic-assisted oxidative degradation (Chen et al., 2011). κ-Carrageenan was dissolved in water at 75 °C by stirring to form 2% aqueous solution. The solution was cooled down to 50 °C, and a certain volume of 30% H_2O_2 was added dropwise under ultrasonic condition. After the degradation was completed, 20 wt% $NaBH_4$ was added, and kept to rest with pH > 10 for 2 h under room temperature.

The reduced κ-carrageenan was concentrated by rotary evaporator for 0.5 h, precipitated by triple volume of EtOH, classified with different dialysis bags (cut-off 3500∼12,000) for 3d, and freeze-dried to give degraded samples with different molecular weights. The resulting degraded carrageenan was dissolved in water and separated by centrifugal ultrafiltration (cut-off 3000) repeatedly. The solution containing carrageenan with $M_w \approx 3000$ was dialyzed (cut-off 1000) for 3d and freeze-dried to give κ-carrageenan oligosaccharide ($M_w \approx 3000$).

2.3. Preparation of sulfated agarose, κ-carrageenan, and desulfurated κ-carrageenan

The agarose and κ-carrageenan were sulfated by pyridine-chlorosulfonic acid method (Jie, Zhang, Chen, Mao, & Tang, 2012). Chlorosulfonic acid was slowly added dropwise into anhydrous pyridine with vigorous stirring in ice bath to form the SO_3-pyridine complex. After the reaction was completed, the formamide solution containing 1.5% carrageenan or agarose was added under higher temperature. The pH of the resulting solution was adjusted to slightly alkaline with 2.5 mol/L NaOH. Then the solution was concentrated, precipitated by triple volumes of EtOH, and centrifuged. The precipitate was dialyzed (cut-off 3500), washed and centrifuged for multiple times, and freeze-dried.

The κ-carrageenan was desulfurated through a mild desulfurization method (Miller & Blunt, 1998). κ-Carrageenan (0.5 g) was dissolved in anhydrous DMSO (100 mL). Then 15 (v/v) % pyridine, 0.06 mol 1,2,4,5-pyromellitic acid and 0.024 mol NaF was added in one portion at 100 °C by stirring for several hours. The resulting solution was neutralized to pH 7–8 with 2.5 mol/L NaOH solution. Then the product was precipitated by triple volume of EtOH,

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