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Preparation and in vitro antioxidant activity of κ-carrageenan oligosaccharides and their oversulfated, acetylated, and phosphorylated derivatives

Huamao Yuan,[a,b] Weiwei Zhang,[a] Xuegang Li,[a] Xiaoxia Lü,[c] Ning Li,[a,b] Xuelu Gao[a,b] and Jinming Song[a,*]

[a]*Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, PR China*
[b]*The Graduate School of the Chinese Academy of Science, Beijing 100039, PR China*
[c]*Marine Geology College, Ocean University of China, Qingdao 266003, PR China*

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Abstract—In order to study the relationship between chemical structure and properties of modified carrageenans versus antioxidant activity in vitro, κ-carrageenan oligosaccharides were prepared through mild hydrochloric acid hydrolysis of the polysaccharide, and these were used as starting materials for the partial synthesis of their oversulfated, acetylated, and phosphorylated derivatives. The structure and substitution pattern of the oligosaccharides and their derivatives were studied using FTIR and ^{13}C NMR spectroscopy, and their in vitro antioxidant activities were investigated. Certain derivatives of the carrageenan oligosaccharides exhibited higher antioxidant activity than the polysaccharides and oligosaccharides in certain antioxidant systems. The oversulfated and acetylated derivatives, which scavenge superoxide radicals, the phosphorylated and low-DS acetylated derivatives, which scavenge hydroxyl radicals, and the phosphorylated derivatives, which scavenge DPPH radicals, all exhibited significant antioxidant activities in the systems examined. The effect of the molecular weight of the carrageenan on antioxidant activities, however, is not obvious from these studies.

Keywords: Carrageenan oligosaccharide; Oversulfated; Acetylated; Phosphorylated; Antioxidant activity; In vitro

1. Introduction

Cell walls from marine algae characteristically contain sulfated polysaccharides, which are not found in land plants and which may have specific functions in the regulation of ionic materials.[1] Carrageenan is a collective term for a group of sulfated polysaccharides extracted from marine red algae. Carrageenans consist of alternating 3-linked β-D-galactose (G units) and 4-linked α-D-galactose (D units) or 4-linked 3,6-anhydro-D-galactose (An units). Carrageenans can be divided into different types depending on the number and position of the sulfate groups, giving the economically important κ-(An-G4S), ι-(An2S-G4S), and λ-(D2, 6S-G2S) carrageenans.[2–4]

Sulfated polysaccharides from marine algae are known to exhibit many biological and physiological activities including anticoagulant, antithrombotic, anti-inflammatory, antiviral, and antitumor activities.[5–8] In recent years, algal polysaccharides have been demonstrated to play an important role as free-radical scavengers in vitro and antioxidants for the prevention of oxidative damage in living organisms.[9–12] Their activity depends on several structural parameters such as the degree of sulfation (DS), the molecular weight, the sulfation position, type of sugar and glycosidic branching.[13,14] Moreover, some reports reveal that the sulfate and phosphate groups in the polysaccharides

* Corresponding author. Tel./fax: +86 532 289 8583; e-mail: jmsong@ms.qdio.ac.cn

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lead to differences in their biological activities. Tsipali and co-workers investigated the free-radical scavenging activity of glucan and non-glucan polymers.[15] They observed that phosphorylated and sulfated glucans exhibited antioxidant ability that was greater than that of the glucans and other neutral polysaccharides, which indicate that polyelectrolytes, such as glucan sulfate or phosphate, might have increased scavenging activity. Regarding the relationship between sulfate content and macrophage stimulating activity of polysaccharides from *P. yezoensis*, it was reported that sulfate groups in the molecules probably contribute to the activity.[16,17]

However, studies on oligosaccharides derived from algal polysaccharides, especially studies on their antioxidant activities are comparatively few. Moreover, the relationship between chemical modification of the oligosaccharides and their biological activity, to our knowledge, has never been studied. In this study, carrageenan polysaccharide was degraded by mild acidic hydrolysis, the oligosaccharides and their oversulfated, acetylated, and phosphorylated derivatives were prepared, and their structures were elucidated. The aim of this study was to evaluate their in vitro antioxidant activities and characterize the relationship between chemical structures and properties and antioxidant activity.

2. Experimental

2.1. Materials

Carrageenan was purchased from Yantai Algae Industries (Shandong, China). The carrageenan was treated with NaOH and KCl to increase the content of An residues to enhance gel strength. Properties provided by the manufacturer include: gel strength of 800 g/cm^2 at 1.5% water and a gel point of 41 °C. Nitro blue tetrazolium (NBT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), phenazine methosulfate (PMS), hydrogen peroxide (H_2O_2), thiobarbituric acid (TBA), ethylenediaminetetraacetic acid (EDTA), reduced nicotinamide adenine dinucleotide (NADH), trichloroacetic acid (TCA) and deoxyribose (DR) were purchased from Sigma Chemical Co. All other reagents were of analytical grade.

2.2. Preparation of carrageenan oligosaccharides

κ-Carrageenan polysaccharides (5 g/500 mL) were dissolved at 60 °C in 0.1 M HCl with vigorous stirring and were kept for 4 h. The reaction was terminated by neutralization with 0.1 M NaOH in an ice-water bath, and the mixture was then filtered. The supernatant was concentrated by rotary evaporation under reduced pressure and then desalted on a Sephadex G-25 (superfine, Pharmcia) column (90×1.5 cm), eluting with distilled water. The eluate was concentrated by rotary evaporation and lyophilized to give the powdered oligosaccharide mixture.

2.3. Sulfation of carrageenan oligosaccharides

The sulfation agent, SO_3·DMF, was obtained by dropping 20 mL of $HClSO_3$ into 100 mL of DMF under cooling in an ice-water bath. Lyophilized oligosaccharide (2 g) was added to 84 mL of DMF, and the mixture was stirred at 60 °C for 30 min in order to disperse it into the solvent. Then 16 mL of the SO_3·DMF complex was added. After reaction for 4 h, the mixture was cooled to room temperature by an ice bath, neutralized with 30% NaOH solution, and concentrated under reduced pressure to evaporate the solvent. The residue was added to 20 mL of DMF and filtered, and the filtrate was precipitated with acetone. The precipitate was dissolved in distilled water and freeze dried.

2.4. Acetylation of carrageenan oligosaccharides

2.4.1. Low-DS acetylation. Lyophilized oligosaccharide (2 g) was dispersed in 20 mL of DMF, and the mixture was stirred at 60 °C for 30 min, then 20 mL of a mixture of 50% pyridine and 50% Ac_2O were added. The reaction mixture was kept at 60 °C for 4 h. Distilled water (250 mL) was added to react with the excess Ac_2O, and the mixture was concentrated under reduced pressure. Anhydrous EtOH was added to the concentrated solution, and the mixture was kept overnight at 0–5 °C. The precipitate was filtered off and washed three times with EtOH. The resulting precipitate was dissolved in distilled water and freeze dried.

2.4.2. High-DS acetylation. Lyophilized oligosaccharide (2 g) was added to 20 mL of formamide (FA) and stirred at room temperature for 30 min, then 20 mL of a mixture of 50% pyridine and 50% Ac_2O were added. The reaction mixture was kept for 24 h under continuous stirring at room temperature. Distilled water (250 mL) was added to react with the excess Ac_2O, and the mixture was concentrated under reduced pressure. Anhydrous EtOH was added to the concentrated solution, and the mixture was kept overnight at 0–5 °C. The precipitate was filtered off and washed three times with EtOH. The resulting precipitate was dissolved in distilled water and freeze dried.

2.5. Phosphorylation of carrageenan oligosaccharides

$POCl_3$ (4 mL) was added to 30 mL of pyridine and stirred for 15 min at 0–2 °C (ice-water bath). A formamide solution (15 mL) containing 2 g of carrageenan oligosaccharides was then dripped in. The mixture was stirred for 3 h at 0–2 °C. The reaction mixture was poured into

150 mL of satd aq $Ba(OH)_2$, and the resulting white precipitate was filtered off and washed with water. The filtrate was concentrated under reduced pressure. EtOH was added to the concentrated solution, and the mixture was kept overnight at 0–5 °C. The precipitate was filtered off and washed with EtOH to give a crude product (dark brown). The crude product was dissolved in water, and some insoluble material was removed by centrifugation. The EtOH was added to the supernatant solution, and the resulting precipitate was filtered off and washed with acetone. The purification was re-dissolved in water and freeze dried.

2.6. Analytical methods

The molecular weights of carrageenan oligosaccharides and their derivatives were determined according to the method of Vreeman et al. using a Ubbelohde viscometer at 20 °C.[18] The sulfate content was determined according to the method of Kawai.[19] The degree of acetylation (DS_{Ac}) was determined from the method of Gröndahl et al.,[20] and the total phosphate was determined by the ascorbic acid method.[21]

UV analyses were conducted on an Agilent 6010 UV/vis spectrometer. Fourier-transform infrared (FTIR) spectra were recorded from the polysaccharide and F1 in KBr pellets on a Nicolet Avatar 360 FTIR spectrophotometer. For NMR spectroscopic analyses, ^{1}H NMR, and proton-decoupled ^{13}C NMR and ^{31}P NMR spectra were run on a JEOL 600-MHz spectrometer at 600.01 MHz (^{1}H), 150.9 MHz (^{13}C) and 242.9 MHz (^{31}P) spectra. ^{1}H and ^{13}C chemical shifts are referenced to external DSS, and aq 85% phosphoric acid was used as the external reference (δ_P 0.00 ppm) for ^{31}P NMR spectroscopy.

2.7. Antioxidant activity assays

2.7.1. Superoxide anion scavenging activity. Measurement of superoxide anion scavenging activity was based on the method described by Liu et al. with slight modification.[22] Superoxide radicals were generated in a PMS-NADH system by oxidation of NADH and assayed by the reduction of NBT. In these experiments, the superoxide radicals were generated in 3 mL of Tris–HCl buffer (16 mM, pH 8.0) containing 1 mL of NBT (50 μM) solution, 1 mL of NADH (78 μM) solution and varying concentrations of sample solution in Tris–HCl buffer. The reaction was initiated by adding 1 mL of PMS solution (10 μM) to the mixture. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance at 560 nm was measured against blank samples. In the essential control, NADH was substituted with Tris–HCl buffer. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\%\text{Inhibition} = (1 - A_1/A_0) \times 100\%,$$

where A_0 is the absorbance of the control and A_1 is the absorbance of samples.

2.7.2. Hydroxyl radical scavenging activity. The scavenging activity of hydroxyl radicals was measured according to the method described by Halliwell et al.[23] Reaction mixtures, containing varying concentrations of samples, were incubated with deoxyribose (3.75 mM), H_2O_2 (1 mM), $FeCl_3$ (100 μM), EDTA (100 μM) and ascorbic acid (100 μM) in potassium phosphate buffer (20 mM, pH 7.4) for 60 min at 37 °C. The reactions were terminated by adding 1 mL of TBA (1% w/v) and 1 mL of TCA (2% w/v) and then heating the tubes in a boiling water bath for 15 min. The contents were cooled, and the absorbances of the mixtures were measured at 535 nm against a reagent blank. Decreased absorbance of the reaction mixture indicated decreased oxidation of deoxyribose.

2.7.3. DPPH free-radical scavenging activity. The scavenging activity of the DPPH radical was assayed according to the method of Shimada et al.[24] Briefly, a 0.1 mM solution of DPPH**.** in 1:1 MeOH–water was prepared, and to 1 mL of this solution was added 3 mL of sample solution in 1:1 MeOH–water at different concentrations or negative control (1:1 MeOH–water). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free-radical scavenging activity. The DPPH radical-scavenging activity (%) was calculated by the following equation:

$$\text{Scavenging activity } (\%) = (1 - A_1/A_0) \times 100\%,$$

where A_0 is the absorbance of the control, and A_1 is the absorbance of samples.

3. Results and discussion

Mild-acid hydrolytic depolymerization of κ-carrageenan affords a mixture of oligosaccharides. The average molecular weights of the κ-carrageenan polysaccharide and the oligosaccharide mixtures were estimated to be 37.7 and 1.2 kDa, respectively, as determined by the method of Vreeman et al.[18] (Table 1). At the same time, the sulfate contents of the polysaccharide and the oligosaccharide mixture were 12.95% and 8.98% (w/w), respectively.

Infrared spectra of the crude polysaccharide and the oligosaccharide mixture (Fig. 1) were very similar. The absorption band at 845 cm^{-1} indicated that the sulfate

Table 1. Physical characteristics of κ-carrageenan polysaccharide, oligosaccharides, and their oversulfated, acetylated and phosphorylated derivatives

Sugar	MW (kDa)	SO_4(%)	DS_{Ac}	P(%)
Polysaccharide	37.7	12.95		
Oligosaccharide	1.2	8.98		
Oversulfated derivatives	0.8	47.9		
Low-DS acetylated derivatives	1.2	8.8	1.13	
High-DS acetylated derivatives	1.4	12.8	2.65	
Phosphorylated derivatives	1.1	n.d.[a]		2.89

[a] Not determined.

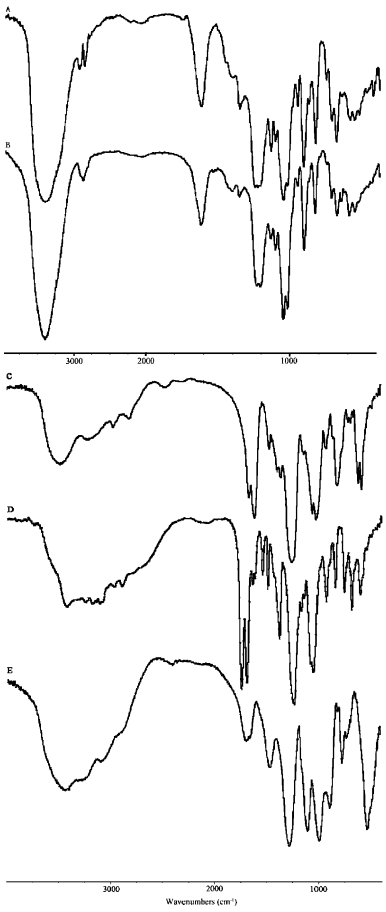

Figure 1. FTIR spectra of carrageenan polysaccharide (A), oligosaccharides (B), and their oversulfated (C), acetylated (D) and phosphorylated (E) derivatives.

group was attached at the C-4 position of galactose. The band at 930 cm^{-1} was attributed to the presence of 3,6-anhydrogalactose. The signal at 1249 cm^{-1} was attributed to the asymmetric stretching of S=O. NMR spectroscopy gave valuable structural information on the carrageenan galactose. The signal assignments in both ^{1}H and ^{13}C NMR spectroscopy of the carrageenan oligosaccharide mixture were made according to our previous work and by comparison with published data (Table 2).[25,26] ^{13}C NMR spectra for oversulfated, high-DS acetylated and phosphorylated derivatives are shown in Figures S1–S4 in the Supplementary data section (available in the electronic edition of this paper).

The solvent in which the oligosaccharide mixture is immersed plays an important role in chemical reactions. In fact, the mixture can be dissolved to form a homogeneous solution in FA and is partly swollen in DMF. In sulfation, the reaction is influenced both by the complex used and by the reaction temperature. According to Alban et al.,[5] the SO_3·DMF complex is more reactive than the SO_3·Py complex at lower temperatures, and when the reaction temperature in DMF reaches 60 °C, the molecular weight of the saccharide under sulfation is only slightly decreased. But when the temperature is higher, degradation is severe. So we chose SO_3·DMF as the sulfation complex, DMF as the solvent, and 60 °C as the reaction temperature. In general, a defined sulfation method established for a certain saccharide cannot be transferred easily to another. However, in applying this procedure to the mixture of carrageenan oligosaccharides, oversulfated and less degraded product was obtained (Table 1). The sulfated content of the oversulfated mixture was 47.93%, which was much higher than that of the starting oligosaccharide mixture (8.98%).

Comparison between the FTIR spectra of the mixture of oligosaccharides and oversulfated derivatives reveals that the S=O vibration band at 1249 cm^{-1} of the oversulfated derivative became wider and stronger. The band at 815 cm^{-1} could be assigned to the sulfate in C-6 of galactose. The presence of a shoulder close to 850 cm^{-1} is the original sulfate at C-4 in the galactose. As no bands or shoulders have been detected at 830 and 805 cm^{-1}, the 2-sulfated galactose and the sulfate on C-2 of 3,6-anhydrogalactose were not present, or these bands were covered by the wide band at 815 cm^{-1}.[27]

The position of sulfation was determined by analysis of the ^{13}C NMR spectra. Downfield displacements of the signals for C-6 of G4S to δ 68.5, C-2 of G4S to δ 77.2 and C-2 of the An residue to δ 79.7, as compared with their positions in the corresponding nonsubstituted oligosaccharide at δ 63.5, 73.3, and 74.2, respectively, revealed the substitution pattern in the oversulfated oligosaccharide.[13,28] The existence of strong signals and the disappearance of unmodified signals indicated that the hydroxyl groups at C-6 of the G4S residue were completely sulfated. The significant signals at δ 77.2 for the sulfated C-2 of the G4S residue and δ 73.3 for the unsubstituted C-2, and on the other hand, the significant signals at δ 74.2 for the unsubstituted C-2 of the An residue and δ 79.7 for the sulfated C-2 were observed, indicating that the C-2 hydroxyl groups of both G4S and An residues were partially sulfated.[28] These data also

Table 2. ^{13}C NMR assignments for carrageenan oligosaccharides and their oversulfated, highly acetylated, and phosphorylated derivatives[a]

Samples	Residue	Chemical shifts (ppm)							
		C-1	C-2	C-3	C-4	C-5	C-6	C-2′[a]	C-6′[b]
Oligosaccharides	G4S	104.9	73.3	80.6	76.2	77.2	63.5		
	An	97.0	74.2	81.4	79.2	78.9	71.6		
Oversulfated derivatives	G4S	102.5	71.2	83.4	75.1	77.9		77.2	68.5
	An	93.1	74.5	83.9	79.0	78.4	70.2	79.7	
High-DS acetylated derivatives	G4S	101.7		78.8	74.4	75.2		72.6	66.0
	An	95.0		80.2	77.4	76.7	71.4	73.9	
Phosphorylated derivatives	G4S	104.9	73.3	80.6	75.9	77.1	63.5	78.1	
	An	96.9	74.2	81.4	79.1	78.8	71.7		

[a] The ^{13}C NMR spectra are provided in the Supplementary data section, which is available in the electronic version of this paper.
[b] Substitution position.

independently showed the sulfation activity of different hydroxyl groups occurred in the order C-6 (G4S) > C-2 (G4S) > C-2 (An).

For acetylation, in order to obtain different degrees of substitution for the acetylated mixture of oligosaccharides, the solvent DMF and FA were chosen, respectively, for low- and high-DS products, and the reaction times and temperatures were varied. The degree of acetylation (DS_{Ac}) was determined by ^1H NMR spectroscopy. The signal at 2.00 ppm indicated that the mixture of oligosaccharides was acetylated. The DS_{Ac} was determined from the relative intensities of the signals of the acetyl groups at 2.00 ppm and those of all carbohydrate signals. The following equation was used:

$$DS_{Ac} = \frac{(\text{Sum of integrals for acetyl groups at } 2.0\text{ ppm})/3}{(\text{Sum of integrals for carbohydrate signals at } 3.2\text{–}5.6\text{ ppm})/6}$$

The DS_{Ac} of low- and high-DS acetylated oligosaccharides derivatives was 1.13 and 2.65, respectively. The presence of acetyl groups in the mixture of the modified oligosaccharide was also ascertained by FTIR spectroscopy that showed a new band of strong intensity at 1729 cm^{-1}, which is attributed to the C=O stretching vibration.

In phosphorylation, the reaction was performed using FA as solvent and excess Py as cosolvent. The excess Py can react with the hydrochloric acid produced during the reaction to form Py·HCl so that it can prevent the hydrolysis of the carrageenan oligosaccharides. The total phosphate of the phosphorylated oligosaccharide derivative was 2.98%.

In the FTIR spectrum, the band at 1268 cm^{-1} indicated the P=O stretching vibration, and the band at 988 cm^{-1} was attributed to the P–O vibration. The phosphorylation pattern was also deduced from the ^{13}C NMR spectra. The signal for the G4S C-2 was shifted downfield from δ 73.3 to δ 78.1 in the oligosaccharide due to the α-effect of phosphorylation.[29–31] Very little change was observed in the ^{13}C chemical shifts for any of the unsubstituted positions. The ^{13}C chemical shifts of the phosphorylated oligosaccharide are shown in Table 2. The effect of phosphorylation is very complex, suggesting that conformational changes may accompany phosphorylation. The ^{31}P NMR spectra of the phosphorylated oligosaccharide showed intense signals at δ −11.0 ppm for diphosphate groups.[32,33] Since no other obvious chemical shifts were found in the ^{13}C NMR spectra, we can presume that in the C-2 position of the G4S residue, diphosphoester groups could be attached in the phosphorylated oligosaccharide.

The superoxide radical ($\cdot O_2^-$) is a highly toxic species that is generated by numerous biological and photochemical reactions.[34] Figure 2 shows the inhibitory effect of carrageenan polysaccharides, the mixture of oligosaccharides, and different derivatives of oligosaccharides on superoxide radicals. It can be seen that all the samples exhibited varying degrees of antioxidant activity. However, the oversulfated and acetylated (both low- and high-DS acetylated) derivatives of oligosaccharides showed significant scavenging effects. At a concentration of 400 μg/mL, the inhibitory effect is 92.8%, 90.5%, and 91.1%, respectively, for the oversulfated, low-DS and high-DS acetylated derivatives. This indicates that the sulfation and acetylation can increase the scavenging activity. To our surprise, the phosphorylated derivative showed relatively higher inhibitory effect at low concentration, but the activity decreased when the concentration increased. Although superoxide is a relatively weak oxidant, it decomposes to form stronger, reactive oxidative species, such as singlet oxygen and hydroxyl radicals, which initiate peroxidation of lipids. Further, superoxides are also known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating

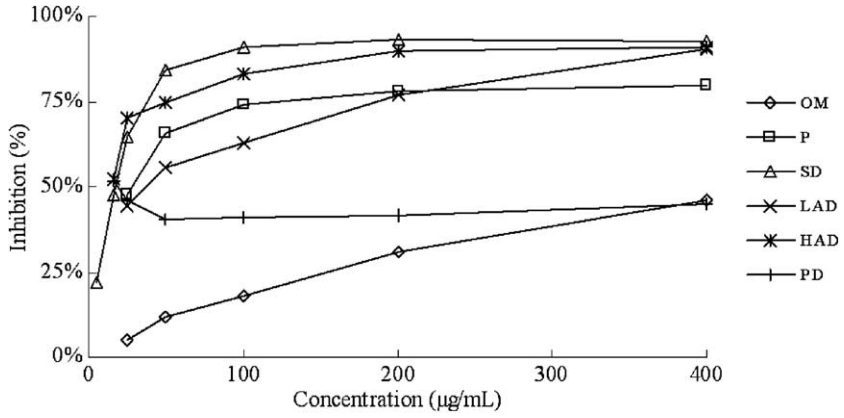

Figure 2. Inhibitory effect of carrageenan polysaccharide (P), oligosaccharides (OM), and their oversulfated (SD), lowly acetylated (LAD), highly acetylated (HAD) and phosphorylated (PD) derivatives on superoxide radicals. Values are means ± S.D. of three determinations.

precursors of hydroxyl radicals.[35] Our results clearly suggest that the antioxidant activity of carrageenan polysaccharide, the mixture of oligosaccharides and different derivatives is also related to their ability to scavenge superoxides.

Among the reactive oxygen species, the hydroxyl radical is the most reactive and induces severe damage to adjacent biomolecules. The scavenging effects of various samples on hydroxyl radicals are shown in Figure 3. A concentration-dependent inhibition against hydroxyl radical-induced deoxyribose degradation was observed. Effect of the carrageenan polysaccharide, the mixture of oligosaccharides, oversulfated and high-DS acetylated derivatives are similar, while the phosphorylated and low-DS acetylated derivatives showed the strong hydroxyl radical scavenging activity. Some workers report that the scavenging activity of hydroxyl radicals was not due to direct scavenging but inhibition of hydroxyl radical generation by chelating ions such as Fe^{2+} and Cu^{+}.[36] Smith et al. reported that molecules that can inhibit deoxyribose degradation are those that can chelate iron ions and render them inactive or poorly active in a Fenton reaction.[37] Iron, a transition metal, is capable of generating free radicals from peroxides by the Fenton reaction and is implicated in many diseases. Fe^{2+} has also been shown to produce oxyradicals and lipid peroxidation, and reduction of Fe^{2+} concentrations in the Fenton reaction would protect against oxidative damage.[35] In the present study, the hydroxyl radical-scavenging activity of oversulfated and phosphorylated derivatives are probably related to the specific chelating groups (sulfate and phosphate) within the molecule due to their high nucleophilic character. However, the significantly different effect on the hydroxyl radicals scavenging between low-DS and high-DS acetylated derivatives is not very clear. The mechanism of different derivatives on the hydroxyl radicals needs to be further investigated.

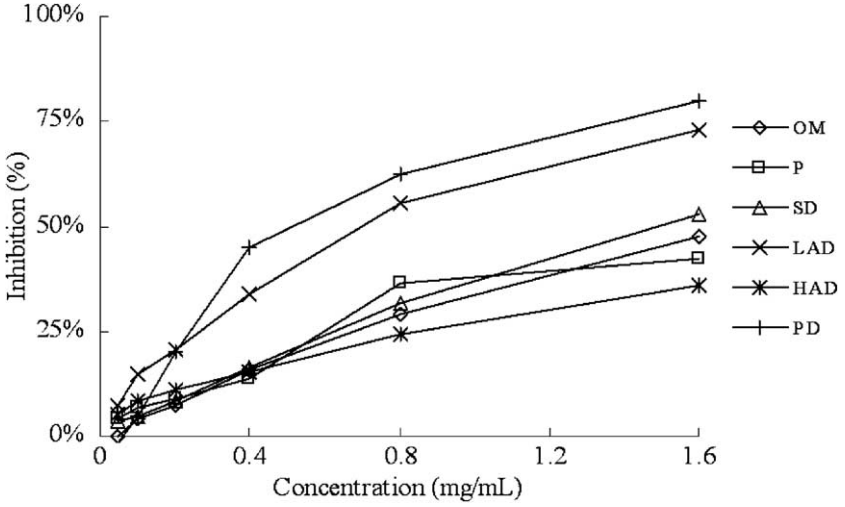

Figure 3. Inhibitory effect of carrageenan polysaccharide (P), oligosaccharides (OM), and their oversulfated (SD), low-DS acetylated (LAD), high-DS acetylated (HAD) and phosphorylated (PD) derivatives on hydroxyl radicals. Values are means ± S.D. of three determinations.

The model of scavenging the stable DPPH radical is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. The DPPH scavenging activity of all samples at 400 μg/mL is depicted in Figure 4. Except for the phosphorylated derivative, other samples showed only a slight scavenging effect on DPPH. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen-donating abilities. DPPH**.** is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule.[38] In the present study, the phosphorylated derivative showed excellent scavenging activity on DPPH radicals, which may be attributable to its strong hydrogen-donating ability compared to other samples.

To summarize, the different derivatives of carrageenan oligosaccharides exhibited higher antioxidant activity than the poly- and oligosaccharides in certain antioxidant systems in vitro. The oversulfated and acetylated derivatives, which scavenged superoxide radicals, the phosphorylated and low-DS acetylated derivatives, which scavenged hydroxyl radicals, and the phosphorylated derivatives, which scavenged DPPH radicals, all exhibited significant antioxidant activities. However, the effect of the molecular weight of the carrageenan in antioxidant activities is not obvious. Both the polysaccharide and the mixture of oligosaccharides exhibited the similar activity against the three antioxidant systems in vitro. This also needs further investigation; moreover, further in vivo experiments are planned to verify relation between chemical structure and properties and antioxidant activity.

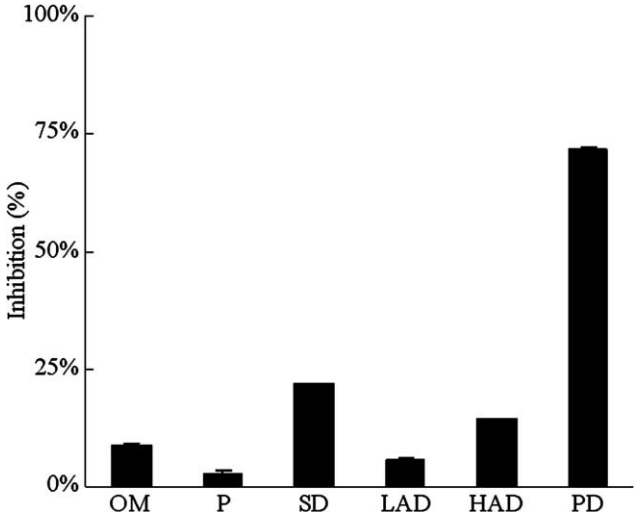

Figure 4. Inhibitory effect of carrageenan polysaccharide (P), oligosaccharides (OM), and their oversulfated (SD), lowly acetylated (LAD), highly acetylated (HAD) and phosphorylated (PD) derivatives on DPPH radicals at 400 μg/mL. Values are means ± S.D. of three determinations.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2004.12.026.

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