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The preparation and antioxidant activity of the sulfanilamide derivatives of chitosan and chitosan sulfates

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Abstract—Chitosan (CS) and chitosan sulfates (CSS) with different molecular weight (Mw) were reacted with 4-acetamidobenzene sulfonyl chloride to obtain sulfanilamide derivatives of chitosan and chitosan sulfates (LSACS, HSACS, LSACSS, HSACSS). The preparation conditions such as different reaction time, temperature, solvent, and the molar ratio of reaction materials are discussed in this paper. Their structures were characterized by FTIR spectroscopy and elemental analyses. The antioxidant activities of the derivatives were investigated employing various established in vitro systems, such as hydroxyl-radical (·OH) superoxide anion ($O_2^{\cdot -}$) scavenging and reducing power. All kinds of the compounds (HCS, LCS, HCSS, LCSS, HSACS, LSACS, HSACSS, LSACSS) showed stronger scavenging activity on hydroxyl radical than ascorbic acid (Vc). The inhibitory activities of the derivatives toward superoxide radical by the PMS-NADH system were obvious. The experiment showed that the superoxide radical scavenging effect of sulfanilamide derivatives of chitosan and chitosan sulfates was stronger than that of original CS and CSS. All of the derivatives were efficient in the reducing power. The results indicated that the sulfanilamide group were grafted on CS and CSS increased the reducing power of them obviously.

1. Introduction

Chitosan (CS) is an abundant natural biopolymer obtained from the exoskeletons of crustaceans and arthropods which is a nontoxic copolymer consisting of β-(1,4)-2-acetamido-2-deoxy-D-glucose and β-(1,4)-2-anaino-2-deoxy-D-glucose units.[1] As a natural renewable resource, chitosan has a number of unique properties such as biocompatibility, biodegradability, nontoxicity, and antimicrobial activity, which have attracted much scientific and industrial interest in such fields as biotechnology, pharmaceutics, wastewater treatment, cosmetics, agriculture, food science, and textiles.[2–9] Although chitosan is soluble in aqueous dilute acids below pH 6.5, it is insoluble in water and most organic solvents. The poor solubility of chitosan is a major limiting factor to its utilization. Therefore, special attention has been paid to its chemical modification and depolymerization to obtain derivatives soluble in water over a wider pH range.[10]

Sulfanilamide derivatives were successfully employed as effective chemotherapeutic agents for the prevention and cure of bacterial infections in human biological systems.[11] Sulfonamide compounds are used to cure the bacterial infectious cells as they neither interfere with the development of specific antibodies as a response to infection nor the antigenic properties of the infective organism are significantly affected. These drugs act on the bacteria themselves and either prevent their growth (bacteriostatic) or act as germicides (bactericide) and have no effect on the smooth muscles, heart, blood pressure or respiration.[12] To the best of our knowledge, no people have studied the sulfanilamide derivatives of chitosan and chitosan sulfates. When sulfanilamide group was polymerized on chitosan, the solubility of chitosan increased in wide pH range. We synthesized new sulfanilamide derivatives of chitosan (SACS) and sulfanilamide derivatives of chitosan sulfates (SACSS).

In recent years, there has been increasing interest in finding natural antioxidants, since they can protect progress

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of many chronic diseases.[13] The antioxidant activity of chitosan and its derivatives has attracted the most attention. Xing et al. had found that all kinds of sulfated chitosans possessed antioxidant activities and free radical scavenging activities.[14] Yin et al. reported that low molecular weight chitosan could scavenge superoxide radical and scavenging activity was 80.3% at 0.5 mg/mL.[15] In this paper, we evaluated the antioxidant activity of all compounds (high molecular weight (MW = 20 kDa) chitosan (HCS), low molecular weight (MW = 4 kDa) chitosan (LCS), high molecular weight chitosan sulfates (HCSS), low molecular weight chitosan sulfates (LCSS), 2-(4-acetamido-2-sulfanilamide)-chitosan with high molecular weight (HSACS), 2-(4-acetamido-2-sulfanilamide)-chitosan with low molecular weight (LSACS), 2-(4-acetamido-2-sulfanilamide)-6-sulfo-chitosan with high molecular weight (HSACSS), 2-(4-acetamido-2-sulfanilamide)-6-sulfo-chitosan with low molecular weight (LSACSS)). The results indicated that new sulfanilamide group can increase the solubility and antioxidant activity of chitosan and chitosan sulfates.

2. Chemistry

Synthesis of the object chemicals is via the procedure as outlined in Scheme 1. 4-Acetamidobenzene sulfonyl chloride reacted with $-NH_2$ or –OH (C_6 position) group at chitosan or chitosan sulfates (CSS) with different molecular weight obtained SACS and SACSS. Structure of all the synthesized compounds was confirmed by FT-IR spectrometry and elemental analyses. Characterization data of the compounds are given in the text. The antioxidant activity of the compounds was evaluated in an aqueous system in vitro.

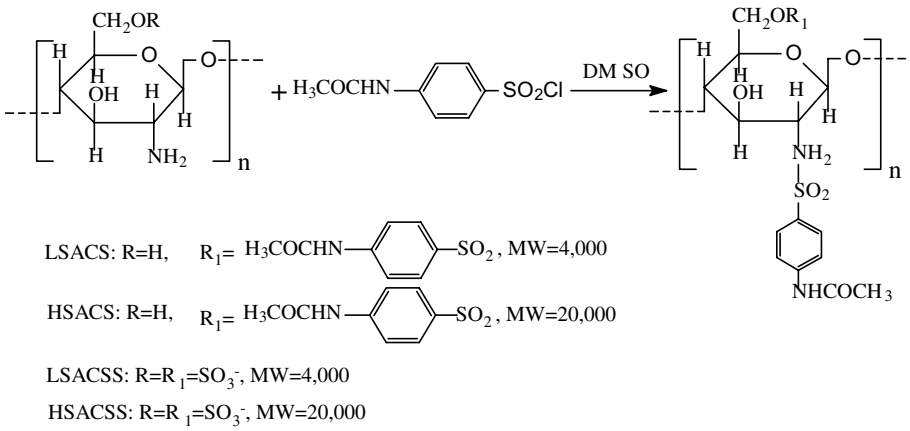
3. Results and discussion

3.1. Chemistry

3.1.1. Effect of reaction time, temperature, and the molar ratio of reaction materials on SACS and SACSS. Table 1 shows the results of all kinds of high molecular weight (MW = 20 kDa) sulfanilamide derivatives of chitosan (HSACS) under different solvent volume and molar ratio. When the volume of solvent and molar ratio changed, color of resultant, solubility in distilled water, yield, and molecular weight changed also. Yield increased and molecular weight decreased with the increase of molar ratio, but they changed slowly after molar ratio beyond 1:2. As a result, 1:2 is the proper molar ratio. Yield changed indistinctively when the volume of solvent changed, but the maximum yield appeared at 60 mL. Therefore, 60 mL is the excellent volume.

As shown in Table 1, it was possible to obtain a wide range of products of different molecular weight, yield, and color of resultant by changing only the time and temperature. An increase in reaction temperature caused a decrease in all the examined parameters such as yield and molecular weight. Yield and molecular weight decreased obviously when the temperature reached 70 °C. This result indicates that the high reaction temperature maybe caused the degradation of chitosan. Therefore, the following experiments were carried out at 65 °C. The yield and molecular weight changed with prolonging reaction time. Yield did not increase after 4 h, so 4 h is the proper time. All these results showed that the reaction depends on both reaction time and temperature.

Table 1 lists the results of high molecular weight (MW = 20 kDa) sulfanilamide derivatives of chitosan sulfates (HSACSS) under different solvent volume and molar ratio. It is possible that a wide range of products with different solubility, yield, molecular weight, and sulfur (which refers to the sulfur in C-6 sulfate) content were obtained. It was found that the sulfur content and molecular weight of products decreased as the molar ratio increased. When the molar ratio was beyond 2:3, sulfur content decreased quickly. It was perhaps caused by the pH value decrease as 4-acetamidobenzene sulfonyl chloride increased, and the chitosan sulfates might decompose at low pH value. We choose 2:3 as the best molar ratio in the experiment in order to get products with high-sulfate-content. In addition, the influence of



Scheme 1. Synthesis pathway of sulfanilamide derivatives of chitosan and chitosan sulfates.

Table 1. Change of HSACS and HSACSS at different solvent volume, molar ratio of reaction materials (*m*:*n* =CS (or CSS): 4-acetamidobenzene sulfonyl chloride), temperature, and time

Entry	DMSO (mL)	*m*:*n*	Time (h)	T (°C)	Color of resultant	Solubility	Yield (%)	Molecular weight ($\times 10^4$)	Sulfur content (%)
HSACS									
1	50	1:1	4	65	Yellow	Partially soluble	53.28	2.312	
2	50	2:3	4	65	Yellow	Partially soluble	54.10	2.295	
3	50	1:2	4	65	White	Soluble	83.61	2.268	
4	50	1:3	4	65	White	Soluble	82.32	2.189	
5	60	1:2	4	65	White	Soluble	84.65	2.256	
6	70	1:2	4	65	White	Soluble	82.97	2.254	
7	80	1:2	4	65	White	Soluble	81.56	2.263	
8	60	1:2	2	60	White	Partially soluble	81.99	2.313	
9	60	1:2	2	65	White	Soluble	80.98	2.298	
10	60	1:2	2	70	Yellow	Soluble	76.44	2.236	
11	60	1:2	2	75	Yellow	Soluble	73.97	2.069	
12	60	1:2	3	65	White	Soluble	80.87	2.156	
13	60	1:2	5	65	White	Soluble	81.42	2.098	
HSACSS									
14	50	1:1	4	65	White	Soluble	75.36	2.251	12.54
15	50	2:3	4	65	White	Soluble	79.65	2.223	12.35
16	50	1:2	4	65	White	Soluble	79.78	2.136	11.65
17	50	1:3	4	65	Yellow	Soluble	79.89	2.104	10.56
18	40	2:3	4	65	White	Soluble	79.23	2.219	12.23
19	60	2:3	4	65	White	Soluble	79.67	2.221	12.31
20	50	2:3	4	60	White	Soluble	77.37	2.163	11.98
21	50	2:3	4	70	Yellow	Soluble	76.69	2.004	10.23
22	50	2:3	5	75	Yellow	Soluble	74.01	1.986	9.55
23	50	2:3	3	65	White	Soluble	78.96	2.164	12.24

solvent volume was indistinctive in the experiment, and the proper volume is 50 mL.

As listed in Table 1, the sulfur content changed obviously with the change of time and temperature. 65 °C and 4 h were the proper reaction conditions for the reason of getting high-sulfate-content and high-yield product. The sulfanilamide derivatives of low molecular weight chitosan (LSACS, MW = 4 kDa) and chitosan sulfates (LSACSS, MW = 4 kDa) were prepared according to the optimal conditions of HSACS and HSACSS.

3.1.2. Structure and physicochemical characteristics of the compounds. Figure 1 presents the comparison of transmission FT-IR spectra data for SACS, SACSS with those for original CS and CSS. As for the FT-IR spectra of high molecular weight chitosan (HCS) with HSACS and low molecular weight chitosan (LCS) with LSACS are concerned, first, obvious translocation at 3500–3200 cm^{-1} due to the O–H and N–H group stretching vibration was observed, at the same time, the breadth of the peak narrowed comparing to the spectra of CS. In addition, the characteristic absorbance of $-NH_2$ at 1600 cm^{-1} changed to 1630 cm^{-1}. This is the result of O–H and N–H reacting with 4-acetamidobenzene sulfonyl chloride. Second, new peaks at about 1540 and 825 cm^{-1} appeared at the spectra of HSACS and LSACS which is the characteristic absorbance of phenyl-group. Third, there are new strong peaks at 1360 and 1165 cm^{-1} at SACS spectra, which are assigned to the characteristic absorbance of SO_2–N. All of the above

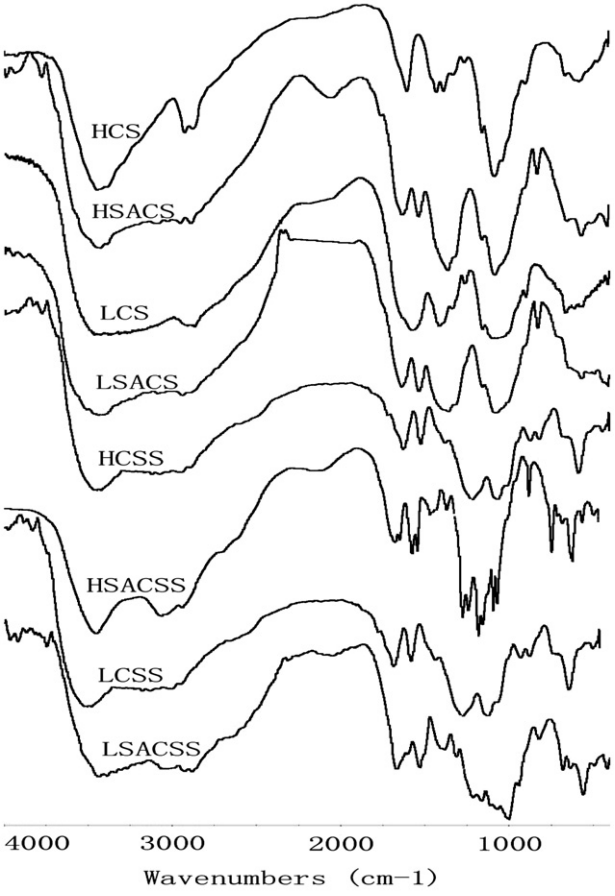


Figure 1. FT-IR spectra of the compounds.

results showed SACS were obtained. As for the spectra of SACSS and CSS were concerned, in the same reason, obvious translocation at 1630 and 3500–3200 cm^{-1} was observed, and new peaks at 1385, 1170, 1533.34, and 830 showed SACSS were also obtained. Above-mentioned results demonstrated that SACSS were formed successfully.

The results of elemental analyses, yield, and color of the sulfanilamide derivatives are listed in Table 2. The elemental analyses indicate that the C_6–O-substitution of CSS is about 0.147. Furthermore, the substitution degree of sulfanilamide group of HSACS, HSACSS is calculated as 0.45, but LSACS and LSACSS is 0.55 and 0.49, respectively, per glucosamine unit from the results of the elemental analyses.

3.2. Antioxidant activities

3.2.1. Hydroxyl radical scavenging activity of CS, CSS, SACS, and SACSS. 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 hydroxyl radical scavenging competes with deoxyribose for the resulted hydroxyl radicals and diminishes tint formation.[16] Above-mentioned model was used to measure inhibitory activities of all of the compounds on hydroxyl radicals. The result is shown in Table 3 and Figure 2. As shown in Figure 2, apart from HCS, LCS, LCSS, and HCSS, others had obvious scavenging activity. The scavenging rate increased with increasing concentration. IC_{50} of HSACS, LSACS, HSACSS, and LSACSS was 0.485, 0.262, 0.091, and 0.067 mg/mL, respectively. These results are stronger than that of ascorbic acid (Vc), for earlier reported that the Ic_{50} of Vc was 1.537 mg/mL.[14] The antioxidant activity of HSACS and LSACS increased notably than HCS and LCS. The scavenging effect on hydroxyl radical of LSACSS and HSACSS was nearly 100% at 0.356 mg/mL. This result was stronger than those of HCSS and LCSS. The mechanism is the grafted sulfanilamide group contained active NH group, which can react with hydroxyl radical to form stable macromolecular radicals. Chitosan has many hydrogen bonds on O_3–O_5 and N_2–O_6. When CS and CSS reacted with 4-acetamidobenzene sulfonyl chloride, the intramolecular and intermolecular hydrogen bonds would be weak. Therefore, the hydroxyl groups in the polysaccharide unit can react with ·OH by the typical H-abstraction reaction, and ·OH can react with the residual free amino groups $-NH_2$ to form stable macromolecule radicals. The NH_2 groups can form ammonium groups NH_3^+ by absorbing hydrion from the solution, then reacting with ·OH through addition reaction.[17] Above theory explains why the scavenging activities of SACS and SACSS toward hydroxyl radicals were stronger. Besides, the antioxidant activity of low molecular weight SACS and SACSS was stronger than that of the high molecular weight ones. This result may be caused by intramolecular and intermolecular hydrogen bonds of low molecular weight SACS and SACSS which were weaker than those of high molecular weight ones, for LSACS and LSACSS 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 chain. LSACS and LSACSS have more free hydroxyl and amino groups than HSACS and HSACSS, thus their scavenging activities toward hydroxyl radical were stronger. Furthermore, HSACSS and LSACSS showed

Table 2. The elemental analyses results and the color of CS, CSS, SACS, and SACSS

Compound	Elemental analyses			Yield (%)	Color
	C	N	H		
HCS	44.28	8.52	7.36		Ivory
HSACS	46.39	8.00	5.54	84.65	Orange
LCS	44.29	8.50	7.37		Yellow
LSACS	46.54	7.95	5.40	82.08	Brown
HCSS	41.44	8.06	6.91		Yellow
HSACSS	43.83	5.93	7.72	79.89	Brown
LCSS	41.43	8.05	6.92		Yellow
LSACSS	43.90	5.87	7.69	75.54	Brown

Table 3. The absorbance value of CS, CSS, SACS, and SACSS on hydroxyl radical

Sample	Concentration (mg/mL)				
	0.044	0.088	0.178	0.356	0.711
HCS	0.096 ± 0.0	0.099 ± 0.0	0.101 ± 0.001	0.103 ± 0.001	0.105 ± 0.001
LCS	0.146 ± 0.001	0.148 ± 0.0	0.149 ± 0.001	0.165 ± 0.0	0.231 ± 0.001
HCSS	0.104 ± 0.0	0.105 ± 0.001	0.105 ± 0.0	0.106 ± 0.001	0.107 ± 0.0
LCSS	0.116 ± 0.001	0.129 ± 0.001	0.158 ± 0.001	0.167 ± 0.0	0.247 ± 0.0
HSACS	0.117 ± 0.001	0.147 ± 0.001	0.149 ± 0.001	0.297 ± 0.0	0.697 ± 0.001
LSACS	0.176 ± 0.0	0.218 ± 0.0	0.306 ± 0.001	0.576 ± 0.001	0.985 ± 0.0
HSACSS	0.150 ± 0.001	0.442 ± 0.001	0.726 ± 0.001	0.898 ± 0.0	
LSACSS	0.332 ± 0.001	0.550 ± 0.001	0.809 ± 0.001	0.924 ± 0.0	

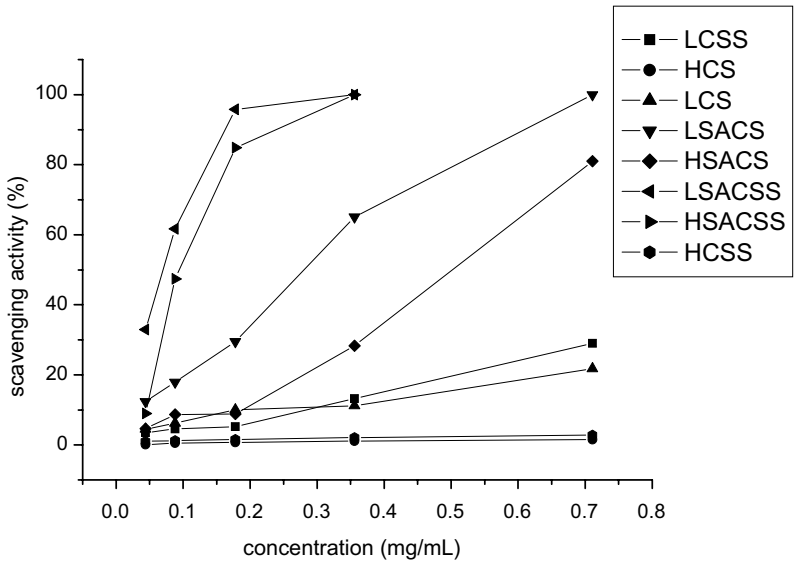


Figure 2. Scavenging effects of all kinds of compounds on hydroxyl radical.

higher scavenging activity than HSACS and LSACS. All of the results indicated that the sulfanilamide derivatives of chitosan or chitosan sulfates have stronger scavenging effect than chitosan or chitosan sulfates. It may be helpful for chitosan employed extensively in medical field.

3.2.2. Scavenging activity of superoxide radical by CS, CSS, SACS, and SACSS. Although relatively weak oxidants, superoxide exhibits limited chemical reactivity, but can generate more dangerous species, including singlet oxygen and hydroxyl radicals, which cause the peroxidation of lipids.[18] In the present study, sulfanilamide derivatives of chitosan or chitosan sulfates effectively scavenged superoxide radical in a concentration-dependent manner. Figure 3 and Table 4 show that the inhibitory effect of all kinds of compounds on superoxide radicals was marked and concentration related. As shown in Figure 3, the scavenging effect of HCS, LCS, HCSS, LCSS, HSACS, LSACS, LSACSS, and HSACSS is 32.13, 35.26%, 66.56%, 52.98%, 62.35%, 75.23%, 86.32%, and 66.56% at 0.80 mg/mL, respectively. Their orders of scavenging activities on superoxide radicals was: LSACSS > HSACSS > LSACS > HSACS > LCSS > LCS > HCSS > HCS. These results showed that the scavenging activity of superoxide radical by SACS and SACSS was stronger than that of CS and CSS. In addition, low molecular weight derivatives showed higher scavenging activity than high molecular weight ones. Furthermore, the scavenging effect of LSACSS and HSACSS was more pronounced than that of LSACS and HSACS, indicating that sulfates can enhance scavenging activity of superoxide radical. Xing et al.[14] reported that the scavenging activity of Vc for superoxide radical was only 25% at 1.75 mg/mL.

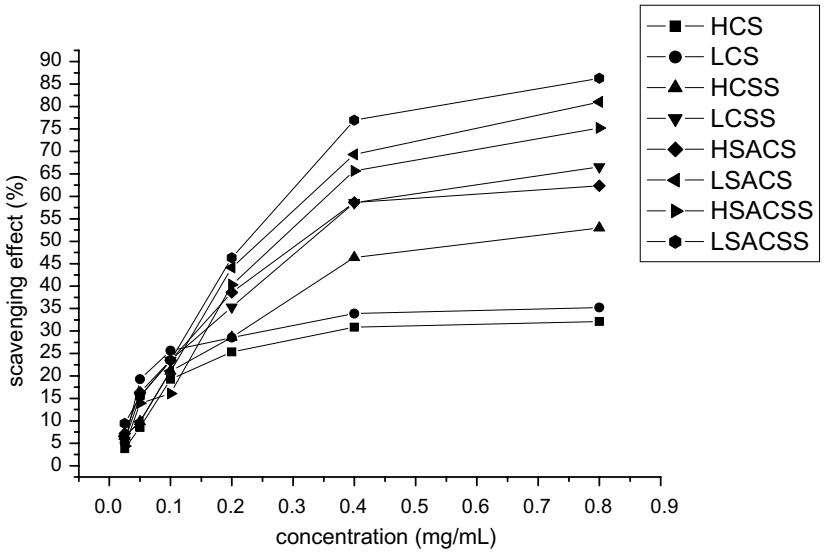


Figure 3. Scavenging effects of all kinds of compounds on superoxide radical.

Table 4. The absorbance value of CS, CSS, SACS, and SACSS on superoxide radical

Compound	Concentration (mg/mL)					
	0.025	0.050	0.100	0.200	0.400	0.800
HCS	1.064 ± 0.001	1.030 ± 0.001	0.886 ± 0.001	0.838 ± 0.001	0.793 ± 0.001	0.777 ± 0.001
LCS	0.995 ± 0.001	0.891 ± 0.001	0.838 ± 0.001	0.812 ± 0.001	0.755 ± 0.0	0.747 ± 0.0
HCSS	1.005 ± 0.001	0.935 ± 0.0	0.865 ± 0.0	0.812 ± 0.0	0.651 ± 0.002	0.577 ± 0.0
LCSS	0.988 ± 0.002	0.926 ± 0.0	0.900 ± 0.001	0.739 ± 0.001	0.490 ± 0.0	0.455 ± 0.0
HSACS	0.999 ± 0.001	0.922 ± 0.001	0.910 ± 0.002	0.750 ± 0.001	0.490 ± 0.001	0.487 ± 0.001
LSACS	1.055 ± 0.002	0.952 ± 0.0	0.930 ± 0.001	0.651 ± 0.001	0.400 ± 0.0	0.302 ± 0.0
HSACSS	1.021 ± 0.0	0.991 ± 0.001	0.936 ± 0.001	0.681 ± 0.0	0.431 ± 0.0	0.350 ± 0.0
LSACSS	0.988 ± 0.001	0.926 ± 0.001	0.900 ± 0.0	0.619 ± 0.001	0.304 ± 0.0	0.208 ± 0.0

Compared to this result, all kinds of compounds had stronger scavenging activity for superoxide radical than Vc. 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. In conclusion, these results suggested that the antioxidant activity of sulfanilamide derivatives of chitosan and chitosan sulfates was related to its ability to scavenge superoxide radical.

3.2.3. Reducing power of CS, CSS, SACS, and SACSS. Figure 4 and Table 5 depict the reducing power of all kinds of compounds. The reducing power of them correlated well with increasing concentration. They have quite well-linear relation. The linear related coefficient of HCS, LCS, HCSS, LCSS, LSACS, HSACS, LSACSS, and HSACSS is 0.99672, 0.99736, 0.99456, 0.99823, 0.99769, 0.99853, 0.99703, and 0.99822. Moreover, the reducing power of the SACS and SACSS was relating more pronounced than CS and CSS. Low molecular weight derivatives had obvious reducing power than high molecular weight ones. In addition, the reducing power of HSACSS and LSACSS was stronger than that of HSACS and LSACS. All of the data indicated that the sulfanilamide group was polymerized on CS and CSS increasing the reducing power of them obviously. Mau et al.[19] reported reducing powers were 0.80, 0.89, and 0.92 at 1.0 mg/mL for ascorbic acid. However, as shown in Figure 4 and Table 4, the reducing power of chitosans and sulfanilamide chitosan with different molecular weight was lower than that of ascorbic acid. Earlier authors[20] 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,[21] which have been shown the exert antioxidant action by breaking the free radicals chain by donating a hydrogen atom.[22] Reductones are also reported to react with certain precursors of peroxide, thus prevent peroxide formation. Our results were mainly caused by the sulfanilamide group grafted on CS and CSS containing active –NH groups, which can react with certain precursors of peroxide, explaining the derivatives' reducing power increases obviously. Our results on the reducing power of SACS and SACSS suggested that it likely contributed significantly toward the observed antioxidant effect.

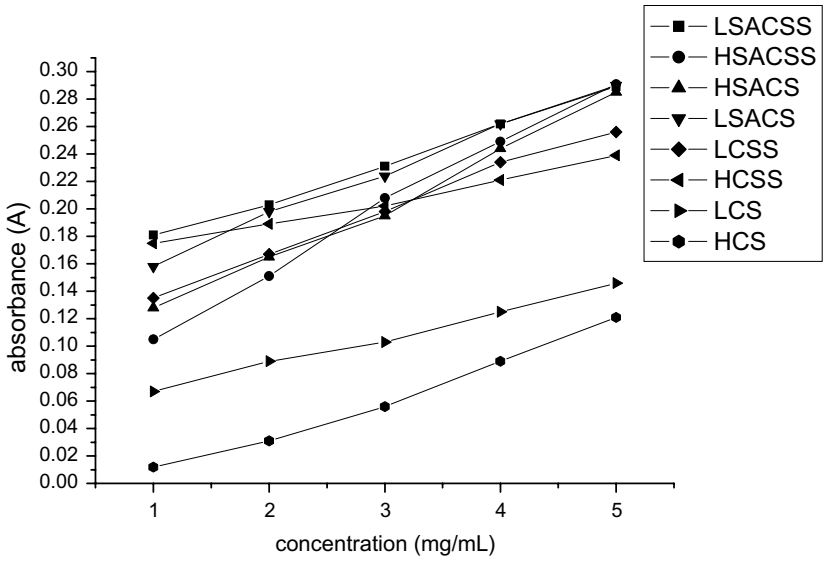


Figure 4. Reducing power of all kinds of compounds (each value is expressed as mean ± SD (n = 3)).

Table 5. The absorbance value of the reducing power of CS, CSS, SACS, and SACSS

Compound	Concentration (mg/mL)				
	1.00	2.00	3.00	4.00	5.00
HCS	0.012 ± 0.001	0.031 ± 0.001	0.056 ± 0.001	0.089 ± 0.001	0.121 ± 0.001
LCS	0.067 ± 0.001	0.089 ± 0.001	0.103 ± 0.001	0.125 ± 0.001	0.146 ± 0.001
HCSS	0.175 ± 0.001	0.189 ± 0.0	0.202 ± 0.001	0.221 ± 0.001	0.239 ± 0.002
LCSS	0.135 ± 0.001	0.167 ± 0.001	0.198 ± 0.001	0.234 ± 0.001	0.256 ± 0.001
HSACS	0.128 ± 0.001	0.165 ± 0.001	0.195 ± 0.002	0.244 ± 0.001	0.285 ± 0.0
LSACS	0.158 ± 0.001	0.198 ± 0.001	0.224 ± 0.001	0.262 ± 0.001	0.290 ± 0.002
HSACSS	0.105 ± 0.001	0.151 ± 0.001	0.208 ± 0.001	0.249 ± 0.001	0.291 ± 0.001
LSACSS	0.181 ± 0.0	0.203 ± 0.001	0.231 ± 0.002	0.262 ± 0.001	0.289 ± 0.001

4. Conclusion

In this paper, new sulfanilamide derivatives of chitosan or chitosan sulfates were prepared according to the optimal synthesis conditions: DMSO as solvent, reactions were carried out at 65 °C, the proper reaction time was 4 h. The molar ratio of SACS was 1:2, and that of SACSS was 2:3. Furthermore, as expected, we obtained several satisfying results of the antioxidant action of the compounds, as follows: First, all kinds of sulfanilamide derivatives of chitosan and chitosan sulfates possessed obvious antioxidant activities and free radical scavenging activities than original chitosan and chitosan sulfates. Second, low molecular weight sulfanilamide derivatives of chitosan and chitosan sulfates had stronger scavenging effect on $(O_2^{\cdot -})$ and ·OH than high molecular weight ones, and the reducing power was more pronounced also. Third, the antioxidant action of sulfanilamide derivatives of chitosan sulfates was stronger than that of sulfanilamide derivatives of chitosan. All of the results indicated that the sulfanilamide group polymerized on chitosan and chitosan sulfates can increase their antioxidant activity obviously. These assays had important applications for the pharmaceutical and food industries.

5. Experimental

5.1. General procedure for chemistry

5.1.1. Materials. High molecular weight chitosan is a commercial material supplied by Qingdao Baicheng Biochemical Corp. (China). It has deacetylation of 96%, average molecular weight (MW) 20 kDa. Water-soluble chitosan with a molecular weight of 4 kDa was prepared in our laboratories by the method of acetic acid and hydrogen peroxide (H_2O_2) hydrolysis. Chitosan sulfates were prepared according to previous work.[23] Nitro blue tetrazolium (NBT), phenazine mothosulfate (PMS), hydrogen peroxide (H_2O_2), thiobarbituric acid (TBA), ethylene diamine tetraacetic acid (EDTA), nicotinamide adenine dinucleotidereduced (NADH), trichloroacetic acid (TCA), 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.

5.1.2. Analytical methods. Fourier transform infrared (FTIR) spectra of all of the compounds were measured in the 4000–400 cm^{-1} regions using a Nicolet Magna-Avatar 360 FT-IR spectrometer with KBr disks. The elemental analyses (C, H, N) were 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 of the derivatives was estimated from the intrinsic viscosity determined in the solvent 0.1 mol L^{-1} CH_3COOH/0.2 mol L^{-1} 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}.

5.2. General procedure for the synthesis of SACS and SACSS

Two grams (0.012 mol) of chitosan or 3.84 g (0.016 mol) of chitosan sulfates was dissolved in a proper volume dimethylsulfoxide (DMSO). When the temperature was 65 °C, a DMSO solution containing 5.58 g (0.024 mol) 4-acetamidobenzene sulfonyl chloride was added to the system. After stirring for 4 h, the reaction mixture was cooled to room temperature and poured into a beaker containing 400 mL acetone, giving a white precipitate. After placed at 4 °C for 10 h. The mixture of products was filtered through a Buchner funnel under reduced pressure. The precipitate was rinsed with acetone, redissolved in distilled water. The solution was dialyzed against distilled water for 48 h using a 3600 Da MW cut-off dialysis membrane. The product was then concentrated and lyophilized to give SACS or SACSS. The yield of SACS and SACSS varied according to different conditions including time, temperature, solvent, and the molar ratio of reaction materials. The sulfur content of SACSS changed from 9.55 to 12.54.

5.2.1. 2-(4-Acetamidobenzene sulfonyl)-chitosan with high molecular weight (HSACS). IR (Nicolet): 3427.95, 2883.64, 1627.83, 1531.15 (–Ar), 1358.32 (SO_2–N), 1163.02 (SO_2–N), 1078.69, 824.67 (–Ar).

5.2.2. 2-(4-Acetamidobenzene sulfonyl)-chitosan with low molecular weight (LSACS). IR (Nicolet): 3438.67, 2945.09, 1635.32, 1532.41 (–Ar), 1357.64 (SO_2–N), 1168.14 (SO_2–N), 1077.72, 823.33 (–Ar).

5.2.3. 2-(4-Acetamidobenzene sulfonyl)-6-sulfo-chitosan with high molecular weight (HSACSS). IR (Nicolet): 3443.54, 2915.41, 1645.56, 1528.13 (–Ar), 1358.46

(SO_2–N), 1222.00 (S=O), 1188.31 (SO_2–N), 1099.03, 819.06 (–Ar).

5.2.4. 2-(4-Acetamidobenzene sulfonyl)-6-sulfo-chitosan with low molecular weight (LSACSS). IR (Nicolet): 3436.59, 2886.71, 1669.28, 1533.34 (–Ar), 1386.32 (SO_2–N), 1221.19 (S=O), 1165.51 (SO_2–N), 1094.96, 826.01 (–Ar).

5.3. Bioactivity

5.3.1. Hydroxyl radical assay. The reaction mixture, containing all kinds of the prepared compounds (0.04–0.75 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 30 min at 37 °C.[24] The reaction was terminated by adding 1 mL TBA (1% W/V) and 1 mL 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 blank. Decreased absorbance of the reaction mixture indicated oxidation of deoxyribose. The scavenging ability was calculated as follows:

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

5.3.2. Superoxide-radical scavenging assay. The superoxide radical scavenging ability of the compounds was assessed by the method of Nishikimi et al.[25] The reaction mixture, containing sample(0.04–0.45 mg/mL), PMS (30 μM), NADH (338 μM), and NBT (72μM) 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 superoxide radical was calculated using the following equation:

$$\text{Scavenging effect } (\%) = (1 - \frac{A_{\text{sample560nm}}}{A_{\text{control560nm}}}) \times 100$$

5.3.3. Measurement of reducing power. The reducing power of the compounds was quantified by the method described earlier by Yen and Chen with minor modifications.[26] Briefly, 1 mL of reaction mixture, containing different concentrations of sample 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 of the reaction mixture indicated increased reducing power.

5.4. 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 tests. The results were processed by computer programs: Excel and Statistic software (1999).

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