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Relevance of molecular weight of chitosan and its derivatives and their antioxidant activities in vitro

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Abstract—The antioxidant potency of different molecular weight (DMW) chitosan and sulfated chitosan derivatives was investigated employing various established in vitro systems, such as superoxide ($O_2^{\bullet-}$)/hydroxyl ($^{\bullet}OH$) radicals scavenging, reducing power, iron ion chelating. As expected, we obtained several satisfying results, as follows:

Firstly, low molecular weight chitosan had stronger scavenging effect on $O_2^{\bullet-}$ and $^{\bullet}OH$ than high molecular weight chitosan. For example the $O_2^{\bullet-}$ scavenging activity of low molecular weight chitosan (9 kDa) and high molecular weight chitosan (760 kDa) were 85.86% and 35.50% at 1.6 mg/mL, respectively. Secondly, comparing with DMW chitosan, DMW sulfated chitosans had the stronger inhibition effect on $O_2^{\bullet-}$. At 0.05 mg/mL, the scavenging activity on $O_2^{\bullet-}$ reached 86.26% for low molecular weight chitosan sulfate (9 kDa), but that of low molecular weight chitosan (9 kDa) was 85.86% at 1.6 mg/mL. As concerning chitosan and sulfated chitosan of the same molecular weight, scavenging activities of sulfated chitosan on superoxide and hydroxyl radicals were more pronounced than that of chitosan. Thirdly, low molecular weight chitosan sulfate had more effective scavenging activity on $O_2^{\bullet-}$ and $^{\bullet}OH$ than that of high molecular weight chitosan sulfate. Fourthly, DMW chitosans and sulfated chitosans were efficient in the reducing power, especially LCTS. Their orders were found to be LCTS > CTS4 > HCTS > CTS3 > CTS2 > CTS1 > CTS. Fifthly, CTS4 showed more considerable ferrous ion-chelating potency than others. Finally, the scavenging rate and reducing power of DMW chitosan and sulfated derivatives increased with their increasing concentration. Moreover, change of DMW sulfated chitosans was the most pronounced within the experimental concentration. However, chelating effect of DMW chitosans were not concentration dependent except for CTS4 and CTS1.

1. Introduction

In recent years, there has been increasing interest in finding natural antioxidants, since they can protect the human body from free radicals and retard the progress of many chronic diseases.[1] In general, the natural antioxidants mainly constitute a broad range of compounds including phenolic compounds, nitrogen compounds and carotenoids.[2] 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. Marine plants and invertebrates characteristically contain sulfated polysaccharides which are not found in land plants and which may have specific functions in ionic regulation. Xue et al.[3] reported several marine polysaccharides such as alginate, alginate sulfate, propylene gucolaginate sodium sulfate, *N*,*O*-carboxymethyl chitosan and hydroxypropylated chitosan inhibited the oxidation of phosphatidylcholine-liposomal initiated by addition of APPH. Xie et al.[4] reported that water-soluble chitosan derivatives prepared by graft copolymerization of maleic acid sodium onto

Abbreviations: HCTS, high molecular weight sulfated chitosan of $C_{2,3,6}$ sulfation; LCTS, low molecular weight sulfated chitosan of $C_{2,3,6}$ sulfation; DMW, different molecular weight; CTS–CTS4, different molecular weight chitosan; NBT, nitro blue tetrazolium; PMS, phenazine methosulfate; H_2O_2, hydrogen peroxide; TBA, thiobarbituric acid; EDTA, ethylene diamine tetra-acetic acid; NADH, nicotinamide adenine dinucleotide-reduced; TCA, trichloroacetic acid; DR, deoxyribose.

Keywords: DMW chitosans; DMW sulfated chitosans; Radical scavenging effect; Reducing power; Chelating effect; Comparative relevance.

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hydroxypropyl chitosan and carboxymethyl chitosan sodium, showed radical scavenging activity against hydroxyl radical. Matsugo et al.[5] reported that three different water-soluble chitosan derivatives obtained by the acylation of chitosan inhibited thiobarburic acid reactive substance of formation in *t*-butylhydroperoxide and benzoyl peroxide induced lipid peroxidations. Park et al.[6] studied chitosan with relatively higher degree of deacetylation among three kinds of partially deacetylated hetero-chitosans showed the higher radical scavenging activity on DPPH, hydroxyl, carbon-centred and superoxide radicals. Je et al.[7] investigated scavenging activities of nine kinds of molecular weight chitosans to DPPH, hydroxyl, superoxide and carbon-centred radicals using electron spin resonance (ESR) spin-trapping technique and revealed that 90-MMWCOS, which was having relatively medium molecular weight prepared from 90% deacetylated chitosan, showed the highest scavenging activity on all tested radicals. Yin et al.[8] reported scavenging activity of low molecular weight chitosan on superoxide radical was more pronounced than that of high molecular weight chitosan. Xing et al.[9] also reported low molecular weight sulfated chitosan had strong scavenging activity on superoxide and hydroxyl radicals. Structure of sulfated chitosan was similarity to heparin. They exhibit a wide variety of physiological activities such as anticoagulant activity,[10] anti-HIV-1 activity,[11] and so on and these activities were better than chitosan itself. However, at present, comparison of different molecular weight (DMW) chitosan and its sulfated derivatives on antioxidant activity had not been reported. By study of their scavenging activities on superoxide/hydroxyl radicals, reducing power and chelating effect, this paper obtained some useful results about above-mentioned questions. Antioxidant activities of chitosans were more pronounced with lower molecular weight. This result was similar to Yin et al.'s[8] report and different to Je et al.'s[7] result. Moreover, antioxidant activities of DMW sulfated chitosan, except for chelating effect, were stronger than that of DMW chitosan. Furthermore, scavenging activities of low molecular weight sulfated chitosan on superoxide/hydroxyl radicals and reducing power were higher than that of high molecular weight sulfated chitosan. However, CTS4 showed more considerable ferrous ion-chelating potency than others, moreover, chelating effect of HCTS was stronger than that of LCTS.

2. Materials and methods

2.1. Chemicals

Nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), hydrogen peroxide (H_2O_2), thiobarbituric Acid (TBA), ethylene diamine tetra-acetic acid (EDTA), ferrozine, nicotinamide adenine dinucleotide-reduced (NADH), trichloroacetic acid (TCA), deoxyribose (DR), potassium ferricyanide and ferric chloride were purchased from Sigma Chemicals Co. All other chemicals and reagents, unless otherwise specified, were not purified, dried or pretreated. DMW chitosans and sulfated chitosans were prepared according to Xing Rong-e's methods[9,12] and Gamzazade's methods,[13] respectively (Table 1).

2.2. Superoxide-radical scavenging assay

The superoxide scavenging ability of DMW chitosans and sulfated chitosans was assessed by the method of Nishikimi et al.[14] The reaction mixture, containing DMW chitosans (0.005–1.6 mg/mL) and sulfated chitosans (0.005–0.4 mg/mL), PMS (30 μM), NADH (338 μM) and NBT (72 μM) in phosphate buffer (0.1 M pH 7.4), was incubated at room temperature for 5 min and the absorbance was read at 560 nm against a blank. The capability of scavenging to superoxide radical was calculated using the following equation:

$$\text{Scavenging effect } (\%) = \left(1 - \frac{A_{\text{sample 560 nm}}}{A_{\text{control 560 nm}}}\right) \times 100$$

2.3. Hydroxyl radical assay

The reaction mixture, containing DMW chitosans and sulfated chitosans (0.1–3.2 mg/mL), was 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.[15] The reaction was 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 absorbance of the mixture was measured at 535 nm against reagent blank. Decreased absorbance of the reaction mixture indicated decreased oxidation of deoxyribose.

2.4. Metal ion chelating assay

The ferrous ion-chelating potential of DMW chitosans and sulfated chitosans was investigated according to the method of Decker and Welch,[16] wherein the Fe^{2+}-chelating ability of DMW chitosans and sulfated chitosans was monitored by absorbance of the ferrous iron–ferrozine complex at 562 nm. Briefly, the reaction mixture, containing DMW chitosans and sulfated chitosans of different concentration, $FeCl_2$ (2 mM), and ferrozine (5 mM) was adjusted to a total volume of 0.8 mL with water, shaken well and incubated for 10 min at room temperature. The absorbance of the mixture was measured at 562 nm against blank. The

Table 1. Symbols of DMW chitosans and sulfated chitosans

Symbols	CTS	CTS1	CTS2	CTS3	CTS4	HCTS	LCTS
Molecular weight ($\times 10^4$)	76	12	6	2	0.9	12.4	0.9
Sulfur content (%)	0	0	0	0	0	14.7	14.5

ability of DMW chitosans and sulfated chitosans to chelate ferrous ion was calculated using the following equation:

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

2.5. Measurement of reducing power

The reducing power of DMW chitosans and sulfated chitosans was quantified by the method described earlier by Yen and Chen[17] with minor modifications. Briefly, 1 mL of reaction mixture, containing different concentration of DMW chitosans and sulfated chitosans in phosphate buffer (0.2 M, pH 6.6), was incubated with potassium ferricyanide (1% W/V) at 50 °C for 20 min. The reaction was terminated by TCA solution (10% W/V) and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant was mixed with distilled water and 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.

2.6. Statistical analysis

All data are expressed as means ± SD. Data were analyzed by an analysis of variance ($P < 0.05$) and the means separated by Duncan's multiple range test. The results were processed by computer programmes: Excel and Statistica software (1999).

3. Results and discussion

3.1. Scavenging activity of superoxide radical by DMW chitosans and sulfated chitosans

Figure 1 showed that the inhibitory effect of DMW chitosans (0.005–1.6 mg/mL) and sulfated chitosans (0.005–0.4 mg/mL) on superoxide radicals was marked and concentration related. Significant scavenging of superoxide radical was evident at all the tested concentrations of sulfated chitosans and CTS4. However, the scavenging rate of others increased with increasing concentration, which was not obvious. Moreover, as shown in Figure 1, IC_{50} of LCTS, HCTS, CTS4, CTS3, CTS2 was 0.010, 0.012, 0.1, 0.75, 1.6 mg/mL, respectively. However, IC_{50} of CTS and CTS1 could not be read. This result showed LCTS, HCTS had the highest activity upon the elimination of superoxide radicals compared to DMW chitosan. Moreover, scavenging activity of LCTS on superoxide radical was higher than that of HCTS. Scavenging activities of low molecular weight chitosan on superoxide radical was more pronounced than that of high molecular weight chitosan. This result was similar to Yin et al.'s[8] result. Yin et al.[8] estimated this result might be the effect of intramolecular hydrogen bond. Chitosans own a lot of hydrogen bonds on N_2–O_6 and O_3–O_5. High molecular weight chitosans had compact structures and the effect of their intramolecular hydrogen bonds is stronger. Strong effect of intramolecular hydrogen bonds caused activities of hydroxyl and amino groups to become soft. On the contrary, low molecular weight chitosan own incompact structure. It means to be the soft effect of intramolecular hydrogen bonds. However, superoxide radical is a zwitterionic radical. It could react with free hydroxyl and amino groups in chitosan, then superoxide radical was eliminated by this reaction. Because low molecular weight chitosans have more free hydroxyl and amino groups than high molecular weight chitosans, their scavenging activities on superoxide radical were more pronounced than that of high molecular weight chitosans.[8] Furthermore, scavenging activities of sulfated chitosans on superoxide radical were higher than that of chitosans. It might be they have the better solubility than DMW chitosan. However, the mechanism of these resultants on superoxide radical needs to be further researched. Although superoxide was a relatively weak oxidant, it decomposed to form stronger reactive oxidative species, such as singlet oxygen and hydroxyl radicals, which initiate peroxidation of lipids.[18] In the present study, DMW chitosans and sulfated chitosans effectively scavenged superoxide in a concentration-dependent manner. Further, superoxides were also known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals.[19] These results clearly suggested that the antioxidant activity of DMW chitosans and sulfated chitosans was also related to their ability to scavenge superoxide radical.

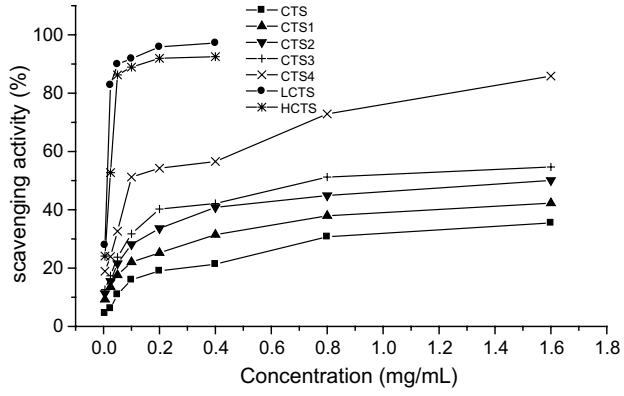

Figure 1. Scavenging effect of DMW chitosans and sulfated chitosans on superoxide radical. Values are means ± SD of three determinations.

3.2. Hydroxyl radical scavenging activity of DMW chitosans and sulfated chitosans

Hydroxyl radicals, generated by reaction of iron–EDTA complex with H_2O_2 in the presence of ascorbic acid, attack deoxyribose to form products that, upon heating with 2-thiobarbituric acid under acid conditions, yield a pink tint. Added hydroxyl radical scavengers compete with deoxyribose for the resulted hydroxyl radicals and diminish tint formation.[20] The above-mentioned model was used to measure inhibitory activities of DMW chitosans and sulfated chitosans on hydroxyl radicals. The result was plotted in Figure 2. As shown in Figure 2, apart from CTS and CTS1, others had obvious scavenging

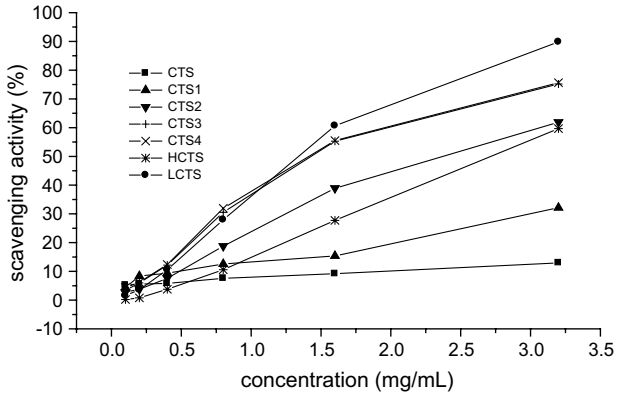

Figure 2. Inhibiting effect of DMW chitosans and sulfated chitosans on deoxyribose oxidative damage. Values are means ± SD of three determinations.

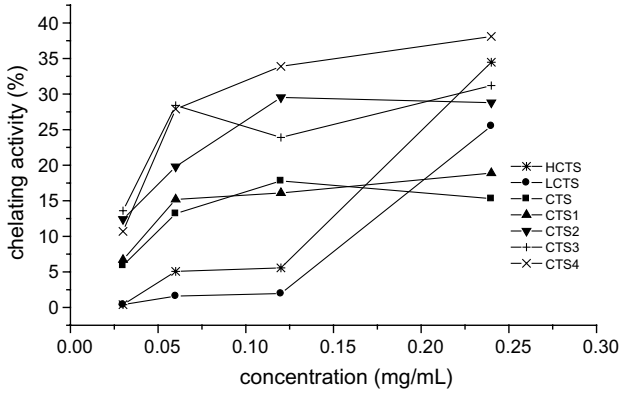

Figure 3. Chelating effect of DMW chitosans and sulfated chitosans on ferrous ions. Each value is expressed as mean ± SD ($n = 3$).

activity on hydroxyl radical. Scavenging activities of low molecular weight chitosans on hydroxyl radical at a little high concentration were stronger than that of high molecular weight chitosans. LCTS had the strongest scavenging activity on hydroxyl radical. Furthermore, as shown in Figure 2, scavenging activity of HCTS was between high molecular weight chitosans and low molecular weight chitosans. However, as concerning chitosan and sulfated chitsoan of the same molecular weight, scavenging activities of sulfated chitosans on hydroxyl radical were more pronounced than that of chitosans. On the other hand, the scavenging rate increased with their increasing concentration. IC_{50} of LCTS, HCTS, CTS4, CTS3, CTS2 was 1.32, 3.269, 1.40, 1.435, 2.355 mg/mL, respectively. IC_{50} of CTS and CTS1 could not be read. Earlier, numerous workers[15] had employed above-mentioned system to assess the biological activity of various natural plant derived biomolecules. Smith et al.[21] earlier reported that molecules that could inhibit deoxyribose degradation were those that could chelate iron ions and render them inactive of poorly active in a Fenton reaction. In the present study, in another assay system, we found DMW chitosans and sulfated chitosans have soft chelating ability, it might be likely that the chelating effect of DMW chitosans and sulfated chitosans on metal ions might be responsible for the inhibition of deoxyribose oxidation. However, the mechanism of these resultants on hydroxyl radical needs to be further researched by plural experimental methods.

3.3. Chelating effects on ferrous ions

The ferrous ion-chelating effect of DMW sulfated chitosans was concentration related and that of DMW chitosans was not concentration dependent as shown in Figure 3. The chelating effects of DMW chitosans and sulfated chitosans on ferrous ions were low. At low concentration, the chelating effects of DMW chitosans were more pronounced than that of DMW sulfated chitosans. At high concentration, DMW sulfated chitosans showed higher chelating ability but their effects were lower than that of CTS4. Furthermore, as shown in Figure 3, the chelating ability of LCTS was almost lowest except for beyond CTS and CTS1 at high concentration. The most effective pro-oxidants present in food systems are ferrous ions.[22] Because DMW chitosans and sulfated chitosans own the chelating ability to ferrous ions and various physiological activities, it would be beneficial if they were formulated into foods.

3.4. Reducing power of DMW chitosans and sulfated chitosans

Figure 4 depicted the reducing power of DMW chitosans and sulfated chitosans. The reducing power of DMW chitosans and sulfated chitosans correlated well with increasing concentrations. Figure 4 showed the reducing power increased with increasing DMW chitosans and sulfated chitosans concentration. Moreover, the reducing power of LCTS, CTS4 was relating more pronounced than that of others and that of LCTS was the most pronounced. The reducing power of HCTS was lower LCTS and CTS4 and higher others. Reducing power of chitosan was more pronounced with lower molecular weight. Mau et al.[23] reported reducing powers were 0.80, 0.89 and 0.92 at 1.0 mg/mL for ascorbic acid, α-tocopherol and BHA, respectively. However, as shown in Figure 4, the reducing power of DMW chitosans and sulfated chitosans was lower than that of ascorbic acid, α-tocopherol and BHA. Earlier authors[24]

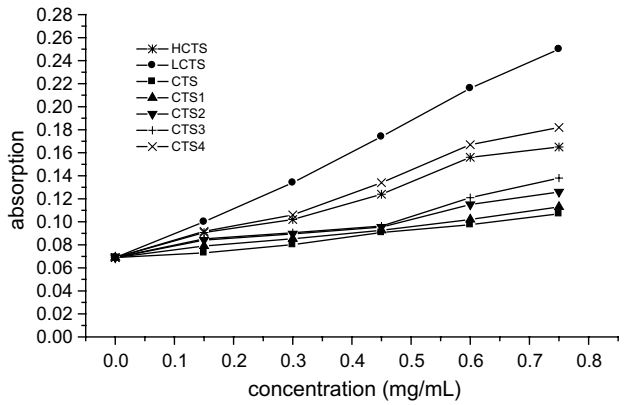

Figure 4. Reducing power of DMW chitosans and sulfated chitosans. Each value is expressed as mean ± SD ($n = 3$).

have observed a direct correlation between antioxidant activities and reducing power of certain plant extracts. The reducing properties are generally associated with the presence of reductones,[25] which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom.[26] Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Our data on the reducing power of DMW chitosans and sulfated chitosans suggested that it was likely to contribute significantly towards the observed antioxidant effect.

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