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Antioxidant activity and cytoprotective effect of κ-carrageenan oligosaccharides and their different derivatives

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Abstract—Antioxidant activity of κ-carrageenan oligosaccharides (OM) and their chemical modification derivatives was investigated employing various established in vitro systems, such as reducing power, iron ion chelation, and total antioxidant activity using β-carotene–linoleic acid system. The oversulfated (SD), lowly (LAD), and highly acetylated derivatives (HAD) in reducing power assay, the phosphorylated derivative (PD) in metal chelating assay, and oversulfated and phosphorylated derivatives in total antioxidant activity assay exhibited antioxidant activity higher than that of carrageenan oligosaccharides. The results indicated that the chemical modification of carrageenan oligosaccharides can enhance their antioxidant activity in vitro. The protective effects of the carrageenan oligosaccharides and their chemically modified derivatives against H_2O_2 and UVA (long-wave ultraviolet radiation) induced oxidative damage on rat thymic lymphocyte were investigated by measuring cell viability via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Thymic lymphocyte exposure to H_2O_2 and UVA, a marked reduction in cell survival was observed, which was significantly prevented by carrageenan oligosaccharides and their derivatives (preincubated for 2 h) at 66.7–2000 μg/mL. But both the carrageenan oligosaccharides and their different derivatives showed the similar protective effects on intracellular level. Taken together, these results suggest that carrageenan oligosaccharides and their derivatives show relevant antioxidant activity both in vitro and in a cell system.

Research in recent years has shown the implication of oxidative and free radical-mediated reactions in degenerative processes related to aging and diseases such as atherosclerosis, dementia, and cancer.[1–3] When oxygen is supplied in excess or its reduction is insufficient, reactive oxygen species (ROS) in the form of superoxide anion $^{\bullet}O_2^-$, hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2) are generated. Membrane lipids, proteins, and deoxyribonucleic acid (DNA) are the targets of such species and can suffer oxidative damage, causing tissue injury.[4,5] Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates. In recent years, there has been increasing interest in finding natural antioxidants since the synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are suspected of being responsible for liver damage and carcinogenesis.[6,7] A multitude of natural antioxidants have already been isolated from different kinds of plant materials such as seeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs.[8] Those natural antioxidants constitute a broad range of compounds including phenolic compounds, nitrogen-containing compounds, and carotenoids which have the capacity to protect the human body from radicals and retard the progress of many chronic diseases.[9]

However, natural antioxidants are not limited to terrestrial sources. In the search of new antioxidants, exploration of aquatic habitats has led to the discovery that marine plants and invertebrates also contain antioxidants.[10] Seaweed is considered to be a rich source of antioxidants.[11–13] Cell walls from marine algae characteristically contain sulfated polysaccharides, which are not found in land plants and which may have specific functions in ionic regulation.[14] Carrageenan is a collective term for a group of sulfated polysaccharides extracted from marine red algae. They consist of alternating 3-linked β-D-galactose (G units) and 4-linked

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α-D-galactose (D units) or 4-linked 3,6-anhydro-D-galactose (An units). They can be divided into different types depending on the number and position of sulfate groups giving the economically important κ- (An-G4S), ι- (An2S-G4S), and λ- (D2, 6S-G2S) carrageenan.[15–17] They are natural ingredients, which have been used for decades in food applications and regarded as safe.[18]

In recent years, algal polysaccharides were reported to be useful candidates in the search for an effective, nontoxic substance and 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.[19–22] However, the study on the oligosaccharides derived from algal polysaccharides, especially on the antioxidant activity, was comparatively deficient. Moreover, the relationship between chemical modification of the oligosaccharides and their biological activity was never studied before to our knowledge. In our previous work, κ-carrageenan oligosaccharides and their oversulfated, acetylated, and phosphorylated derivatives were prepared, their structures elucidated, and their antioxidant activities in vitro also preliminarily studied.[23] In the present study, the antioxidant activities were further investigated employing both in vitro assay systems and cell system, in order to evaluate whether the chemical modification will have any influence on the antioxidant activity.

Butyl hydroxy anisole (BHA), nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), hydrogen peroxide (H_2O_2), 1,1-diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), ethylenediaminetetraacetic acid (EDTA), ferrozine, nicotinamide adenine dinucleotide-reduced (NADH), trichloroacetic acid (TCA), deoxyribose (DR), and potassium ferricyanide were purchased from Sigma/Aldrich Chemicals Co. All other reagents were of analytical grade.

Carrageenan polysaccharide 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 gel point, 41 °C. Carrageenan oligosaccharides and their oversulfated, acetylated, and phosphorylated derivatives were prepared in our laboratory.

The reducing power of all the carrageenan samples was quantified by the method described earlier by Yen and Chen[24] with slight modifications. Briefly, different concentrations of samples in a 3.5 mL phosphate buffer (0.2 M, pH 6.6) were incubated with 2.5 mL potassium ferricyanide (1% w/v) at 50 °C for 20 min. The reaction was terminated by 2.0 mL TCA solution (10% w/v) and the mixture was centrifuged at 5000 rpm for 10 min. The 2.5 mL supernatant was mixed with 2.5 mL distilled water and 0.5 mL ferric chloride (0.1% w/v) solution and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power of the samples.

The ferrous ion-chelating potential of all carrageenan samples was investigated according to the method of Decker and Welch,[25] wherein the Fe^{2+}-chelating ability of samples was monitored by measuring the absorbance of the ferrous iron–ferrozine complex at 562 nm. Briefly, the reaction mixture, containing carrageenan samples of different concentrations, 0.25 mL $FeCl_2$ (2 mM), and 1 mL ferrozine (5 mM), was adjusted to a total volume of 4 mL with distilled water, shaken well, and incubated for 10 min at room temperature. The absorbance of the mixture was measured at 562 nm against blank. EDTA was used as a positive control. The ability of all samples to chelate ferrous ion was calculated using the following equation:

$$\text{Chelating effect } (\%) = (1 - A_{\text{sample 562 nm}}/A_{\text{control 562 nm}}) \times 100$$

The antioxidant activity of samples was evaluated by the method of Jayaprakasha et al. with some modifications using the β-carotene–linoleic acid system.[26] Four milliliters of a solution of β-carotene in chloroform (1 mg/mL) was pipetted into a flask containing 40 mg of purified linoleic acid and 400 mg of Tween 40. The chloroform was removed by a rotary vacuum evaporator at 40 °C for 4 min, and 100 mL distilled water was added slowly to the semisolid residue with vigorous agitation to form an emulsion. A 96-well microtiter plate was charged by 50 μL of test sample and 200 μL of the emulsion, and the absorbance was measured at 450 nm, immediately, against a blank consisting of the emulsion without β-carotene. The plate was placed in room temperature (20–23 °C), and the absorbance measurements were conducted again at 30 min intervals up to 270 min. All determinations were carried out in triplicate. The antioxidant activity (AA) of all the samples was evaluated in terms of bleaching of β-carotene using the following formula:

$$\text{AA} = [1 - (A_0 - A_t)/(A'_0 - A'_t)] \times 100,$$

where A_0 and A'_0 are the absorbance values measured at zero time of the sample and the control, respectively, and A_t and A'_t are the absorbance values measured in the test sample and the control, respectively, after 270 min.

Thymus was aseptically removed from sacrificed mice with scissors and forceps in cold phosphate-buffered saline (PBS), gently homogenized with a loose Teflon pestle, and passed through a sterilized mesh (200 mesh) to obtain single-cell suspensions. Thymic lymphocytes were grown in RPMI 1640 supplemented with 5% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. The cells were preincubated with indicated concentrations of carrageenan oligosaccharides and their derivatives for 2 h before exposure to H_2O_2 or UVA.

The cell viability assay is based on the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. Briefly, 2×10^4 cells were seeded in each well

of microtiter plates and were treated with various doses of carrageenan oligosaccharides and their derivatives. After 2 h of incubation, for a H_2O_2 induced model, 10 mM (final concentration) H_2O_2 was added to each well, and for a UVA induced model, the cells were submitted for 30 s at room temperature to UVA exposure, receiving a total equivalent energy of 90 mJ/cm^2 under constant intensity. Both H_2O_2 and UVA treated cells were incubated for another 2 h and then 100 μL of 5 mg/mL MTT in PBS was added to each well. Followed by incubation for 4 h at 37 °C, the plate was then centrifuged at 1000 rpm for 5 min and the supernatants were discarded. A total of 150 μL DMSO was added to each well and shaken until crystals were dissolved. The absorbance A_{490} was detected on the Microplate Reader (Bio-Rad, USA).

Results were expressed as means ± deviation (SD). The statistical significance of the differences between groups was evaluated by the variance analysis, followed by Student's *t*-test. Significant differences were set at $P < 0.05$.

The antioxidant activity has been reported to have a direct, positive correlation with the reducing power.[27] Figure 1 shows the reducing powers of carrageenan oligosaccharides and their different derivatives using the potassium ferricyanide reduction method. In this assay, the yellow color of the test solution changes to various shades of green and blue depending upon the reducing power of each sample. The antioxidant activity of antioxidants has been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity, and radical scavenging.[28,29] The reductive potential of the carrageenan oligosaccharides and their oversulfated and acetylated derivatives increased with increasing concentration and the oversulfated and acetylated derivatives showed a higher reducing power than the oligosaccharides. However, the phosphorylated derivative showed a relatively high inhibitory effect compared to those of other samples at low concentration, but the potential decreased when the concentration increased. The same situation also occurred in the positive control Vc. The reducing properties are generally associated with the presence of reductones.[26,30,31] Gordon reported that the antioxidant action of reductones is based on the breaking of the free radical chain by donating a hydrogen atom.[32] Reductones also react with certain precursors of peroxide, thus preventing peroxide formation. The sulfated and acetylated derivatives appear to function as good electron and hydrogen atom donors and therefore should be able to terminate radical chain reactions by converting free radicals to more stale products.

Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted with the result that the red color of the complex is decreased. Measurement of color reduction therefore allows estimation of the chelating activity of the coexisting chelator.[33,34] In this assay, the phosphorylated derivative and positive control of EDTA interfered with the formation of ferrous and ferrozine complex (Fig. 2), suggesting that they have chelating activity and capture ferrous ion before ferrozine. Other samples showed little metal chelating activity which indicated that phosphorylation of carrageenan oligosaccharides can significantly enhance their metal chelating activity while any other modification cannot.

Iron can stimulate lipid peroxidation by the Fenton reaction and also accelerate peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation.[35] Metal chelating capacity was significant since it reduced the concentration of the catalyzing transition metal in lipid peroxidation.[36] It was reported that chelating agents, which form σ-bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion.[32] In the present study, the phosphorylated derivative demonstrated a moderate capacity for iron binding, suggesting that its action as peroxidant protector may be related to its iron binding capacity.

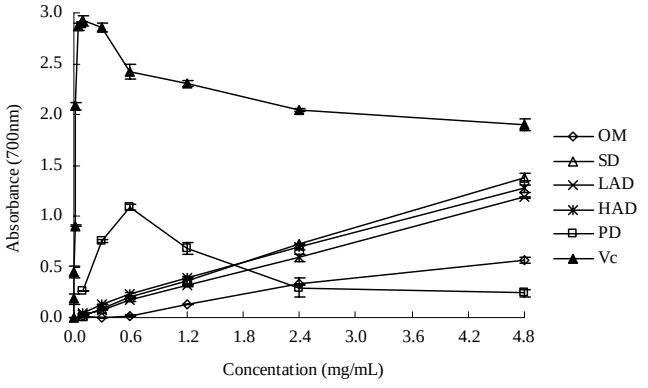

Figure 1. Reducing power of carrageenan oligosaccharides (OM) and their oversulfated (SD), lowly acetylated (LAD), highly acetylated (HAD) and phosphorylated (PD) derivatives. Values are means ± SD of three determinations.

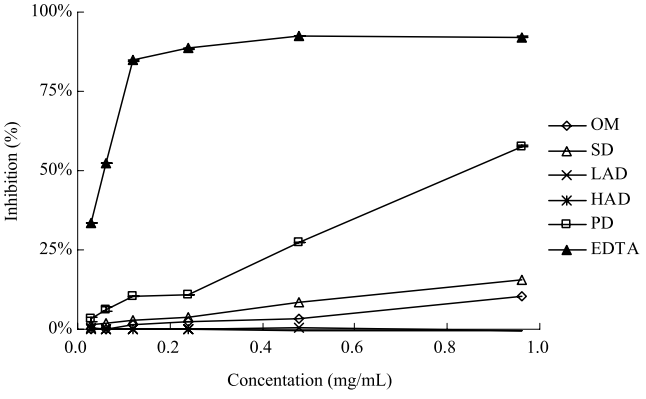

Figure 2. Chelating effect of carrageenan oligosaccharides (OM) and their oversulfated (SD), lowly acetylated (LAD), highly acetylated (HAD) and phosphorylated (PD) derivatives. Values are means ± SD of three determinations.

In our previous study, the phosphorylated derivative showed high inhibitory effect on hydroxyl radicals. Some workers report that hydroxyl radicals scavenging activity was not due to direct scavenging but inhibition of hydroxyl radical generation by chelating ions such as Fe^{2+} and Cu^{+}, and rendering them inactive or poorly active in a Fenton reaction.[37,38] Since the phosphorylated derivative was demonstrated to have a moderate iron binding capacity, it is likely that the chelating effect of the phosphorylated derivative on metal ions might be responsible for its hydroxyl radical scavenging activity due to its inhibition of hydroxyl radical generation by chelating ions.

The total antioxidant activity, which reflected the ability of the carrageenan oligosaccharides and their derivatives to inhibit the bleaching of β-carotene, was measured and compared with that of the control which contained no antioxidant component and is presented in Figure 3. It can be seen that carrageenan oligosaccharides and their different derivatives exhibited varying degrees of antioxidant activity. SD and PD were found to have antioxidant activities similar to that of BHA, while other samples showed no antioxidant activity compared to the control. However, SD showed high antioxidant activity at low concentration, and the activity decreased with the concentration increased (data not showed). But PD showed a similar total antioxidant activity in a dose-dependent manner at different time intervals (Fig. 4). The mechanism of bleaching of β-carotene is a free radical-mediated phenomenon resulting from the hydroperoxides formed from linoleic acid. β-Carotene, in this model system, undergoes rapid discoloration in the absence of an antioxidant. The linoleic acid free radical, formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsaturated β-carotene molecules. As β-carotene molecules lose their double bonds by oxidation, the compound loses its chromophore and characteristic orange color, which can be monitored spectrophotometrically.[26] The presence of the sulfated, especially phosphorylated derivatives can hinder the extent of β-carotene-bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system.

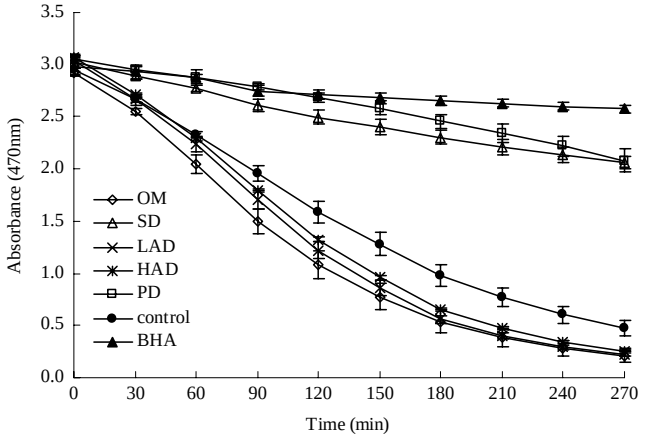

Figure 3. Total antioxidant activity of carrageenan oligosaccharides (OM) and their oversulfated (SD), lowly acetylated (LAD), highly acetylated (HAD), and phosphorylated (PD) derivatives and BHA at 10 μg/mL in β-carotene–linoleic system. Values are means ± SD of three determinations.

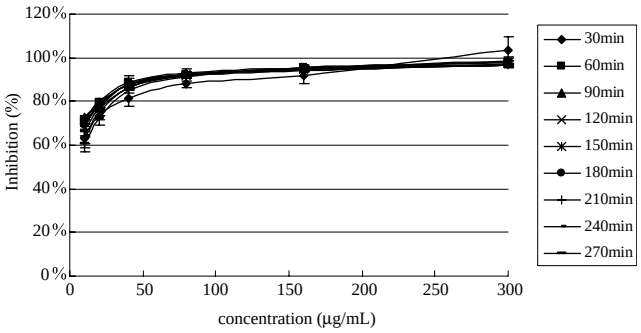

Figure 4. Total antioxidant activity of phosphorylated derivatives (PD) at different time intervals in β-carotene–linoleic system. Values are means ± SD of three determinations.

H_2O_2, an endogenous genotoxic risk factor, causes DNA damage by decomposing to the hydroxyl radical. This may result in DNA instability, mutagenesis, and ultimately carcinogenesis.[39] To test the effect of carrageenan oligosaccharides and their derivatives on the protection of thymic lymphocyte cells against H_2O_2-induced cell death, cells were exposed to H_2O_2 (10 mM) for 2 h or pretreated with various concentrations of carrageenan oligosaccharides and their derivatives (66.7, 182, 400, or 2000 μg/mL) for 2 h prior to H_2O_2 exposure. As seen in Figure 5, cell viability significantly decreased after 2 h of H_2O_2 exposure. As compared to H_2O_2 alone, cell viability significantly increased when pretreated with carrageenan oligosaccharides and their derivatives. However, they cannot increase the cell viability in dose-dependent manner, except the PD-treated group. Cell viability increased from 37.3 ± 1.1% in H_2O_2 exposed cells to 68.7 ± 2.3%, 71.5 ± 4.4%, 74.5 ± 3.4% and 74.7 ± 3.2%, respectively. Meanwhile, carrageenan oligosaccharides and their derivatives showed a similar protective effect against H_2O_2 induced oxidative damage, indicating that the chemical modification of carra-

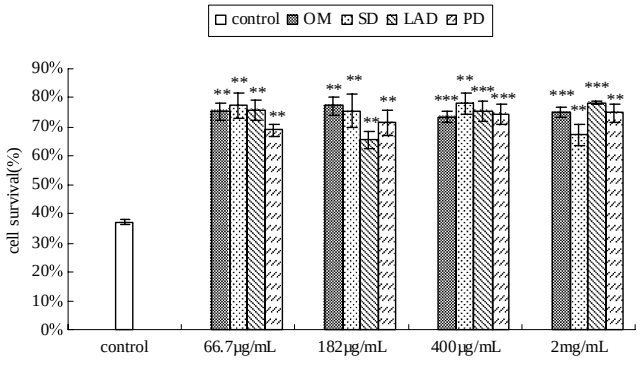

Figure 5. Effect of carrageenan oligosaccharides and their derivatives on cell viability after H_2O_2 exposure. Cells pretreated with carrageenan oligosaccharides and their derivatives for 2 h were exposed to 10 mM H_2O_2. Cell viability was determined 2 h after exposure. Each value represents the means ± SD obtained from four separate experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to H_2O_2 alone.

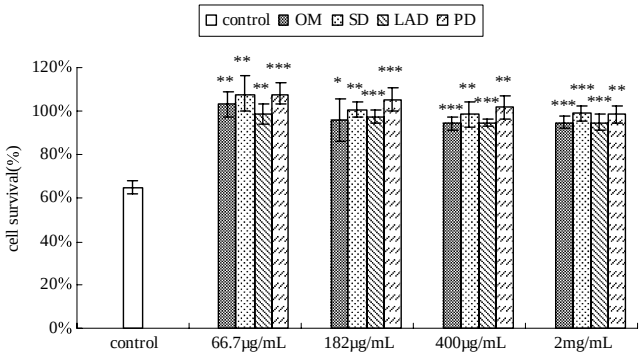

Figure 6. Effect of carrageenan oligosaccharides and their derivatives on cell viability after UVA exposure. Cells pretreated with carrageenan oligosaccharides and their derivatives for 2 h were exposed to 90 mJ/cm^2 UVA. Cell viability was determined 2 h after exposure. Each value represents the means ± SD obtained from four separate experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to UVA alone.

geenan oligosaccharides had no effect on their protective effect. Nevertheless, these results demonstrated that carrageenan oligosaccharides and their derivatives showed the potential scavenging effects on intracellular oxidative damage directly induced by H_2O_2, and the antioxidant activities were associated with the improvement of cell viability.

Exposure of mammalian cells to ultraviolet (UV) light induces various deleterious responses. Damage to cells by UVA is thought to involve reactive oxygen species, including singlet oxygen, the superoxide and hydroxyl radicals, and hydrogen peroxide. Some of the major harmful effects are DNA damage, systemic immune suppression, and accelerated aging.[40,41] As shown in Figure 6, when thymic lymphocytes were exposed to a dose of 90 mJ/cm^2 UVA, their viability was decreased by about 60%. However, the viability of lymphocytes was significantly increased compared with the control group when carrageenan oligosaccharides and their derivatives were preadministered to cells before UVA radiation. This phenomenon showed that carrageenan oligosaccharides and their derivatives can protect the lymphocytes against UVA injury. As like a H_2O_2 induced oxidative stress model, the chemical modification of carrageenan oligosaccharides also had no significant effect on their protective effect. SD and PD showed a little higher protective effect than carrageenan oligosaccharides, while LAD showed a similar protective effect on lymphocytes.

The different derivatives of carrageenan oligosaccharides exhibited higher antioxidant activity than the carrageenan oligosaccharides in certain antioxidant systems in vitro, which indicated that the chemical modification of carrageenan oligosaccharides could enhance their antioxidant activity. There are few reports on the structure–antioxidant activity relationship of saccharides. Tsipali et al. investigated the free radical scavenging activity of glucan and nonglucan polymers. They observed that phosphated and sulfate glucan exhibited antioxidant ability that was greater than that of glucan and other neutral polysaccharides, which indicated that polyelectrolytes, such as glucan sulfate or phosphate, might have increased scavenging activity.[42] This report revealed that the sulfate and phosphorylated groups in a polysaccharide led to differences in their biological activities. In our opinion, the antioxidant activity may have originated from their hydrogen atom donating capacity. The sulfate, phosphate, and acetyl groups, which substituted in C-2 of the galactose (G4S) in carrageenan oligosaccharide, could activate the hydrogen atom of the anomeric carbon. The higher activated capacity of the group, the stronger hydrogen atom-donating capacity the derivatives have. The antioxidant activity of the derivatives in different systems is different but generally the antioxidant activity of the derivatives is in the order PD > SD > LAD, HAD which indicates the activated capacity of the substituted group. Meanwhile, carrageenan oligosaccharides and their derivatives showed effective protection against H_2O_2 and UVA induced oxidative damage on rat lymphocytes. But the chemical modification of carrageenan oligosaccharides had no effect on their protective effect. A major component of the antioxidant system in mammalian cells consists of the three enzymes, namely, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). These enzymes work in concert to detoxify ROS such as $^{\bullet}O_2^-$ and H_2O_2 in cells. Higher levels of the antioxidant enzymes have been correlated with decreased susceptibility to cell damage. Mou et al. found that the oral administration of κ-carrageenan oligosaccharides was advantageous to promote the activities of SOD and CAT in mouse.[43] The cytoprotective effect of κ-carrageenan oligosaccharides and their different derivatives may be due to the improvement in the reduced activities of antioxidant enzymes. Further investigation needs to be conducted including on intracellular ROS, lactate dehydrogenase, and the activities of antioxidant enzymes involved in the antioxidant mechanism to overall assess their cytoprotective effect.

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