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In Vitro Antioxidant Activities of Low-Molecular-Weight Polysaccharides with Various Functional Groups

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The objectives of this study were to evaluate the effect of different functional groups of sulfate, amine, and hydroxyl and/or their ionized groups on *in vitro* antioxidant capacities of low-molecular-weight polysaccharides (LMPS) prepared from agar (LMAG), chitosan (LMCH), and starch (LMST), respectively, and to elucidate their structure−activity relationship. Ascorbic acid and ethylenediaminetetraacetic acid (EDTA) were used as positive controls. The *in vitro* antioxidant capacities of LMAG and LMCH were higher than that of LMST in the DPPH radical, superoxide radical, hydrogen peroxide, and nitric oxide radical scavenging and ferrous metal-chelating capacities. The different scavenging capacities may be due to the combined effects of the different sizes of the electron-cloud density and the different accessibility between free radical and LMPS, which, in turn, depends upon the different hydrophobicities of the constituent sugars.

KEYWORDS: Antioxidant; free-radical scavenging; agar; chitosan; starch

INTRODUCTION

Oxidative stress, induced by free radicals, is believed to be a primary factor in various degenerative diseases, such as atherosclerosis, inflammation, carcinogenesis, Alzheimer's disease, and aging (*1, 2*). Free radicals can be divided into reactive oxygen species (ROS), such as superoxide anion ($\bullet O_2^-$), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2), and reactive nitrogen species (RNS), such as nitric oxide (NO•) and peroxynitrate ($\bullet ONOO^-$). These reactive species are generated from normal metabolic processes or produced after stimulation from exogenous factors and/or agents, such as ultraviolet light, ionizing radiation, and chemical reactions (*3*). They initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. These reactive species are capable of damaging a wide range of essential biomolecules (*2*). Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates. They exert their effects by scavenging free radicals, activating a battery of detoxifying proteins, or preventing the generation of free radicals.

In recent years, polysaccharides have been demonstrated to scavenge free radicals *in vitro* and to be used as antioxidants for the prevention of oxidative damage in foods (*4*) and living organisms. The antioxidant activity of polysaccharides depends upon several structural parameters, such as the molecular weight (*5, 6*), type and position of functional groups, such as hydroxyl (*7*), sulfate (*8, 9*), amine (*7*), carboxyl (*1*), and phosphate (*8, 9*), type of saccharide, and glycosidic branching (*10*).

Chitosan, a cationic polysaccharide, is made up of D-glucosamine and *N*-acetylglucosamine linked through β-1,4 glycosidic linkages. It is produced by the deacetylation of chitin obtained from crab and shrimp shells. Chitosan has several reactive groups, such as −OH and $-NH_2$, which can react with many compounds (*11*). Recently, the antioxidant activity of chitosan has attracted much attention. Xue et al. (*12*) showed that the oligosaccharide of chitosan inhibited lipid peroxidation of phosphatidylcholine liposomes *in vitro*. Je et al. (*5*) showed that medium molecular weight (5−1 kDa) of 90% deacetylated chitooligosaccharide scavenged DPPH, hydroxyl, superoxide, and carbon-centered radicals stronger than that of higher molecular-weight (10−5 kDa) ones or lower molecular-weight (below 1 kDa) ones. With regard to the effect of the degree of deacetylation (DD), the 90% deacetylated ones were higher than those that were 75 or 50% deacetylated. The chitooligosaccharides quench various radicals by action of nitrogen on the C-2 position of the chitooligosaccharide. The free-radical-scavenging activity of chitosan was dependent upon their degree of deacetylation and molecular weights. Kim and Thomas (*4*) showed that chitosan inhibited lipid peroxidation of salmon during a storage period, and the 30 kDa chitosan showed the highest scavenging DPPH activity compared to either 90 or 120 kDa chitosan. Ji et al. (*11*) reported that the antioxidant activity

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of the 1,3,5-thiadiazine-2-thione derivatives of chitosan oligomers was stronger than that of chitosan oligomers and the antioxidant activity of chitosan oligomers (2.4 and 3.5 kDa) was stronger than chitosan (700 kDa).

Agar extracted from red seaweeds is composed of agarose and agaropectin. Agarose, a neutral polysaccharide, consists of β-D-galactose and 3,6-anhydro-α-L-galactose. Agaropectin, a negative-charged sulfated polysaccharide, consists of β-1,3-glycosidically linked D-galactose units, some of which are sulfated at the C-6 position. Wang et al. (*6*) reported that the fragment of agar oligosaccharide with the sulfate group showed higher antioxidative activity than that without the sulfate group and the oligosaccharide with a high molecular mass (3.8−2.0 kDa) had higher antioxidation activity than that with a low molecular mass. The sulfate content had a very important effect on the activity of polysaccharides. Wu et al. (*13*) showed that algal oligosaccharides, prepared by degradation of commercial agar with agarase, showed DPPH and hydrogen peroxide scavenging, ferrous ion chelation, and lipid peroxidation inhibition. The antioxidant activities of agar were related to molecular weight and sulfate groups (*6*). The molecular weights of saccharides also influence their antioxidant activities. Yuan et al. (*9*) show that inhibition of DPPH radicals of κ-carrageenan oligosaccharide (1.2 kDa) was stronger than that of κ-carrageenan polysaccharide (37.7 kDa). Xing et al. (*14*) reported that antioxidant activities of chitosan were increased by decreasing the molecular weight (760 kDa to 9 kDa). This may be due to the fact that low-molecular-weight chitosans have a noncompact structure and more free hydroxyl and amine groups that could react with free radicals (*11*). This might be the reason that the antioxidant activities of low-molecular-weight polysaccharides (LMPS) were stronger than those of high-molecular-weight saccharides.

However, the antioxidant mechanisms of carbohydrates are not yet fully understood. Tsiapali et al. (*8*) reported that phosphorylated and sulfated glucan exhibited higher antioxidant ability than glucan without any functional groups. Yuan et al. (*9*) showed that inhibition of DPPH radicals by κ-carrageenan oligosaccharide phosphorylated and oversulfated derivatives were stronger than κ-carrageenan oligosaccharide. The above reports showed that factors such as molecular weight and functional groups on the backbone of the polymer affect their activity; however, the real mechanisms are still not revealed.

The purposes of this study were to evaluate the effect of different functional groups of sulfate, amine, and hydroxyl and/or their ionized groups on *in vitro* antioxidant activities of LMPS prepared from agar, chitosan, and starch, respectively, and an electron-transfer (ET) mechanism was used to explain different antioxidation activities to elucidate the structure−activity relationship.

MATERIALS AND METHODS

Polysaccharide Materials. Agar was purchased from Laboratorios, S. A. (Madrid, Spain). Chitosan (MW, 500 kDa; DD, 90%) was obtained from Lytone Enterprise, Inc. (Taipei, Taiwan). Starch was purchased from Sigma-Aldrich Co. (St Louis, MO).

Preparation of LMPS. Agar and starch were hydrolyzed in 0.1 N HCl (10 g/1 L) with stirring at 60 °C for 4 h. After acidic hydrolysis, the reaction was terminated by neutralization with 0.1 M NaOH in an ice−water bath. The hydrolysates were obtained by centrifugation at 6000*g* for 15 min and filtered to remove insoluble particles. Chitosan was suspended in 10% hydrogen peroxide (10 g/100 mL) at 60 °C with stirring for 4 h. The mixture was centrifuged at 6000*g* for 15 min and filtered to remove insoluble particles. The supernatant was desalted by dialyzing against distilled water using 1000 Da MW cutoff dialysis membranes (Membrane Filtration Products, Inc., Seguin, TX) and then filtered by 5000 Da MW ultrafilter membranes (Millipore Co., Billerica, MA). The filtrate was concentrated by rotary evaporation and lyophilized to give samples of LMPS.

Molecular-Weight (MW) Determination. The MWs of hydrolysates were determined by size-exclusion high-performance liquid chromatography (SE-HPLC) (*15*) using a column packed with TSK gel G4000 PW_{XL} and G5000 PW_{XL} (Tosoh Co. Ltd., Japan). The elution peak was detected with a model M132 RI detector (Gilson, Middleton, WI). The molecular weights of the samples were calculated from the pullulan standards (Shodex, Kawasaki, Japan) calibration curve with SISC-LAB software (Scientific Information Service Co., Taipei, Taiwan).

DD Measurement. Infrared spectrometry was used to determine the DD of the chitosans (*16*). LMCH powder was mixed with KBr (1:100) and pressed into a pellet. The absorbances of amide 1 (1655 cm^{-1}) and the hydroxyl band (3450 cm^{-1}) were measured using a Bio-Rad FTS-155 infrared spectrophotometer. The band of the hydroxyl group at 3450 cm^{-1} was used as an internal standard to correct for disk thickness and differences in chitosan concentration in making the KBr disk. The acetylation percentage of the amine group in a sample is given by $(A_{1655}/A_{3450}) \times 115$, where A_{1655} and A_{3450} are the absorbances at 1650 and 3450 cm^{-1}, respectively.

Sulfate Content Measurement. The sulfate content was determined by the rhodizonate method (*17*) with a sulfate standard. A total of 0.5 mL of 100 μg/mL LMAG aqueous solution was mixed with 0.5 mL of 1 N HCl then heated at 100 °C for 1 h. The mixture was dried by evaporation at 65 °C, and then 0.5 mL of distilled water was added to prepare hydrolyzed LMPS solution. A total of 0.5 mL of hydrolyzed LMPS solution was mixed with 2 mL of ethanol (95%), 1 mL of $BaCl_2$ solution (10 mL of 2 M acetic acid, 0.2 mL of 0.05 M $BaCl_2$, and 0.8 mL of 0.02 M $NaHCO_3$, diluted to 100 mL with 95% ethanol), and 1.5 mL of sodium rhodizonate reagent (1 mg of sodium rhodizonate and 10 mg of ascorbate dissolved in 20 mL of deionized water and then diluted to 100 mL with 95% ethanol). The mixture was shaken and kept at room temperature in the dark. After 20 min, the absorbance was measured at 520 nm by a spectrophotometer.

***In Vitro* Antioxidant Activity Assays.** *α,α-Diphenyl-β-picryhydrazyl (DPPH) Radical-Scavenging Assay.* The DPPH radical-scavenging activity of LMPS was assayed by the method of Espin et al. (*18*), with slight modification. A total of 1 mL of sample solution in distilled water at different concentrations was mixed with 4 mL of methanolic DPPH solution (100 μM). Distilled water was used as a control, and ascorbic acid was used for comparison. The mixture was shaken and kept at room temperature in the dark. After 60 min, the absorbance was measured at 517 nm by a spectrophotometer. The DPPH radical-scavenging activity (%) was calculated by the following equation:

$$\text{scavenging activity (\%)} = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100$$

where A_{control} is the absorbance of the control and A_{sample} is the absorbance of samples.

Superoxide Radical-Scavenging Assay. The superoxide radical-scavenging activity of LMPS was assayed by the method of Liu and Ng (*19*), with slight modification. The superoxide radical was generated by the PMS−NADH system (phenazine methosulfate and reduced nicotinamide adenine dinucleotide) and assayed by the reduction of NBT. A total of 1 mL of sample solution in distilled water at different concentrations was added in sequence to 1 mL of 300 μM nitroblue tetrazolium, 1 mL of 936 μM NADH, and 1 mL of 120 μM PMS in 100 mM phosphate buffer (pH 7.4). Distilled water was used as a control, and ascorbic acid was used for comparison. The mixture was incubated at room temperature for 5 min in the dark. The absorbance was measured at 560 nm by a spectrophotometer. The superoxide radical-scavenging activity (%) was calculated as follows:

$$\text{scavenging activity (\%)} = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100$$

where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of samples.

Hydrogen Peroxide Scavenging Assay. The hydrogen peroxide (H_2O_2) scavenging activity of LMPS was assayed by the method of Yen and Chung (*20*), with slight modification. A total of 1 mL of sample solution in distilled water at different concentrations was mixed with 400 μL of H_2O_2 solution (5 mM) and incubated for 20 min at room temperature. Distilled water was used as a control, and ascorbic acid was used for comparison. The mixture was supplemented with 600 μL of HRPase−phenol red solution (300 μg/mL HRPase and 4.5 mM phenol red in 100 mM phosphate buffer). After 10 min, the absorbance was measured at 610 nm by a spectrophotometer. The hydrogen peroxide scavenging activity (%) was calculated as follows:

$$\text{scavenging activity (\%)} = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100$$

where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of samples.

Nitric Oxide Radical-Scavenging Assay. The nitric oxide (NO) radical-scavenging activity of LMPS was assayed by the method of Sumanont et al. (*21*), with slight modification. The nitric oxide radical was generated by the sodium nitriprusside (SNP) and assayed by the Griess reagent. A total of 950 μL of sample solution in distilled water at different concentrations was mixed with 50 μL of 100 mM SNP in 100 mM phosphate buffer (pH 7.4). Distilled water was used as a control, and ascorbic acid was used for comparison. The mixture was incubated at 20 °C for 2.5 h. The reaction mixture was diluted with 2 mL of Griess reagent [1 mL of 1.2 M sulfanilamide and 1 mL of 80 mM *N*-(1-naphthyl)-ethylenediamine dihydrochloride (NED)] at room temperature for 10 min. The absorbance was measured at 540 nm by a spectrophotometer. The NO radical-scavenging activity (%) was calculated as follows:

$$\text{scavenging activity (\%)} = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100$$

where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of samples.

Ferrous Metal-Ion-Chelating Ability. The chelating of ferrous ions by LMPS was assayed by the method of Dinis et al. (*22*), with slight modification. A total of 1 mL of sample solution in distilled water at different concentrations was mixed with 0.1 mL of $FeCl_2$ (0.2 mM) for 30 s, and then the mixture was reacted with 0.2 mL of ferrozine (5 mM) for 10 min. Distilled water was used as a control, and EDTA was used for comparison.. The absorbance of the solution was measured at 562 nm by a spectrophotometer. The ferrous ion-chelating ability (%) was calculated as follows:

$$\text{chelating ability (\%)} = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100$$

where $A_{control}$ is the absorbance of control and A_{sample} is the absorbance in the presence of the sample.

Statistical Analysis. All antioxidative results are expressed as mean ± standard deviation (SD) ($n = 6$). Data were analyzed by a one-way analysis of variance (ANOVA). When the ANOVA identified differences among groups, multiple comparisons among means were made using Duncan's new multiple range test. Statistical significance was determined by setting the aggregate type I error at 5% ($p < 0.05$), for each set of comparisons, using Statistical Analysis System software.

RESULTS AND DISCUSSION

The molecular weights of LMAG, LMCH, and LMST were 3573, 3767, and 3643 Da, respectively. The DD of LMCH was 89.6 ± 7.1%. The sulfate content of LMAG was 11.8 ± 0.3%.

The DPPH scavenging capacities of LMAGs, LMCHs, LMSTs, and ascorbic acid are depicted in **Figure 1**. LMAGs and LMCHs had a dose-dependent scavenging activity and displayed 83% and 71% scavenging activity at 8.0 mg/mL, whereas the DPPH scavenging capacity of ascorbic acid displayed over 97% at 0.2−2.0 mg/mL and higher than that of LMAG and LMCH by 16% and 28% at 8.0 mg/mL, respectively. However, LMSTs scarcely had any scavenging activity on the DPPH radical between 0.2 and 8.0 mg/mL.

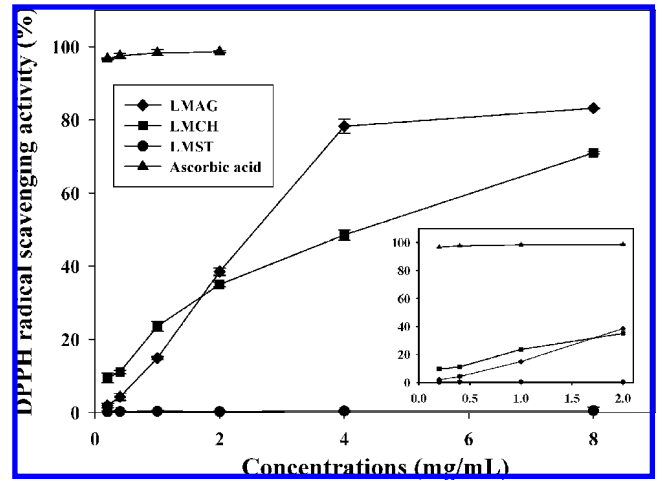

Figure 1. Scavenging activity of LMAG, LMCH, LMST, and ascorbic acid on DPPH radicals. Values are means ± SD of six determinations.

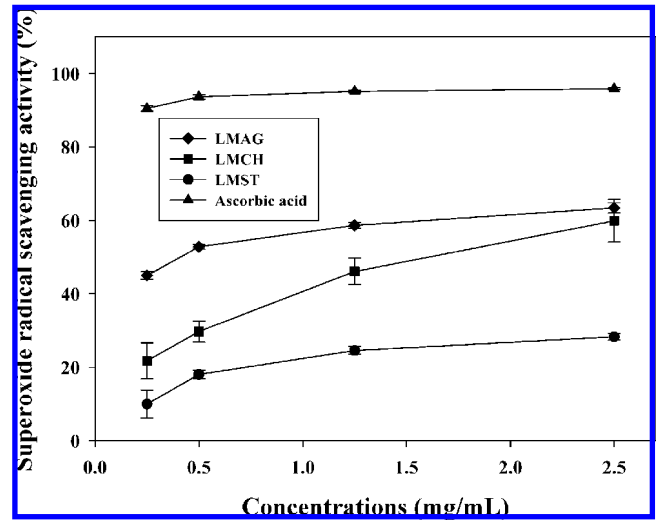

Figure 2. Scavenging activity of LMAG, LMCH, LMST, and ascorbic acid on superoxide radicals. Values are means ± SD of six determinations.

The superoxide radical-scavenging capacities of LMAGs, LMCHs, LMSTs, and ascorbic acid are depicted in **Figure 2**. The more pronounced dose-dependent scavenging activities of LMCHs were observed compared to those of LMAGs and LMSTs, with the activities of 63%, 60%, and 28% at 2.5 mg/mL for LMAGs, LMCHs, and LMSTs, respectively. The scavenging activities of LMAGs and LMCHs were significantly higher compared to that of LMSTs (all below 30%). The superoxide radical-scavenging capacity of ascorbic acid displayed over 90% at 0.25−2.5 mg/mL and higher than that of LMAG, LMCH, and LMST by 32%, 36%, and 67% at 2.5 mg/mL, respectively.

The dose-dependent hydrogen peroxide scavenging capacities of LMAGs, LMCHs, and LMSTs are depicted in **Figure 3**. The scavenging activities on hydrogen peroxide were 46%, 91%, and 10% at 5.0 mg/mL, respectively. The hydrogen peroxide scavenging capacity of ascorbic acid displayed over 92% at 0.5−5.0 mg/mL and higher than that of LMAG, LMCH, and LMST by 49%, 3%, and 85% at 5.0 mg/mL, respectively.

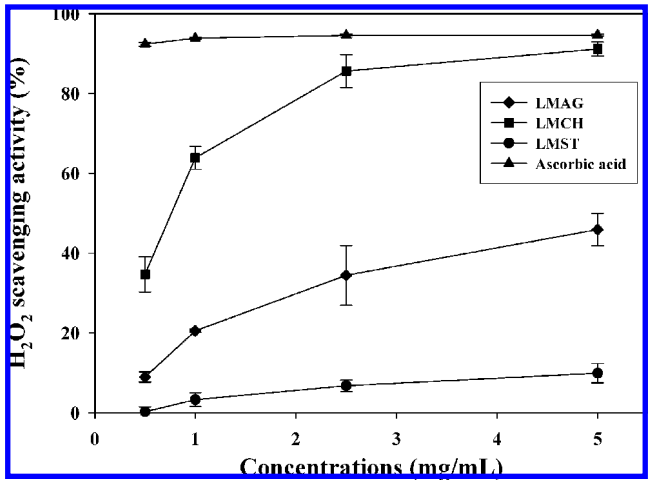

Figure 3. Scavenging activity of LMAG, LMCH, LMST, and ascorbic acid on hydrogen peroxide. Values are means ± SD of six determinations.

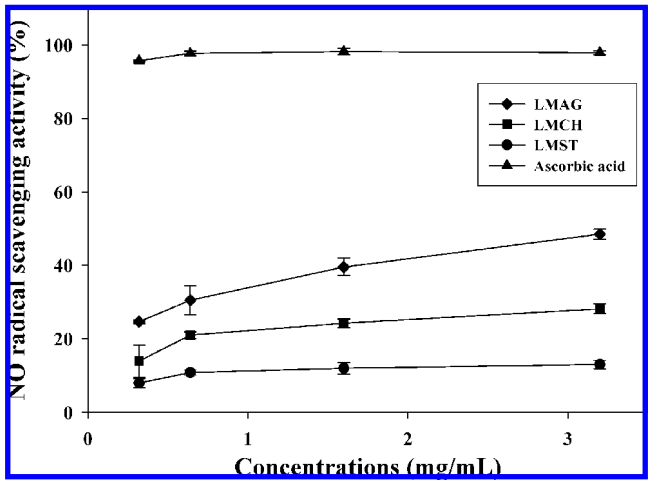

Figure 4. Scavenging activity of LMAG, LMCH, LMST, and ascorbic acid on nitric oxide radicals. Values are means ± SD of six determinations.

LMCHs exhibited significantly higher scavenging capacities than that of LMAGs and LMSTs and were close to ascorbic acid at 5 mg/mL.

The dose-dependent nitric oxide radical-scavenging capacities of LMAGs were more pronounced than those of LMCHs and LMSTs (**Figure 4**). The scavenging activities were 49%, 28%, and 13% at 3.2 mg/mL, respectively. The nitric oxide radical-scavenging capacity of ascorbic acid displayed over 95% at 0.3−3.2 mg/mL and higher than that of LMAG, LMCH, and LMST by 49%, 70%, and 85% at 3.2 mg/mL, respectively.

The scavenging capacity of ascorbic acid on DPPH (**Figure 1**), superoxide (**Figure 2**), H_2O_2 (**Figure 3**), and nitric oxide radicals (**Figure 4**) were over 90% at all concentrations and significantly higher than all of the LMPS. Dziezak (*23*) reported that ascorbic acid is a reducing agent in accordance with the antioxidation mechanism. The antioxidant activity of ascorbic acid relies on donating its electrons (*24*). Ascorbic acid can donate two electrons from a double bond between the positions C-2 and C-3 of the 6-carbon molecule. The molecular weight of ascorbic acid (MW = 176 Da) is smaller than LMAGs and LMCHs and, thus, will act with radicals in solution faster than that of LMAGs and LMCHs, therefore rendering ascorbic acid to quench ROS and RNS more efficiency than LMAGs and LMCHs.

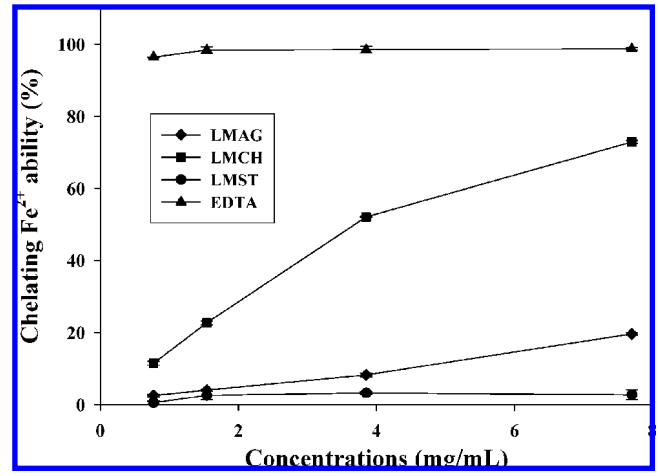

Figure 5. Chelating Fe^{2+} ability of LMAG, LMCH, LMST, and EDTA. Values are means ± SD of six determinations.

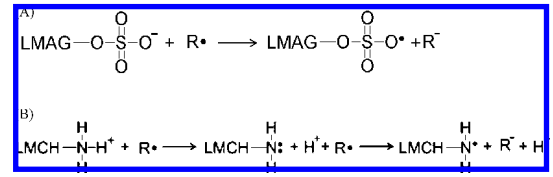

Figure 6. Probable mechanism of ET between (A) LMAG and the free radical (R•) and (B) LMCH and the free radical to form the stable LMPS radicals.

Wu et al. (*13*) showed that, from scavenging activities of the agar oligosaccharide and monosaccharide mixtures with a polymerization degree from 2 to 6, galactose and 3,6-anhydro-galactose algal oligosaccharide prepared from commercial agar by agarase on DPPH radical and H_2O_2 were 27.7%−10.4% and 26%−7.8% at 1 mg/mL, respectively. The DPPH (**Figure 1**) and H_2O_2 (**Figure 2**) scavenging capacities of LMAG in this study are similar to that of Wu et al. (*13*). The scavenging activity of LMAG on DPPH, superoxide, H_2O_2, and nitric oxide radical may be due to the negative charge of sulfate groups (*15, 19*).

Je et al. (*5*) showed that 90% deacetylated heterochitooligosaccharides (1−5 kDa) scavenged DPPH radicals by 55% and superoxide radicals by 60%, respectively, at concentrations of 2.5 and 0.5 mg/mL. Wang et al. (*1*) showed that scavenging activities of chitosan oligosaccharide (4776 Da) on superoxide radical and H_2O_2 were 41.2% and 80.2% at concentrations of 1.28 and 6.4 mg/mL, respectively. The scavenging capacities of LMCHs used in this study (**Figures 1** and **2**) are similar to those of Je et al. (*5*) and Wang et al. (*1*). This may be due to the molecular weight of LMCHs used in this study being controlled at 1−5 kDa, close to that reported in the previous studies (*1*, *5*). Xie et al. (*7*) reported that chitosan derivatives could scavenge hydroxyl radicals via three mechanisms: the hydroxyl groups in the polysaccharide unit can react with •OH by the typical hydrogen-abstraction reaction; •OH can react with the amine groups ($-NH_2$) to form stable macromolecular radicals; and the amine groups can form ammonium groups ($-NH_3^+$) by absorbing hydrogen ions from the solution and then reacting with •OH through an addition reaction. However, we believe that the antioxidative capacities of LMCH may be due to scavenging free radicals by ET to form stable macromolecular radicals through the amine groups (**Figure 6**). This may be attributed to the free radical accepting an electron from the lone-pair electron of sulfate or amine groups to form a stable free-radical ion, e.g., the ET from the sulfate group of LMAG to the free radical (R•) to form a stable free-radical ion (**Figure**

6A), in the same way as the ET from the amine group of LMCH to the free radical (R•) to form a stable free-radical ion (**Figure 6B**). The LMPS lose an electron from sulfate or amine groups to form stable LMPS radicals.

The antioxidation capacities of LMSTs are weak (**Figures 1**−**4**). Although Tsiapali et al. (*8*) reported that the antioxidant activity of polysaccharides may be due to abstraction of the anomeric hydrogen from one of the internal glucose units. Xie et al. (*7*) reported that the hydroxyl group in the polysaccharide unit can react with •OH by the typical hydrogen-abstraction reaction to scavenge the free radical. Kishk and Al-Sayed (*25*) reported that the radical-scavenging mechanism of polysaccharides was similar to that of phenolic compounds by the hydrogen-abstraction mechanism to form the semiquinones. However, the scavenging capacities of LMSTs on DPPH, superoxide, nitric oxide radicals, and H_2O_2 were very low. This may be due to the radical-scavenging activity of phenolic compounds being a result of hydrogen donation and subsequent radical stabilization by the conjugated double-bond system (*26*). However, the internal pyranose ring of starch has no conjugated double bond to donate a hydrogen atom and, subsequently, stabilize the radical. This may be the reason that LMSTs have little antioxidation activity.

The DPPH assay is believed to involve a hydrogen atom transfer reaction; however, Foti et al. (*27*) suggested that the reaction in fact behaves like an ET reaction. Therefore, the ET mechanism is used to explain why LMPS with sulfate groups, amine groups, and hydroxyl groups showed different antioxidation activities. Results in **Figure 1** showed that, over 2.0 mg/mL, the DPPH radical-scavenging capacities of LMAGs were significantly greater than those of LMCHs and LMSTs; however, below 1.5 mg/mL, LMCHs were significantly greater than those of LMAGs and LMSTs. The DPPH radical is stabilized by accepting an electron (*25*). The scavenging capacity of DPPH will depend upon the capacity of LMCH or LMAG to donate an electron to the DPPH unpaired electron to form a DPPH ion (**Figure 6**), which in turn depends upon the combined effects of the different sizes of the electron-cloud density and different accessibility between DPPH and LMPS. The size of the electron-cloud density of a sulfate group is larger than that of the amine group; however, the 3,6-anhydro-galactose residue of LMAGs is more hydrophobic than that of glucosamine and *N*-acetyl-glucosamine. The electron-cloud density of a functional group affects its electron-donating activity. High electron-cloud density increases the electron-donating activity, and this increases the scavenging effect on free radicals and the reducing power (*28*). The hydrophobic residue of 3,6-anhydro-galactose may interfere with the accessibility between DPPH and LMAGs. In contrast, the structure of LMCHs facilitated the accessibility between DPPH and LMCHs. This may be the reason that the scavenging DPPH capacities of LMCHs were higher than LMAGs below 1.5 mg/mL (**Figure 1**); however, over 2 mg/mL, the effect of different sizes of electron-cloud density overwhelms the effect of different accessibility between DPPH and LMAGs. Therefore, the DPPH scavenging capacity of LMAGs is higher than that of LMCHs.

The reasons that LMAGs showed higher superoxide radical-scavenging activity could be attributed to results of Xing et al. (*14*). They reported that the superoxide radical-scavenging activity of the chitosan sulfate was stronger than those of chitosans. Their results indicated that the scavenging activity of sulfate groups on the superoxide radical were stronger than amine groups. At the lower concentration of 1.25 mg/mL, results are consistent with Xing et al. (*14*) (**Figure 2**), but at the higher concentration of 2.5 mg/mL, the combined effects of different sizes of electron-cloud density and different accessibility between superoxide radicals and LMPS were mentioned previously by ET. In addition, the different slopes of superoxide radical-scavenging activity between LMAG and LMCH reached a plateau between 0.5 and 1.25 mg/mL by LMAG versus the dose dependent between 0.5 and 2.5 mg/mL by LMCH. These features resulted in a similar scavenging effect on superoxide by LMAGs and LMCHs at 2.5 mg/mL. Weak scavenging activity of LMSTs on superoxide radicals may be due to LMSTs containing only hydroxyl groups and a pyranose ring without a conjugated double bond on the molecular structure; therefore, the ability of electron delocalization is weak for scavenging superoxide radicals.

Figure 5 shows the dose-dependent ferrous ion-chelating abilities of LMAGs and LMCHs. However, LMSTs do not show ferrous ion-chelating ability. The chelating abilities of LMAGs and LMCHs are 20% and 73%, respectively, at a concentration of 7.7 mg/mL, respectively. The ferrous ion-chelating capacity of EDTA displayed over 96% at 0.7−7.7 mg/mL and higher than that of LMAG, LMCH, and LMST by 79%, 26%, and 96% at 2.5 mg/mL, respectively. EDTA had a significantly higher chelating ability to all of the LMPS. This may be due to EDTA complexing with pro-oxidative metal ions, such as iron (Fe^{2+}) and copper (Cu^{2+}) (*23*). There are six unshared pairs of electrons from two nitrogen atoms and four negatively charged oxygen atoms in EDTA, creating a hexadentate ligand to complex with metal ions. This may be the reason that Fe^{2+}-chelating capacities of EDTA were significantly greater than those of LMPS. LMCHs chelate Fe^{2+} by the amine groups and hydroxyl groups at positions C-2 and C-3, respectively, to form the chelating ligand. This creates the excellent chelating capacities of chitosan with heavy metals (*29*). LMAGs chelate Fe^{2+} by electrostatic interactions between sulfate groups at position C-6 and metal ions (*30*). Wang et al. (*1*) showed that the ferrous ion-chelating ability of their chitosan oligosaccharides is 57.6% at the concentration of 12.8 mg/mL. Our results (**Figure 5**) were significantly higher than those of Wang et al. (*1*). The hydroxyl groups of the starch have no charged group to chelate the metal ions.

ABBREVIATIONS USED

DD, degree of deacetylation; DPPH, α,α-diphenyl-β-picryhydrazyl radical; HRPase, horseradish peroxidase; LMPS, low-molecular-weight polysaccharides; LMAG(s), low-molecular-weight agar; LMCH(s), low-molecular-weight chitosan; LMST(s), low-molecular-weight starch; MW, molecular weight; NADH, reduced nicotinamide adenine dinucleotide; NBT, nitro blue tetrazolium; NED, *N*-(1-naphthyl)-ethylenediamine dihydrochloride; NO•, nitric oxide radical; $•O_2^-$, superoxide anion; •OH, hydroxyl radical; $•ONOO^-$, peroxynitrate radical; PMS, phenazine methosulfate; R•, free radical; ROS, reactive oxygen species; RNS, reactive nitrogen species; SNP, sodium nitriprusside.

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