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Antioxidant activity of differently regioselective chitosan sulfates in vitro

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Abstract—Differently regioselective chitosan sulfates were prepared according to Hanno Baumann's methods. Their antioxidant potencies were investigated employing various established in vitro systems, such as 1,1-diphenyl-2-picrylhydrazyl (DPPH)/superoxide/hydroxyl radicals scavenging, reducing power, iron ion chelating and total antioxidant activity. All kinds of sulfated chitosans (HCTS, TSCTS, SCTS, TCTS) showed strong inhibitory activity toward superoxide radical by the PMS-NADH system compared to Vc. According to the above-mentioned order their IC_{50} were 0.012, 0.040, 0.015, 0.022 mg/mL, respectively, however, scavenging activity of Vc on superoxide radical was 68.19% at 2.0 mg/mL. Scavenging activity of superoxide radical was found to be in the order of HCTS > SCTS > TCTS > TSCTS > Vc. Furthermore, all kinds of sulfated chitosans exhibited strong concentration-dependent inhibition of deoxyribose oxidation. Except for HCTS, others had stronger scavenging activity on hydroxyl radical than Vc. Scavenging effect of TSCTS on 1,1-diphenyl-2-picrylhydrazyl radical was little lower than that of BHA, but better than that of others. All kinds of sulfated chitosans were efficient in the reducing power, especially TSCTS. TSCTS and TCTS showed considerable ferrous ion chelating potency. The data obtained in vitro models clearly establish the antioxidant potency of all kinds of sulfated chitosans. These in vitro results suggested the possibility that sulfated chitosans could be effectively employed as ingredient in health or functional food, to alleviate oxidative stress. However, comprehensive studies need to be conducted to ascertain the in vivo safety of sulfated chitosans in experimental animal models.

1. Introduction

Oxidative stress, induced by oxygen radicals, is believed to be a primary factor in various degenerative diseases as well as in the normal process of aging.[1] Reactive oxygen species (ROS) in the forms of superoxide anion ($\cdot O_2^-$), hydroxyl radical (·OH) and hydrogen peroxide (H_2O_2) are generated by normal metabolic process or from exogenous factors and agents, and they can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. These ROS 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 ROS, activating a battery of detoxifying proteins, or preventing the generation of ROS.[1] 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.[3] In general, the natural antioxidants mainly constitute a broad range of compounds including phenolic compounds, nitrogen compounds and carotenoids.[4] However, the antioxidant activities of several biological polysaccharides have recently been described. For example, Zhang et al. reported fucoidan had strong scavenging effect on superoxide radical with IC_{50} of 20.3 μg/mL.[5] Xue et al. found that sulfated fucans had significant scavenging

Abbreviations: HCTS: sulfated chitosan of $C_{2,3,6}$ sulfation; TSCTS: sulfated chitosan of $C_{3,6}$ sulfation; SCTS: sulfated chitosan of C_6 sulfation; TCTS: sulfated chitosan of C_3 sulfation; BHA: butyl hydroxy anisole; NBT: nitro blue tetrazolium; PMS: phenazine methosulfate; H_2O_2: hydrogen peroxide; DPPH: 1,1-diphenyl-2-picrylhydrazyl; TBA: thiobarbituric acid; EDTA: ethylene diamine tetra-acetic acid; NADH: nicotinamide adenine dinucleotide-reduced; TCA: trichloroacetic acid; DR: deoxyribose.

Keywords: All kinds of sulfated chitosans; Antioxidant activity; Reducing power; Chelating effect; Radical scavenging effect.

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effects on free radicals.[6] The antioxidant activity of chitosan and its derivatives also has attracted the most attention. Yin et al. reported that low molecular weight chitosan could scavenge superoxide radical and scavenging activity was 80.3% at 0.5 mg/mL;[7] Esumi et al. showed that the gold-chitosan nanocomposites have an ability to depress the activity of hydroxyl radicals.[8] Its sulfated products-sulfated chitosans, their structures similarity to heparin, have shown multiple biological activities in relation to anticoagulant activity,[9] antitumor activity[10] and antiviral properties.[11] However, few people studied on antioxidant activities of sulfated chitosan, especially none on influence of their structures variety to antioxidant activities. Hence, the present work investigates the possible antioxidant effects of all kinds of regioselective chitosan sulfates. In this study, we studied their antioxidant activities, employing various in vitro assay systems, such as DPPH/superoxide/hydroxyl radicals scavenging, iron ion chelating and so on, in order to understand the mechanisms of their antioxidant activities.

2. Materials and methods

2.1. Chemicals

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), 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. Differently regioselective chitosan sulfates were prepared according to Hanno Baumann's methods (Table 1).[12]

2.2. Superoxide-radical scavenging assay

The superoxide scavenging ability of sulfated chitosans was assessed by the method of Nishikimi et al.[13] The reaction mixture, containing all kinds of 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:

Table 1. Characteristic of all kinds of sulfated chitosans

Species	Molecular weight ($\times 10^4$)	Sulfur content (%)	Color of resultant	Solubility
HCTS	12.4	14.7	Pale yellow	Easy soluble
TSCTS	11.7	12.1	White	Easy soluble
TCTS	12.1	5.2	Yellow	Easy soluble
SCTS	13.5	7.6	White	Soluble
CTS	76	0	Pale yellow	Not soluble

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

2.3. Hydroxyl radical assay

The reaction mixture, containing all kinds of 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.[14] 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. Scavenging of DPPH radical

The effect of sulfated chitosans on DPPH radical was studied, employing the modified method described earlier by Yamaguchi et al.[15] Briefly, 1.5 mL of DPPH solution (0.1 mM, in 95% ethanol or methanol) was incubated with varying concentrations of sulfated chitosans. The reaction mixture was shaken well and incubated for 20 min at room temperature and the absorbance of the resulting solution was read at 517 nm against a blank. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and was calculated using the following equation:

$$\text{Scavenging effect } (\%) = \left(1 - A_{\text{samples (517 nm)}}/A_{\text{control (517 nm)}}\right) \times 100$$

2.5. Measurement of reducing power

The reducing power of the all kinds of sulfated chitosans was quantified by the method described earlier by Yen and Chen[16] with minor modifications. Briefly, 1 mL of reaction mixture, containing different concentration of 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. Metal ion chelating assay

The ferrous ion-chelating potential of sulfated chitosans was investigated according to the method of Decker and Welch,[17] wherein the Fe^{2+}-chelating ability of sulfated chitosans was monitored by absorbance of the ferrous iron-ferrozine complex at 562 nm. Briefly, the reaction mixture, containing 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. EDTA was used as positive control. The ability of sulfated chitosans to chelate ferrous ion was calculated using the following equation:

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

2.7. Antioxidant assay using β-carotene linoleate model system

The antioxidant activity of sulfated chitosans was evaluated by the β-carotene linoleate model system.[18] A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 mL of chloroform. Two milliliters of this solution were pipetted into a 100 mL round-bottom flask. After chloroform was removed under vacuum, 40 mg of purified linoleic acid, 400 mg of Tween 40 emulsifier, and 100 mL of aerated distilled water were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing different concentrations of the sulfated chitosans. BHA was used for comparative purposes. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer. The tubes were placed to 50 °C in a water bath. Measurement of absorbance was continued until the color of β-carotene disappeared; a blank, devoid of β-carotene, was prepared for background subtraction. Antioxidant activity was calculated using the following equation:

$$\text{Antioxidant activity} = (\beta\text{-carotene content after 2 h of assay}/\text{initial}\beta\text{-carotene content}) \times 100$$

2.8. 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 all kinds of sulfated chitosans

Figure 1(a) showed that the inhibitory effect of all kinds of sulfated chitosans on superoxide radicals was marked and concentration related. Significant scavenging of superoxide radical was evident at all the tested concentrations of all kinds of sulfated chitosans except for parent chitosan. Moreover, as shown in Figure 1(a) IC_{50} of HCTS, TSCTS, SCTS, TCTS was 0.012, 0.040, 0.015, 0.022 mg/mL, respectively. However, IC_{50} of CTS could not be read. This result showed HCTS had a highest activity upon the elimination of superoxide radicals.

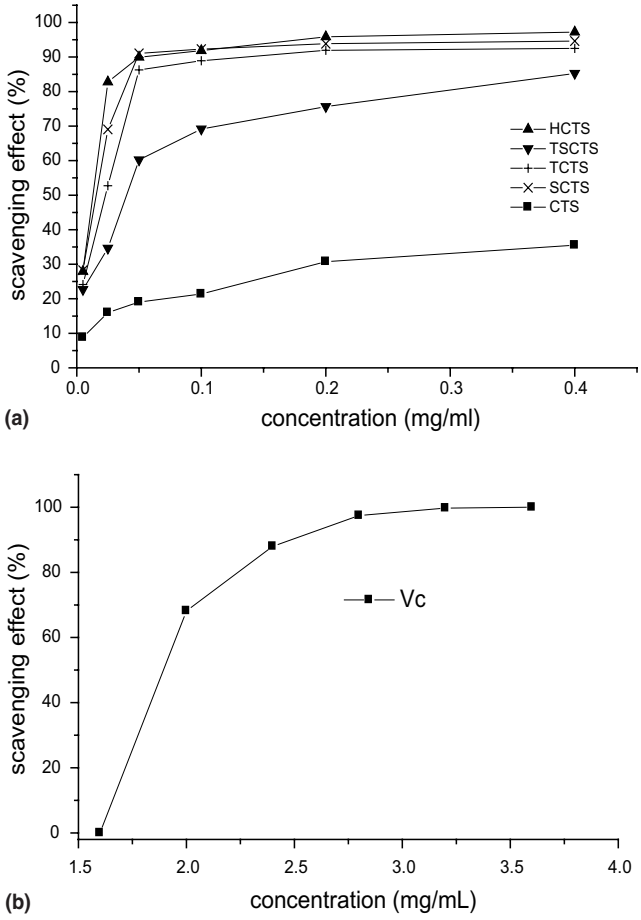

Figure 1. (a) Scavenging effect of all kinds of sulfated chitosans on superoxide radical. (b) Scavenging effect of Vc on superoxide radical. Values are means ± SD of three determinations.

Their orders of scavenging activities on superoxide radicals were: HCTS > SCTS > TCTS > TSCTS > CTS. Furthermore, we studied Vc to scavenging activity of superoxide radical using above-mentioned model, as shown in Figure 1(b). The result showed scavenging activity of Vc for superoxide radical was only 68.19% at 2.0 mg/mL. Compared to this result, all kinds of sulfated chitosans had stronger scavenging activity for superoxide radical than Vc. 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.[19] In the present study, all kinds of sulfated chitosans effectively scavenged superoxide in a concentration-dependent manner. Further, superoxides were also known to indirectly initiate lipid peroxidation as result of H_2O_2 formation, creating precursors of hydroxyl radicals.[20] These results clearly suggested that the antioxidant activity of all kinds of sulfated chitosans was also related to their ability to scavenge superoxide radical.

3.2. Hydroxyl radical scavenging activity of all kinds of 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.[21] Above-mentioned model was used to measure inhibitory activities of all kinds of sulfated chitosans on hydroxyl radicals. The result was plotted in Figure 2. As shown in Figure 2, apart from CTS, others had obvious scavenging activity. The scavenging rate increased with increasing concentration. Moreover, except for HCTS, others had stronger scavenging activity on hydroxyl radical than Vc. IC_{50} of HCTS, TSCTS, SCTS, TCTS, Vc was 3.269, 1.184, 0.925, 0.35, 1.537 mg/mL, respectively. Earlier, numerous workers[14] had employed above-mentioned system to assess the biological activity of various natural plant derived biomolecules. Smith et al.[22] 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 demonstrated the iron chelating effect of sulfated chitosans, especially, TSCTS, TCTS had significant chelating ability on ferrous ions. It was likely that the chelating effect of all kinds of 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.

3.3. Chelating effects on ferrous ions

The ferrous ion-chelating effect of all kinds of sulfated chitosans was concentration related as shown in Figure 3. The chelating effects of HCTS, SCTS on ferrous ions were low. However, TSCTS, TCTS showed excellent chelating ability even if their effects were lower than that of EDTA. In TSCTS and TCTS there were $-OSO_3^-$ and $-NH_2$ groups at position of C_3, C_2, respectively. However, SCTS existed –OH at C_3 and HCTS had considerable $-OSO_3^-$ at C_2. Moreover, chelating effects of TCTS and TSCTS were notably higher than that of HCTS, SCTS. This result might be shown that the chelating effects of $-OSO_3^-$ and $-NH_2$ groups were stronger than

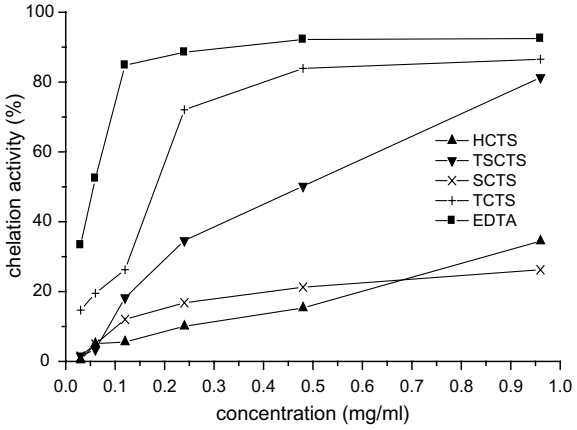

Figure 3. Chelating effect of all kinds of sulfated chitosans on ferrous ions. Each value is expressed as mean ± SD ($n = 3$).

that of –OH and $-NH_2$ and –OH and $-OSO_3^-$. The most effective pro-oxidants present in food systems are ferrous ions[23] and the high chelating effect of TSCTS, TCTS would be beneficial if they were formulated into foods.

3.4. Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl

Total DPPH scavenging potential of all kinds of sulfated chitosans at varying concentrations was measured and the results were depicted in Figure 4. Except for TSCTS the scavenging effect slowly increased with increasing their concentration. However, the scavenging effect abruptly increased with increasing TSCTS concentration up to a certain extent (50 μg/mL) and then almost leveled off with further increase. Figure 4 also showed a little scavenging effect on DPPH was observed with HCTS, SCTS, TCTS. Obviously, TSCTS possessed the strong scavenging ability on DPPH and the scavenging effect at 50 μg/mL was 83.38%. The scavenging effect of BHA on DPPH was not concentration dependent in range of given concentration and strong at considerably low concentration. The result showed BHA had the stronger scavenging activity on DPPH than TSCTS.

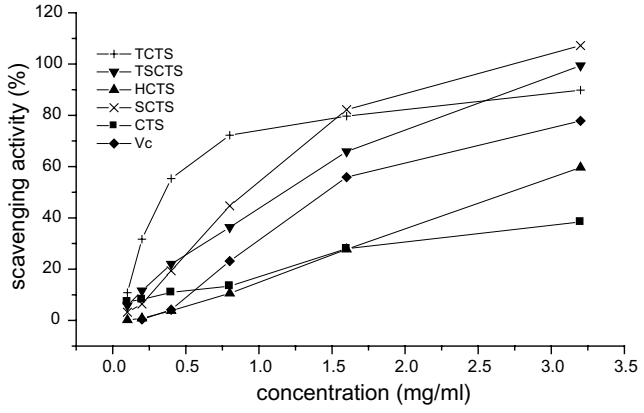

Figure 2. Inhibiting effect of all kinds of sulfated chitosans on deoxyribose oxidative damage. Values are means ± SD of three determinations.

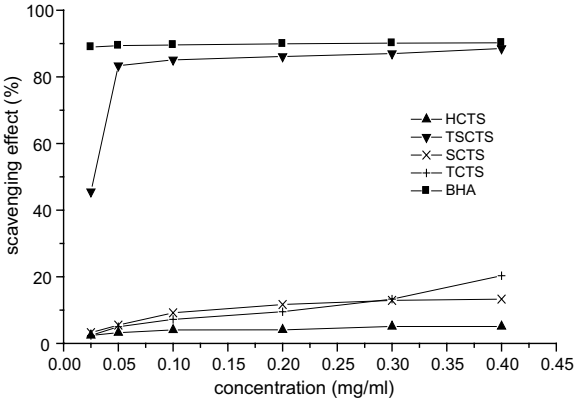

Figure 4. Scavenging effect of all kinds of sulfated chitosans on 1,1-diphenyl-2-picrylhydrazyl radicals. Each value is expressed as mean ± SD ($n = 3$).

But naturally nontoxic TSCTS got the advantage of synthetic minitoxic BHA. In the DPPH test, TSCTS was able to reduce the stable radical DPPH to the yellow-colored diphenyl-picrylhydrazine. DPPH is one of the compounds that possessed a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers.[15] Further it is well accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen-donating ability.[24] In the present study, TSCTS showed excellent scavenging activity of DPPH radical, which may be attributable to its strong hydrogen-donating ability compared to HCTS, SCTS, TCTS. However, the mechanism of all kinds of sulfated chitosans on DPPH radical, especially TSCTS, need to be further researched.

3.5. Reducing power of all kinds of sulfated chitosans

Figure 5 depicted the reducing power of all kinds of sulfated chitosans. The reducing power of all kinds of sulfated chitosans correlated well with increasing concentrations. Figure 5 showed the reducing power increased with increasing all kinds of sulfated chitosans concentration. Moreover, the reducing power of TSCTS, TCTS, SCTS was relating more pronounced than that of HCTS and that of TSCTS was the most pronounced. Jeng-Leun Mau et al.[25] 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 5, the reducing power of TSCTS was 0.92 at 0.08 mg/mL and 1.068 at 0.1 mg/mL. Above-mentioned result showed the reducing power of TSCTS was higher than that of ascorbic acid, α-tocopherol and BHA. Earlier authors[26] 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,[27] which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom.[28] Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Our data on the reducing power of all kinds of sulfated chitosans suggested that it was likely to contribute significantly toward the observed antioxidant effect.

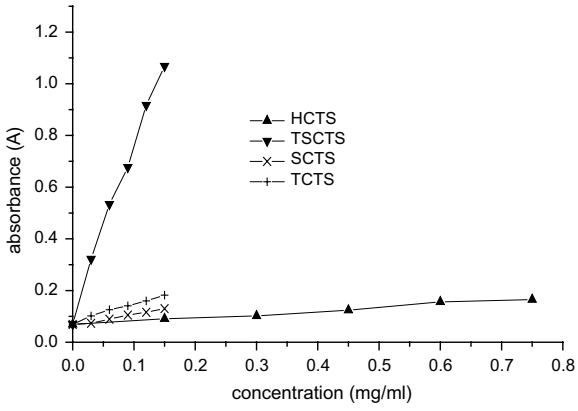

Figure 5. Reducing power of all kinds of sulfated chitosans. Each value is expressed as mean ± SD ($n = 3$).

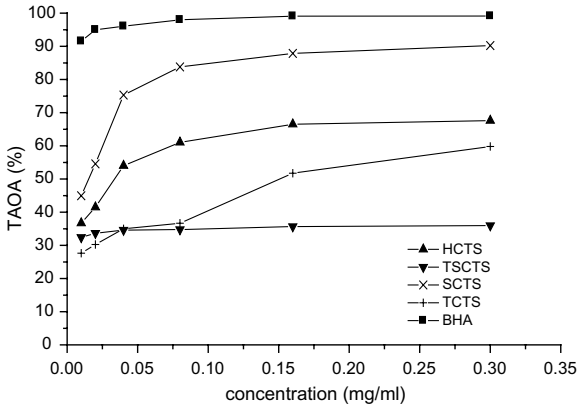

Figure 6. Antioxidant activity of all kinds of sulfated chitosans in the β-carotene-linoleate system. Values are means ± SD of three determinations.

3.6. Antioxidant activity of all kinds of sulfated chitosans in the β-carotene-linoleate system

Figure 6 showed the antioxidant activity of all kinds of sulfated chitosans as measured by the bleaching of β-carotene. Antioxidant activity of TSCTS and TCTS was low, but TCTS was concentration-related. From 10 to 300 μg/mL, its antioxidant activity abruptly changed from 27.63% to 59.79%. However, TSCTS had no notable concentration dependent. Antioxidant activity of SCTS and HCTS increased with their increasing concentration. Their antioxidant activities were 90.23% and 67.60% at 300 μg/mL, but antioxidant activity of BHA reached 99.14% at 300 μg/mL and it was not concentration-related within experimental concentration. Above-mentioned result showed antioxidant activity of sulfated chitosans was lower than that of BHA. But their excellently biological activities had the advantage over BHA and promoted their progress. The antioxidant activity of carotenoid is based on the radical adducts of carotenoid with free radicals from linoleic acid. The linoleic acid free radical attacks the highly unsaturated β-carotene models. The presence of carotenoid shows, not only a decrease of the free radical concentration, but also the reduction of Fe^{3+} to Fe^{2+} by carotenoids. The presence of different antioxidants can hinder the extent of β-carotene-bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system.[29]

4. Conclusion

The results of the present work indicated that all kinds of sulfated chitosans possessed antioxidant activities and free radical scavenging activities. These assays were useful for establishing the abilities of sulfated chitosans to chelate and reduce Fe^{3+} and had important applications for the pharmaceutical and food industries. However, their in vivo antioxidant activity and the different antioxidant mechanisms need to be further researched.

Acknowledgements

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