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Original article

The antioxidant activity of 2-(4(or 2)-hydroxyl-5-chloride-1, 3-benzene-di-sulfanimide)-chitosan

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Abstract

Chitosan (CS) with two different molecular weights was modified by reacting with 4-hydroxyl-5-chloride-1,3-benzene-disulfo-chloride or 2-hydroxyl-5-chloride-1,3-benzene-disulfo-chloride to give new 2-(4(or 2)-hydroxyl-5-chloride-1,3-benzene-di-sulfanimide)-chitosan (2-HCBSAHCS, 2-HCBSALCS, 4-HCBSAHCS, 4-HCBSALCS). The structure of the derivatives was characterized by FT-IR and ^{13}C NMR spectroscopy. The antioxidant activities of the derivatives were investigated employing various established systems, such as hydroxyl radical ($^{\bullet}OH$)/superoxide anion ($O_2^{\bullet-}$) scavenging/reducing power and chelating activity. All the derivatives showed stronger scavenging activity on hydroxyl radical than chitosan and ascorbic acid (Vc), and IC_{50} of 4-HCBSAHCS, 4-HCBSALCS, 2-HCBSAHCS and 2-HCBSALCS was 0.334, 0.302, 0.442, 0.346 mg/mL, respectively. The inhibitory activities of the derivatives toward superoxide radical by the PMS–NADH system were strong. The results showed that the superoxide radical scavenging effect of 2-(4(or 2)-hydroxyl-5-chloride-1,3-benzene-di-sulfanimide)-chitosan was higher than chitosan. The derivatives had obviously reducing power and slight chelating activity. The data obtained in in vitro models clearly establish the antioxidant potency of 2-(4(or 2)-hydroxyl-5-chloride-1,3-benzene-disulfanimide)-chitosan.

Keywords: 2-(4(or 2)-Hydroxyl-5-chloride-1,3-benzene-di-sulfanimide)-chitosan; Chitosan; Radical scavenging effect; Reducing power; Chelating effect

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 ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$) 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. 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]. The antioxidant activity of chitosan and its derivatives has attracted the most attention due to their nontoxic nature and natural abundance. Xing et al. [4,5] had studied the antioxidant activity of chitosan sulfate using a spectroscopic technique. Chitosan sulfate has a strong negatively charged nature that was obtained via modification of hydroxyl or amino groups. Yin et al. [6] reported that low molecular weight chitosan could scavenge superoxide radical and scavenging activity was 80.3% at 0.5 mg/mL. Zhong et al. [7] showed that sulfanilamide derivatives of chitosan and chitosan sulfates display various antioxidant activities.

Antioxidant activities of chitosan and its derivatives are attributed to their ability to abstract hydrogen atoms easily from

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free radicals. Further this ability directly correlates to their structural properties that they have amino and hydroxyl groups attached to C-2, C-3 and C-6 positions of the pyranose ring [8]. In order to improve antioxidant activity of chitosan, we prepared new 2-(4(or 2)-hydroxyl-5-chloride-1,3-benzene-di-sulfanimide)-chitosan (HCBSACS) in this paper. Their antioxidant activities were evaluated in the experiment, and the results indicated that HCBSACS showed higher antioxidant activity than chitosan.

2. Chemistry

2-(4(or 2)-Hydroxyl-5-chloride-1,3-benzene-di-sulfanimide)-chitosan (HCBSACS) was synthesized as shown in Scheme 1. Chitosan with two different molecular weights reacted with 4-hydroxyl-5-chloride-1,3-benzene-di-sulfo-chloride or 2-hydroxyl-5-chloride-1,3-benzene-di-sulfo-chloride in formamide solution at 70 °C to give the derivatives. All the products gave satisfactory spectroscopic data, which are in full accordance with their assigned structures.

3. Results and discussion

3.1. Structure and physicochemical characteristics of the derivatives

Fig. 1 presents the comparison of transmission FT-IR spectra data for HCBSAHCS with original CS. Firstly, obvious translocation at 3500–3200 cm^{-1} due to the O–H and N–H group stretching vibration was observed. In addition, the characteristic absorbance of $-NH_2$ at 1600 cm^{-1} changed to 1685 cm^{-1}. This is the result of N–H reacting with 2-hydroxyl-5-chloride-1,3-benzene-disulfo-chloride. Secondly, new peaks at about 1535 and 870 cm^{-1} appeared in the spectra of HCBSACS are the characteristic absorbance of phenyl group. Thirdly, there are new strong peaks at 1375 and 1165 cm^{-1} in HCBSAHCS spectra, which are assigned to the characteristic absorbance of SO_2–N. All the above results showed that HCBSAHCS were obtained.

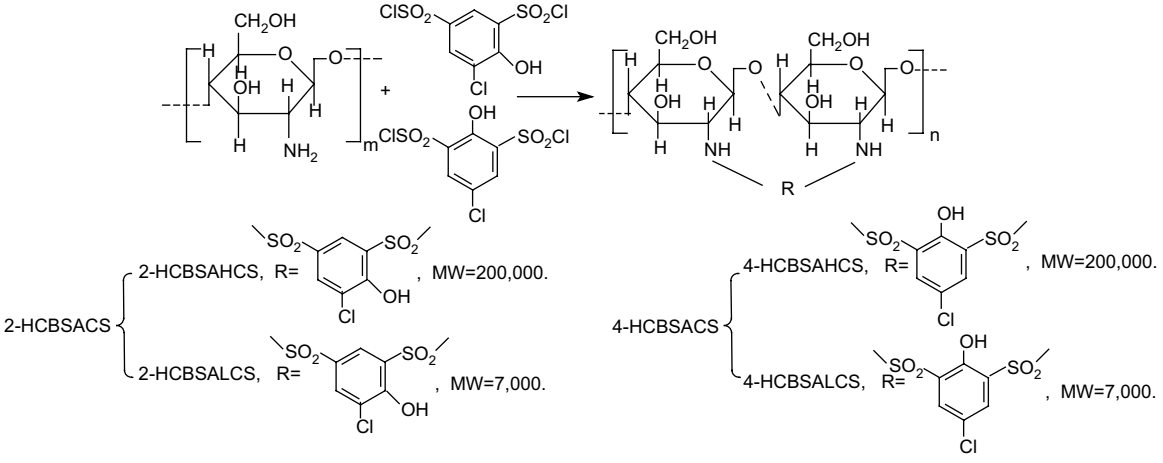
Fig. 2 depicts the ^{13}C NMR spectrum of 2-HCBSAHCS. The peaks at $\delta = 100.1$, 62.9, 74.5, 78.5, 77.2 and 58.4 ppm were attributed to the C-1, C-2, C-3, C-4, C-5 and C-6 in chitosan molecule, respectively. The peaks at $\delta = 133.4$, 167.5, 169.2, 172.3 were the characteristic absorbance of C-a, C-b, C-c, C-d in phenyl group. The peaks appearing at 177.2 and 25.9 ppm were the peaks of the residual C=O and $-CH_3$, respectively. Based on all the above results, we can conclude that 2-HCBSAHCS was obtained successfully. As for the spectra of 4-HCBSAHCS was concerned, the peaks at $\delta = 105.5$, 62.7, 76.0, 78.5, 77.3 and 58.5 ppm were attributed to the C-1, C-2, C-3, C-4, C-5 and C-6 in chitosan molecule, respectively. The peaks at $\delta = 130.9$, 167.2, 168.9, 173.3, 133.8, 131.4 were the characteristic absorbances of C-a, C-b, C-c, C-d, C-e, C-f in phenyl group. The peaks appearing at 184.1 and 25.9 ppm were the peaks of the residual C=O and $-CH_3$ in chitosan molecule, respectively. Based on all the above results, we can conclude that 4-HCBSAHCS was obtained successfully.

The FT-IR and ^{13}C NMR spectra of the derivatives with low molecular weight were similar to the high molecular ones, besides that the intensity of the peaks is different. So we can conclude that 4-HCBSACS and 2-HCBSACS were obtained successfully.

The results of elemental analyses, yield, grafted degree and color of the derivatives are listed in Table 1. The elemental analyses indicate that the substitution degree of 4-HCBSAHCS, 4-HCBSALCS, 2-HCBSAHCS and 2-HCBSALCS is about 75.6, 79.8, 73.2 and 77.2, respectively. Furthermore, color of the high molecular weight derivatives was lighter than that of low molecular ones, and yield changed from 65.38 to 73.92%.

3.2. Hydroxyl radical scavenging activity of CS and HCBSACS

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 pin tint. Added



Scheme 1. Synthesis pathway of 2-(4 (or2)-hydroxyl-5-chloride-1,3-benzene-di-sulfanimide)-chitosan.

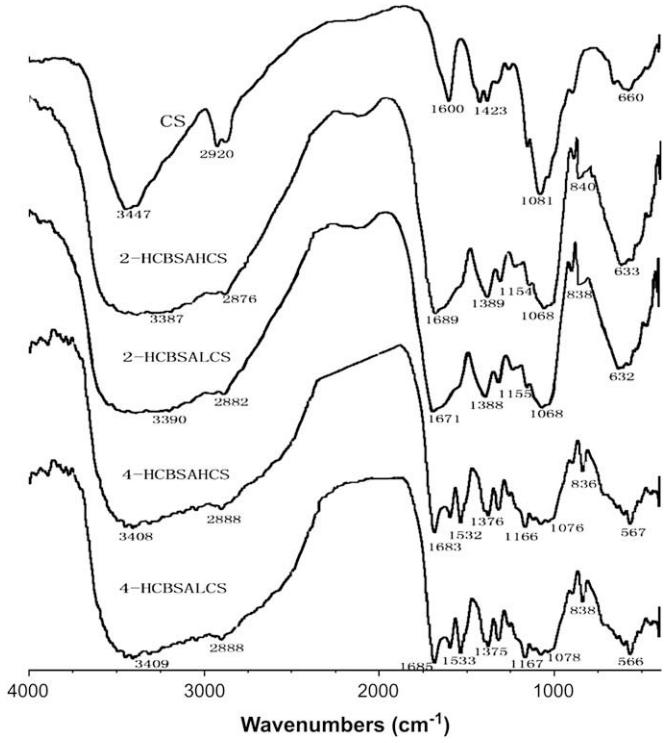


Fig. 1. FT-IR spectra of HCS and HCBSACS.

hydroxyl radical scavenging competes with deoxyribose for the resulted hydroxyl radicals and diminishes tint formation [9]. The above-mentioned model was used to measure inhibitory activities of CS and HCBSACS. The results are plotted in Fig. 3. As shown in Fig. 3, all the compounds had strong scavenging activity. The scavenging rate of the derivatives increased with increasing concentration. IC_{50} of 4-HCBSAHCS, 4-HCBSALCS, 2-HCBSAHCS and 2-HCBSALCS were 0.334, 0.302, 0.442 and 0.346 mg/mL, respectively. These results are higher than ascorbic acid (Vc), for the IC_{50} of Vc is higher than 0.711 mg/mL. The antioxidant activity of HCBSACS increased notably than chitosan (HCS and LCS). The mechanism is the grafted 4(or 2)-hydroxyl-5-chloride-1,3-benzene-di-sulfanimide group containing active OH group, which can react with hydroxyl radical to form stable macromolecular radicals. Besides, CS has many hydrogen bonds on O_3—O_5 and N_2—O_6. When CS reacted with 4(or 2)-hydroxyl-5-chloride-1,3-benzene-di-sulfo-chloride, the intramolecular and intermolecular hydrogen bonds would be weak. Therefore, the hydroxyl groups in the polysaccharide unit can react with $^{\bullet}OH$ by the typical H-abstraction reaction, and $^{\bullet}OH$ can react with the residual free amino groups $-NH_2$ to form stable macromolecular radicals. The NH_2 groups can form ammonium groups NH_3^+ by absorbing hydrogen from the solution, then reacting with $^{\bullet}OH$ through addition reaction [8]. The above theory explains why the scavenging activities of HCBSACS toward hydroxyl radicals were stronger. Besides, the antioxidant activity of low molecular weight derivatives (HCBSALCS) was stronger than high molecular weight derivatives (HCBSAHCS). This result may be caused by intramolecular and intermolecular hydrogen bonds of HCBSALCS that were weaker than that of HCBSAHCS, for HCBSALCS have less-compact structure, and the effect of the intramolecular hydrogen bonds is weak, so hydroxyl radical can react with free hydroxyl and amino group in chitosan. Furthermore, 4-HCBSACS showed higher antioxidant activity on hydroxyl radical than 2-HCBSACS, this result may be caused by steric effect, and steric hindrance of hydroxyl group of 4-HCBSACS was higher than that of 2-HCBSACS. All the results indicated that 2-(4(or 2)-hydroxyl-5-chloride-1,3-benzene-di-sulfanimide)-chitosan has stronger scavenging effect than chitosan. It may be helpful for chitosan employed extensively in medical field.

3.3. Scavenging activity of superoxide radical by Vc, CS and HCBSACS

Although relatively a weak oxidant, superoxide exhibits limited chemical reactivity, but can generate more dangerous species, including singlet oxygen and hydroxyl radicals, which cause the peroxidation of lipids [10]. In the present study, 2-(4(or 2)-hydroxyl-5-chloride-1,3-benzene-di-sulfanimide)-chitosan effectively scavenged superoxide in a concentration-dependent manner. Fig. 4 showed that the inhibitory effect of all kinds of 2-(4(or 2)-hydroxyl-5-chloride-1,3-benzene-di-sulfanimide)-chitosan on superoxide radicals was marked and concentration related. As shown in Fig. 4, the scavenging effect of HCS, LCS, 4-HCBSAHCS, 4-HCBSALCS, 2-HCBSAHCS and 2-HCBS ALCS is 28.44, 40.7, 89.66, 97.91, 87.59 and 96.82% at 0.40 mg/mL, respectively. Their orders of scavenging activities on superoxide radicals were: 4-HCBSALCS > 2-HCBSALCS > 4-HCBSAHCS > 2-HCBSAHCS > LCS > HCS. These results showed that the scavenging activity of superoxide radical by HCBSACS was stronger than CS. In addition, low molecular weight derivatives showed higher scavenging activity than high molecular ones. Furthermore, all kinds of derivatives had stronger scavenging activity for superoxide radical than Vc, for the scavenging effect of Vc is 33.86% at 0.40 mg/mL. Ji et al. [11] had reported that superoxide radical is a zwitterionic radical. It could react with free hydroxyl and amino groups in chitosan and its derivatives. So the mechanism of these results is that the inner structure of chitosan was severely disrupted by the introduction of grafted polymer chains after modification. The ability to form hydrogen bond declines sharply, and the hydroxyl and amino groups are activated, so this is helpful to the reaction with superoxide anion. Moreover, the grafted 4(or 2)-hydroxyl-5-chloride-1,3-benzene-di-sulfanimide group contained active —OH, which can react with superoxide anion. In conclusion, these results suggested that the antioxidant activity of HCBSACS was related to its ability to scavenge superoxide radical.

3.4. Reducing power of CS and HCBSACS

Fig. 5 depicts the reducing power of all kinds of chitosan and 2-(4(or 2)-hydroxyl-5-chloride-1,3-benzene-di-sulfanimide)-chitosan. The reducing power of all the compounds

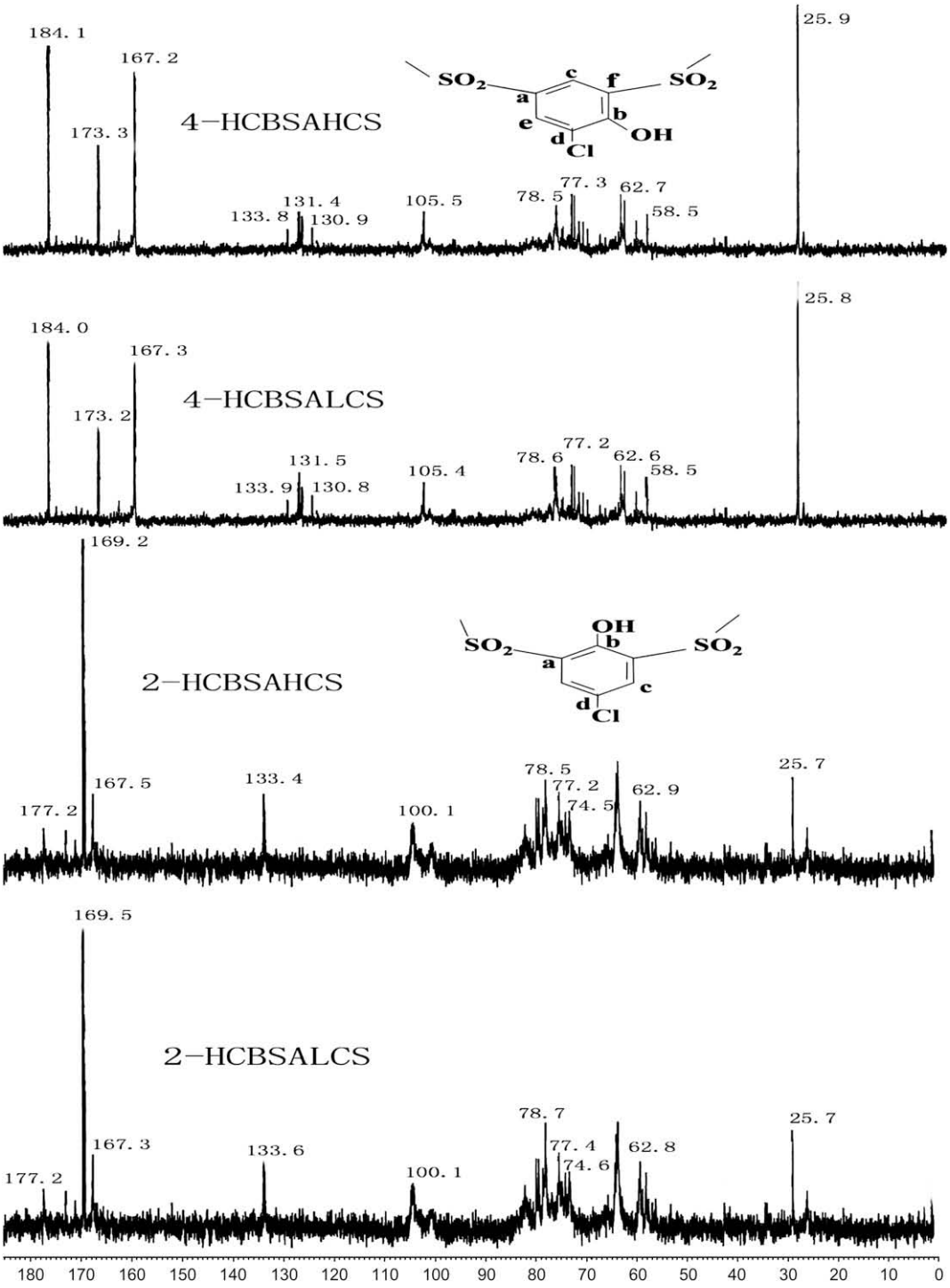


Fig. 2. ^{13}C NMR spectra of HCBSACS.

correlated well with increasing concentration, and they have quite a well linear relation. The linear related coefficients of HCS, LCS, 4-HCBSAHCS, 4-HCBSALCS, 2-HCBSAHCS and 2-HCBSALCS are 0.99284, 0.99171, 0.98622, 0.95094, 0.99276 and 0.98632, respectively. Moreover, the reducing power of 2-(4(or 2)-hydroxyl-5-chloride-1,3-benzene-disulfanimide)-chitosan was relatively more pronounced than that of chitosan. Low molecular weight derivatives had obviously more reducing powers than high molecular weight ones. In addition, the reducing powers of 4-HCBSACS were stronger than 2-HCBSACS. All the data indicated that the 4(or 2)-hydroxyl-5-chloride-1,3-benzene-disulfanimide group polymerized on chitosan increased its reducing power obviously. Mau et al. [12] reported reducing powers were 0.80, 0.89 and 0.92 at 1.0 mg/mL for ascorbic acid. As shown in Fig. 5, the reducing powers of 4-HCBSALCS and

Table 1
The elemental analysis results, yield and the grafted degree of chitosan derivatives

Compounds	Yield (%)	Color	Elemental analysis (%)				Grafted degree (%)
			C	N	H	S	
CS	–	White	44.28	8.52	7.36	–	–
4-HCBSAHCS	73.92	Yellow	38.60	5.45	4.82	9.41	75.6
4-HCBSALCS	69.82	Orange	39.39	5.34	4.73	9.73	79.8
2-HCBSAHCS	67.23	Yellow	38.71	5.51	4.87	9.22	73.2
2-HCBSALCS	65.38	Orange	38.52	5.40	4.78	9.54	77.2

2-HCBSALCS were higher than that of ascorbic acid. Earlier authors [13] have observed a direct correlation between antioxidant activity and reducing power of certain plant extracts. The reducing properties are generally associated with the presence of reductones [14], which have been shown to exert antioxidant action by breaking the free radicals' chain by donating a hydrogen atom [15]. Reductones are also reported to react with certain precursor of peroxide, thus preventing peroxide formation. Our results mainly caused by the 4(or 2)-hydroxyl-5-chloride-1,3-benzene-di-sulfanimide group grafted on CS contained active −OH groups, which can react with certain precursor of peroxide, explaining the obvious increase in derivatives' reducing power. Our results on the reducing power of HCBSACS suggested that it was likely to contribute significantly toward the observed antioxidant effect.

3.5. Chelating activity

The ferrous ion chelating effect of the derivatives is shown in Fig. 6. The chelating effect of them was low, especially when compared to EDTA. This result may be caused by $-NH_2$ group in chitosan that had reacted with 4(or 2)-hydroxyl-5-chloride-1,3-benzene-di-sulfo-chloride, and the chelating effect of −OH contained in the 4(or 2)-hydroxyl-5-chloride-1,3-benzene-di-sulfanimide group being lower than that of $-NH_2$ group.

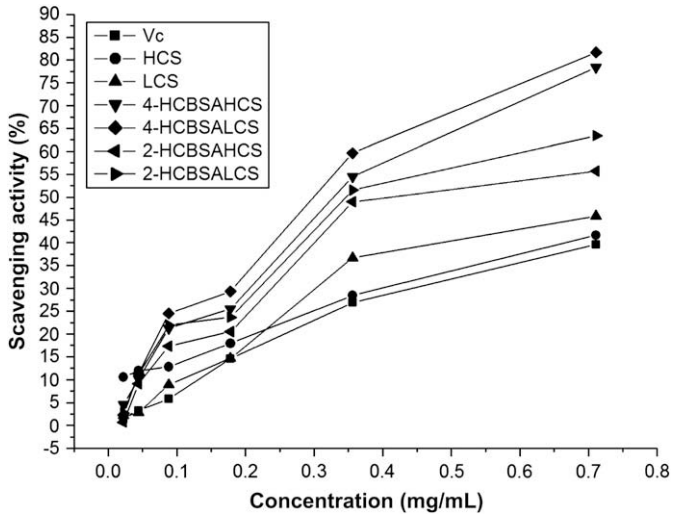


Fig. 3. Scavenging effects of Vc, CS and HCBSACS on hydroxyl radical.

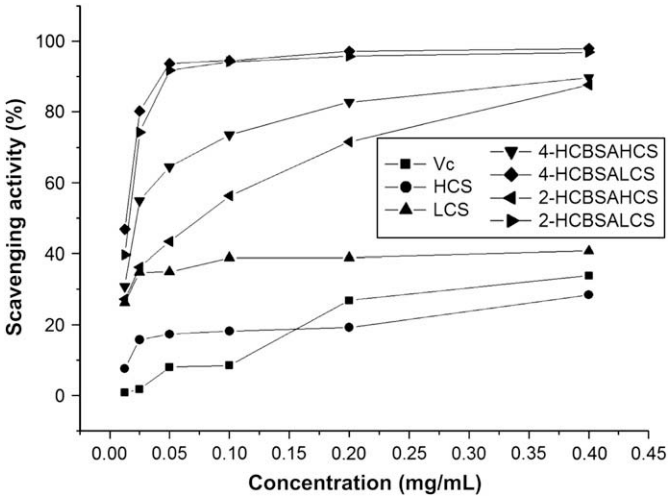


Fig. 4. Scavenging effects of Vc, CS and HCBSACS on superoxide radical.

4. Conclusion

In this paper 2-(4(or 2)-hydroxyl-5-chloride-1,3-benzene-di-sulfanimide)-chitosan was prepared. Their antioxidant activities were studied in the experiment, and several satisfying results were obtained as follows: firstly, all the derivatives possessed obviously greater antioxidant activities and free radical scavenging activities than chitosan. Secondly, low molecular weight 2-(4(or 2)-hydroxyl-5-chloride-1,3-benzene-di-sulfanimide)-chitosan had stronger scavenging effect on $O_2^{\bullet-}$ and $^{\bullet}OH$ than high molecular ones, and the reducing powers were more pronounced also. Thirdly, different position of −OH in the 4(or 2)-hydroxyl-5-chloride-1,3-benzene-di-sulfanimide group had effect on the antioxidant activity of the derivatives. All the results indicated that the 4(or 2)-hydroxyl-5-chloride-1,3-benzene-di-sulfanimide group polymerized on chitosan can increase its antioxidant activity obviously. These assays had important applications for the pharmaceutical and food industries.

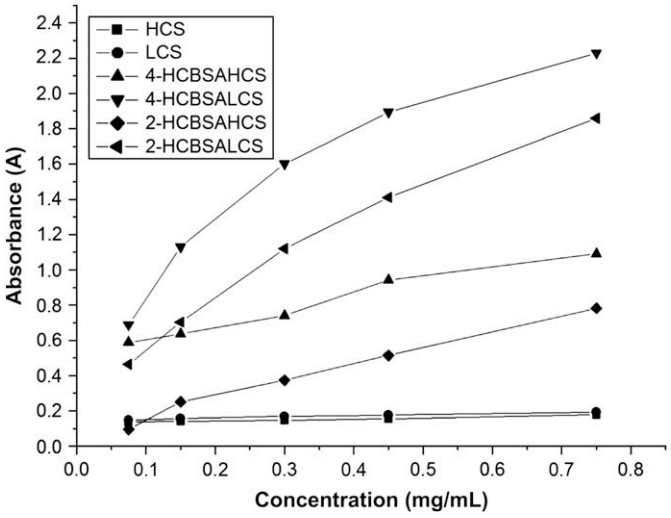


Fig. 5. Reducing power of CS and HCBSACS. Each value is expressed as mean ± SD ($n = 3$).

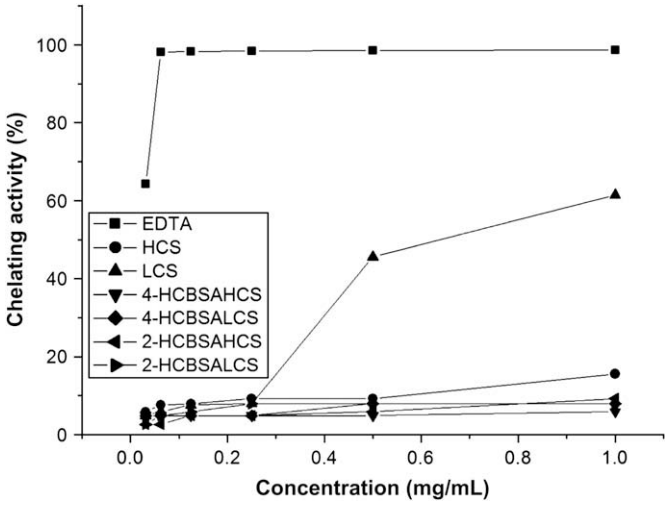


Fig. 6. Chelating effect of EDTA, CS and HCBSACS on ferrous ions.

5. Experimental

5.1. Materials

High molecular weight chitosan (HCS) is a commercial material supplied by Qingdao Baicheng Biochemical Corp. (China). It has deacetylation of 96%, average molecular weight (MW) 200,000 Da. Water-soluble chitosan (LCS) with a molecular weight of 7000 Da was prepared in our laboratories by the method of acetic acid and hydrogen peroxide (H_2O_2) hydrolysis. Nitro blue tetrazolium (NBT), phenazine mothosulfate (PMS), hydrogen peroxide (H_2O_2), thiobarbituric acid (TBA), ethylene diamine tetra-acetic acid (EDTA), nicotinamide adenine dinucleotidereduced (NADH), trichloroacetic (TCA), ferrozine, potassium ferricyanide and ferric chloride were purchased from Sigma Chemicals Co. All other chemicals and reagents were of analytical grade and used without further purification.

5.2. Analytical methods

Fourier transform infrared (FT-IR) spectra of the derivatives were measured in the 4000–400 cm^{-1} region using a Nicolet Magna-Avatar 360 FT-IR spectrometer with KBr disks. ^{13}C NMR spectra were recorded on a Jam-Ecp600 (600 MHz) NMR spectrometer, in D_2O solvent. The elemental analysis (C, H, N) was performed on a Carlo-Erba 1106 elemental analyzer. Sulfate content % was measured in a SC-132 sulfur meter (LECO). The average viscometric molecular weight of chitosan and all the derivatives was estimated from the intrinsic viscosity determined in the solvent 0.1 M CH_3COOH/0.2 M NaCl using the Mark–Houwink parameter $\alpha = 0.96$, $K_\eta = 1.424$ at 25 °C when the intrinsic viscosity is expressed in mL g^{-1}.

5.3. The preparation of HCBSACS

HCBSACS were synthesized as shown in Scheme 1. Chitosan (3 mmol) with two different molecular weights was dissolved in 30 mL formamide. When temperature of the water bath reached 70 °C, a formamide solution containing 2 mmol 4-hydroxyl-5-chloride-1,3-benzene-di-sulfo-chloride or 2-hydroxyl-5-chloride-1,3-benzene-di-sulfo-chloride was added to the system. After stirring for 8 h, the reaction mixture was cooled to room temperature and poured into a beaker containing 400 mL acetone. After cooling at 4 °C for 10 h, the mixture of products was filtered through a Bucher funnel under reduced pressure. The precipitate was rinsed with acetone, Soxhlet-extracted with cyclohexane for 24 h. Then a yellow precipitate was produced.

5.4. Hydroxyl radical assay

The hydroxyl radicals' scavenging ability of CS and HCBSACS were assessed by the method of Halliwell et al. [16]. The scavenging ability was calculated as follows:

$$E\% = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}}} \times 100$$

5.5. Superoxide radical scavenging assay

The superoxide radicals' scavenging ability of CS and HCBSACS were assessed by the method of Nishikimi et al. [17]. The capability of scavenging superoxide radical was calculated using the following equation:

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

5.6. Measurement of reducing power

The reducing power of CS and HCBSACS was quantified by the method described earlier by Zhong et al. [7].

5.7. Metal ion chelating assay

The ferrous ion chelating potential of CS and HCBSACS was investigated according to the method of Decker and Welch [18]. The ability of HCBSACS to chelate ferrous ion was calculated using the following equation:

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

5.8. Statistical analysis

All data are expressed as mean ± SD. Data were analyzed by an analysis of variance ($P < 0.05$) and the means separated by Duncan's multiple range tests. The results were processed by computer programs: Origin (6.1).

Acknowledgements

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