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Carbohydrate Polymers 61 (2005) 148–154
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Preparation of high-molecular weight and high-sulfate content chitosans and their potential antioxidant activity in vitro

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Received 25 September 2004; accepted 21 April 2005
Available online 2 June 2005

Abstract

The influence of molecular weight and substitution degree of sulfated polysaccharides on their biological activity is considered in majority of works involving the anticoagulant or antiviral properties of these substances. Therefore, the present paper describes the effect of preparation conditions of sulfated chitosans on their molecular weight and sulfur content, such as different reaction time, acid solvent and temperature. Foregoing literature expounded the action of dichloroacetic acid (DCAA) as acid solvent in homogeneous reaction. However, DCAA is expensive and noxious, therefore, in the present paper cheap and non-noxious formic acid (88%) was in place of DCAA. Furthermore, during reaction formic acid was not dehydrated. Under formic acid we obtained the satisfying results that was higher yield and equivalent sulfur contents compared to DCAA. IR and ^{13}C NMR spectrums proved the structure of the resultant obtained under formic acid or DCAA to be same. Now, it has not been reported for formic acid as acid solvent in homogeneous reaction of chitosan sulfatation. In this present paper, we also determined antioxidant activity of high-molecular weight and high-sulfate-content chitosans (HCTS). The results showed that HCTS could scavenge superoxide and hydroxyl radical. Its IC_{50} is 0.012 and 3.269 mg/mL, respectively. It had obviously reducing power and slight chelating activity. The data obtained in in vitro models clearly establish the antioxidant potency of HCTS. It is a potential antioxidant in vitro.

Keywords: Chitosans; High-molecular weight; High-sulfate-content; Dichloroacetic acid; Formic acid; Reducing power; Chelating activity; Radical scavenging effect

1. Introduction

Chitosan [(1-4)-2-amino-2-deoxy-β-D-glucan] is found in all arthropods, in some other invertebrata (e.g. squid and cuttlefish) and in some microorganisms and is readily prepared from chitin by N-deacetylation with alkali. Chitosan shows some biological activities such as immunological activity (Lu, Shi, & Chen, 2001), antibacterical activity (Zheng, Zhu, & Sun, 2000). Moreover, chitosan itself is non-toxic and biodegradable. Therefore, chitosan is an appealing bioactive polymer for further development. Since chitosan itself is insoluble in water at neutral or high pH region, the application of chitosan is quite limited. Therefore, chemical modification of chitosan to provide water-soluble materials is of prime interest to generate novel biomaterials.

Among various derivatives, chitosan sulfates have attracted most attention because of their obvious anticoagulant activity, antitumor activity, etc. Moreover, the influence of molecular weight and substitution degree of sulfated polysaccharides on their biological activity is considered in majority of works involving the anticoagulant or antiviral properties of these substances (Murata, Saiki, & Matsuno, 1990). Previous studies have reported their methods of preparation. Xu and Xiao (1994) reported that chitosan was sulfated in sulfuric acid (95, 90, 80%) at −10–0 °C for 3 h, and molecular weight of the resulting product was 2.51×10^4; Wu, Li, Wu, Cao, Chen (2000) prepared chitosan sulfates with chlorosulfonic acid at 70 °C for 4 h; Vongchan, Sajomsang, and Subyen (2002) droped solvated chitosan to the sulfating complex (4.5 mL of $HClSO_3$ in

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doi:10.1016/j.carbpol.2005.04.007

cold DMF) and stirred at RT for 5 h. However, they did not systemically study the preparation conditions of chitosan sulfates. Therefore, the present paper describes the effect of preparation conditions of sulfated chitosans on their molecular weight and sulfur content and researched the influence of different acid solvent in homogeneous reaction. Foregoing literature (Gamzazade et al., 1997) expounded the action of DCAA as an acid solvent in homogenous reaction. However, in the present paper we use cheap and non-noxious formic acid to replace the expensive and noxious DCAA. Under formic acid we obtained the satisfying results that was higher yield, molecular weight and equivalent sulfur contents compared to DCAA. It was showed that it was possible to place formic acid instead of DCAA as an acid solvent of homogeneous reaction. The results of the chitosan sulfates were characterized by FTIR and ^{13}C NMR spectroscopy. Now, it has not been reported for formic acid as acid solvent in homogeneous reaction of chitosan sulfatation.

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 (Halliwell, Gutteridge, & Cross, 1992). Reactive oxygen species (ROS) in the forms of superoxide anion ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$) 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 (Halliwell & Gutteridge, 1990). 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 (Halliwell et al., 1992). 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 (Kinsella, Frankel, German, & Kanner, 1993). For example, Yin, Lin, Zhang, and Yang (2002) reported that low molecular weight chitosan could scavenge superoxide radicals, and its scavenging activity was 80.3% at 0.5 mg/mL. Esumi, Takei, and Yoshimura (2003) showed that gold–chitosan nanocomposites have an ability to depress the activity of hydroxyl radicals. In this paper, HCTS was studied as a new antioxidant. We found that it could scavenge superoxide/hydroxyl radicals and had obviously reducing power and slight chelating activity. It was a potential antioxidant in vitro.

2. Experimental

2.1. Materials and apparatus

Chitosan was bought from Qingdao Lida Sea Biochem. Corp. (Shandong, China). The degree of deacetylation was 87% and viscosity average molecular of 7.6×10^5. Nitro Blue tetrazolium (NBT), phenazine methosulphate (PMS), hydrogen peroxide (H_2O_2), thiobarbituric acid (TBA), ethylene diamine tetra-acetic acid (EDTA), ferrozine, nicotinamide adenine dinucleotide-reduced (NADH), trichloroacetic acid (TCA), and deoxyribose (DR) were purchased from Sigma Chemicals Co. Potassium ferricyanide and ferric chloride were purchased from Sigma Chemicals Co. All other chemicals and reagents including formic (88%), dichloroacetic acid (DCAA), unless otherwise specified, were not purified, dried or pretreated. Dialysis Membranes were bought from Sigma Chemicals, molecular weight cut-off was 8000 Da.

2.2. Analytical methods

FTIR spectra were measured by a Nicolet Magna-Avatar 360 with KBr disks; ^{13}C NMR spectra were recorded on a Bruker Apx 500 (500 MHz) NMR spectrometer, in D_2O solvent. Sulfate content% was measured in a SC-132 sulfur meter (LECO), the average viscometric molecular weight of sulfated chitosan was estimated from the intrinsic viscosity determined in the solvent 0.1 M CH_3COOH/0.2 M NaCl using the Mark–Houwink parameters $\alpha=0.96$, $K_\eta=1.424$ at 25 °C when the intrinsic viscosity is expressed in mL g^{-1}.

2.3. Preparation of sulfating reagent

Five milliliters of $HClSO_3$ was added dropwise with stirring to 30 mL *N*,*N*-dimethylformamide (DMF) which was previously cooled at 0–4 °C. The reaction mixture was stirred without cooling until the solution ($DMF\cdot SO_3$) reached room temperature.

2.4. Preparation of HCTS

Fifty milliliters of $DMF\cdot SO_3$ was added a 500 mL three-necked bottomed flask containing 50 mL of chitosan solution in a mixture of DMF–DCAA or DMF–formic acid with swirling to get gelatinous chitosan. Then the reaction was run at adequate temperature (40–60 °C) for 1–2.5 h, and 95% of EtOH (300 mL) was added to precipitate the product, giving a white precipitate. The mixture of products was filtered through a Buchner funnel under reduced pressure. The precipitate was washed with EtOH, then redissolved in distilled water, and the pH was adjusted to pH 7–8 with 2 M NaOH. The solution was dialyzed against distilled water for 48 h using a 8000 Da MW cut-off dialysis membrane. The product was then concentrated and lyophilized to give chitosan sulfate (2 g chitosan gave 2–3.7 g chitosan sulfates according to different conditions, including time, temperature and acid solvent) with sulfur content of 11.95–16.20%, which corresponds to a degree of sulfation of 1.82–2.46 per glucosamine unit.

2.5. Superoxide radical scavenging assay

The superoxide scavenging ability of HCTS was assessed by the method of Nishikimi, Rao, and Yagi (1972). The reaction mixture, containing HCTS (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 the superoxide radical was calculated using the following equation:

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

2.6. Hydroxyl radical scavenging assay

The reaction mixture containing HCTS (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 (Halliwell, Gutteridge, & Aruoma, 1987). The reaction was terminated by adding 1mL of TBA (1%, w/v) and 1mL 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.

2.7. Measurement of reducing power

The reducing power of HCTS was quantified by the method described earlier by Yen and Chen (1995) with minor modifications. Briefly, reaction mixture 1 mL, containing different concentration of HCTS 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 adding 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.

2.8. Metal ion chelating assay

The ferrous ion-chelating potential of HCTS was investigated according to the method of Decker and Welch (1990), wherein the Fe^{2+}-chelating ability of HCTS was monitored by measuring the ferrous iron–ferrozine complex at 562 nm. Briefly, the reaction mixture, containing HCTS of different concentrations, $FeCl_2$ (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 a positive control. The ability of HCTS to chelate ferrous ion was calculated using the following equation:

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

3. Results and discussion

3.1. Effect of acid solvent, time and temperature on HCTS

Table 1 gives the results of sulfated chitosan under different acid. Compared with the results of DCAA, which showed that the molecular weight of sulfated chitosan was 1.39×10^5, 1.27×10^5, 9.65×10^5, 5.62×10^5, sulfur content was 14.57, 14.17, 13.97, 13.19% and the biggest yield was 176.5% under 40, 45, 50, 55 °C, the result of sulfated chitosan under formic acid showed that the molecular weight of sulfated chitosan was almost equivalent as that under DCAA at below 50 °C and higher than that under DCAA beyond 50 °C. The results showed that the degradation of sulfated chitosan was accelerated under DCAA. Sulfur content of sulfated chitosan was almost the same under DCAA and formic acid. However, the yield of chitosan sulfates under formic acid was much higher than that under DCAA. Above-mentioned results showed formic acid as acid solvent took advantage over DCAA as acid solvent. Moreover, the price of formic acid was considerably lower (10.0 yuan/500 ml) than that of DCAA (92.4 yuan/500 ml) and formic acid was not dehydrated in this experiment, so cost of desiccation was saved and the-quality-price-rate was obviously improved. Previous studies had mainly on DCAA as acid solvent (Gamzazade et al., 1997), and none on formic acid as acid solvent.

As shown in Table 2 it was possible to obtain a wide range of products of different degree of substitution and molecular weight changing only the time or temperature. An increase in reaction time or temperature caused a decrease in all the examined parameters, such as the degree of sulfur substitution, yield and molecular weight. This indicated that

Table 1
Change of HCTS under different acid[a]

T(°C)	DCAA			Formic acid[b]		
	Yield (%)	Sulfur content (%)	Molecular weight ($\times10^4$)	Yield (%)	Sulfur content (%)	Molecular weight ($\times10^4$)
40	176.5	14.57	13.9	181.0	14.52	13.4
45	108.0	14.17	12.7	177.5	14.46	12.5
50	82.5	13.97	9.65	173.0	13.67	10.5
55	90.0	13.19	5.62	154.0	13.45	7.12

[a] Reaction was carried out with 2 g of chitosan, 50 mL of sulfating reagent (DMF·SO_3) and 1 h.
[b] Content of formic acid was 88% and not dehydrated in this experiment.

Table 2
Change of HCTS at different temperature and time[a]

Entry	Time	T (°C)	Color of resultant	Solubility	Yield (%)	Molecular weight ($\times 10^4$)	Sulfur content (%)
1	1	40	Pale yellow	Easy soluble	181.0	13.4	14.52
2	1	45	Pale yellow	Easy soluble	177.5	12.5	14.46
3	1	50	Pale yellow	Easy soluble	173.0	10.5	13.67
4	1	55	Pale yellow	Easy soluble	154.0	7.12	13.45
5	1.5	45	Pale yellow	Easy soluble	131.0	10.2	15.36
6	2.0	45	Pale yellow	Easy soluble	118.0	6.63	14.02
7	2.5	45	Pale yellow	Easy soluble	108.0	4.32	13.33

[a] Reaction was carried out with 2 g of chitosan, 50 mL of sulfating reagent (DMF·SO_3) and 45 °C under formic acid (content of formic acid was 88% and not dehydrated in this experiment).

in a prolonged reaction time or a improved temperature subsequent degradation and slight desulfur reactions can take place. All these results showed that the chitosan sulfatation depends on both reaction time and temperature.

The structure of sulfated chitosan was further investigated by means of FTIR and ^{13}C NMR spectrum (Figs. 1 and 2). In the FTIR spectrum (as shown in Fig. 1), characteristic absorptions at 1222 and 806 cm^{-1}, due to sulfo groups, were assigned to S=O and C–O–S bond stretching, respectively (Nishimura, Kai, & Shinada, 1998; Vongchan et al., 2002). These peaks proved that the result of chitosan sulfates was almost the same under DCAA and formic acid. ^{13}C NMR analysis showed the position of sulfur substitution. In the ^{13}C NMR spectrum (as shown in Fig. 2), the signals of the parent chitosan at 65 and 52 ppm were assigned to the groups of the C-6 hydroxyl group and the C-2 amido group, respectively. After chitosan sulfatation, the two peaks of chitosan were shifted to low field at 69 and 58 ppm, respectively. The existence of strong sulfated and the disappearance of unmodified (parent) signals indicated that the C-6 hydroxyl group and C-2 amido group were both completely sulfated. Two signals at 73 and 76 ppm belong to C-3 of the residue without sulfate and C-3 of the residue with sulfate, respectively. This shows that the C-3 hydroxyl groups were partly sulfated (Gamzazade et al., 1997; Nishimura et al., 1998). As expected, the C-3 hydroxyl groups were incompletly substituted because of steric hindhance limitations. Above-mentioned result showed that the sulfo groups had been successfully introduced to chitosan and testified that it is possible to formic acid as acid solvent in homogeneous reaction in place of DCAA.

3.2. Scavenging activity of superoxide radical by HCTS

In this paper, the product of 45 °C,1 h was chosen for antioxidant assay. Fig. 3 showed that the inhibitory effect of HCTS on superoxide radicals was marked and concentration related. A significant scavenging effect (27.93–97.21%) of superoxide radicals was evident at all tested concentration of HCTS (0.005–0.4 mg/mL). Moreover, as shown in Fig. 3, the scavenging activity of superoxide radicals had reached 90% at 0.05 mg/mL. Compared to low-molecular-weight chitosan and parent chitosan, show scavenging activities for superoxide radicals of 80.3 and 13% at 0.5 mg/mL, respectively

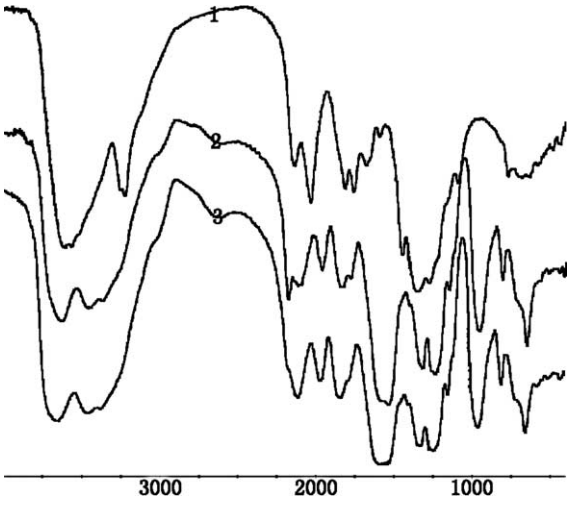

Fig. 1. FTIR of sulfated chitosan (1) chitosan; (2) sulfated chitosan under DCAA; (3) sulfated chitoan under formic acid.

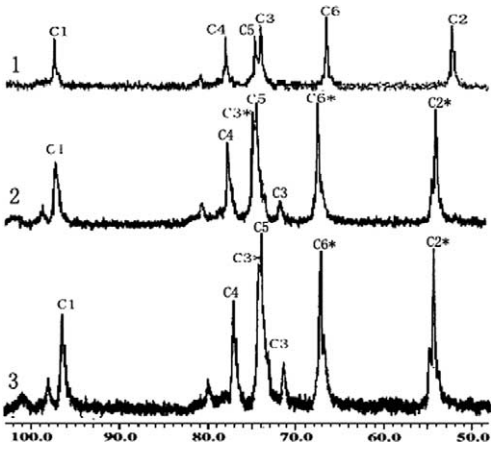

Fig. 2. ^{13}C NMR of sulfated chitosan (1) chitosan; (2) sulfated chitosan under DCAA; (3) sulfated chitoan under formic acid.

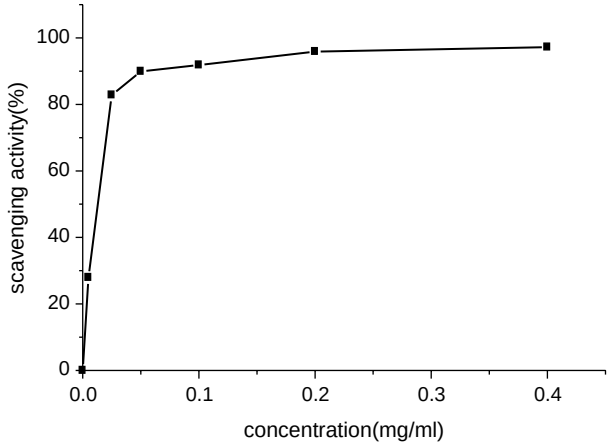

Fig. 3. Scavenging effect of HCTS on superoxide radical. Values are means ± SD of three determinations. HCTS (45 °C, 1 h, formic acid) was chosen for antioxidant assays.

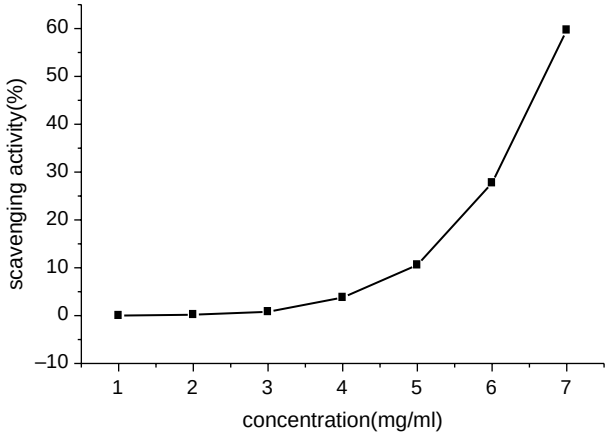

Fig. 4. Inhibitory effect of HCTS on deoxyribose oxidative damage. Values are means ± SD of three determinations. HCTS (45 °C, 1 h, formic acid) was chosen for antioxidant assays.

(Yin et al., 2002). Although superoxide is a relatively weak oxidant, it decomposes to form stronger reactive oxidative species, such as single oxygen and hydroxyl radicals, which initiate peroxidation of lipids (Dahl & Richardson, 1978). In the present study, HCTS effectively scavenged superoxide in a concentration-dependant manner. Further, superoxides are also known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals (Meyer & Isaksen, 1995). These results showed the HCTS has strong scavenging activity for the superoxide radical and clearly suggested that the antioxidant activity of HCTS was also related to its ability to scavenge superoxide radical.

3.3. Hydroxyl radical scavenging activity of HCTS

The effect of HCTS on oxidative damage, induced by Fe^{3+}/H_2O_2 on deoxyribose, as measured by the thiobarbituric acid method, is plotted in Fig. 4. Nearly 59.68% inhibition was observed at the highest concentration (3.2 mg/mL). Hydroxyl radical scavenging activity of HCTS was obtained in the deoxyribose system. In this system, HCTS exhibited a concentration-dependent inhibition of deoxyribose oxidation. Earlier, numerous workers (Halliwell et al., 1987) have employed this system to assess the biological activity of various natural plant-derived biomolecules. Smith, Hallivell, and Aruoma (1992) earlier reported that molecules that can inhibit deoxyribose degradation are those that can chelate iron ions and render them inactive or poorly active in a Fenton reaction. In the present study, in another assay system, we found HCTS had slight chelating effect. So, it is likely that the chelationg effect of HCTS on metal ions may not be responsible for the inhibition of deoxyribose oxidation. Therefore, the mechanism of HCTS on hydroxyl radicals needs to be further researched.

3.4. Chelating effects of HCTS on ferrous ions

The ferrous ion-chelating effect of HCTS was concentration related as shown in Fig. 5. The chelating effect of HCTS was low, especially compared to EDTA.

3.5. Reducing power of HCTS

Fig. 6 depicted the reducing power of HCTS. The reducing power of HCTS correlated well with increasing concentrations. Fig. 6 showed the reducing power increased with increasing HCTS concentration. Mau, Chang, Huang, and Chen (2004) reported that 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 Fig. 6, the reducing power of HCTS was 0.17 at 0.75 mg/mL. According to changed concentration trend we concluded that the reducing power of HCTS was lower than that of

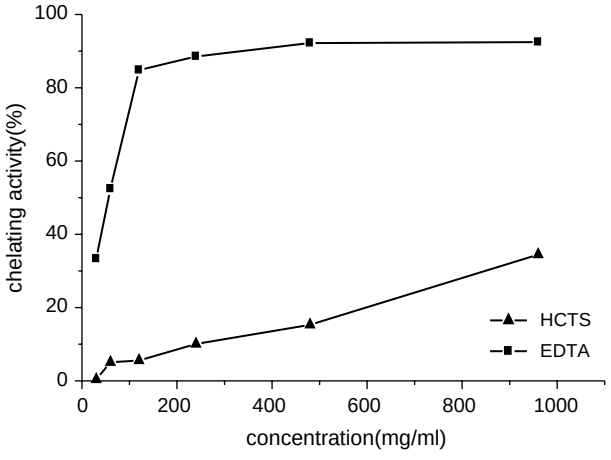

Fig. 5. Chelating effect of HCTS on ferrous ions. Each value is expressed as mean ± SD ($n = 3$), HCTS (45 °C, 1 h, formic acid) was chosen for antioxidant assays.

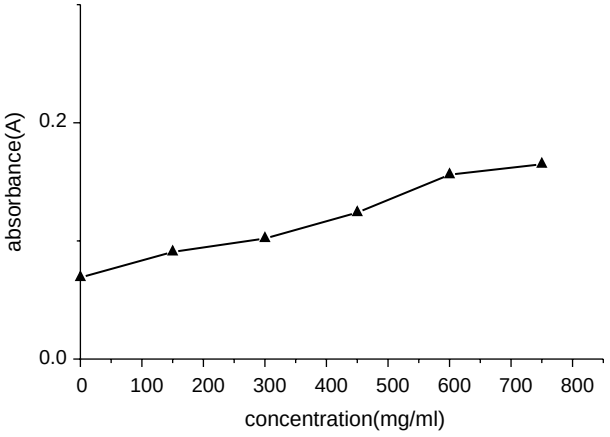

Fig. 6. Reducing power of HCTS. Each value is expressed as mean ± SD ($n = 3$), HCTS (45 °C, 1 h, formic acid) was chosen for antioxidant assays.

ascorbic acid, α-tocopherol and BHA. Earlier authors (Pin-Der Duh, Pin-Chan-Du, & Gow-Chin Yen, 1999) 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 (Pin-Der-Duh, 1998), which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Our data on the reducing power of HCTS suggested that it was likely to contribute significantly towards the observed antioxidant effect.

4. Conclusions

In this paper HCTS was prepared and their antioxidant activity in vitro were studied. Several features of HCTS are noted as follows:

1. In this paper, high sulfate content (16%) and high yield (181%) chitosans were obtained by bolting of experimental conditions, compared to previous research: 11% sulfate content (Huang, Du, Yang, & Fan, 2003) and 87% yield (Vongchan et al., 2002)
2. In this paper, cheap formic acid (10 yuan/500 ml) was in place of expensive DCAA (92.4 yuan/500 ml) (Gamzazade et al., 1997), moreover, formic acid was not dehydrated, so cost of desiccation was saved and the-quality-price-rate was obviously improved. Now, formic acid as acid solvent of chitosan sulfatation has not been reported.
3. HCTS could scavenge superoxide/hydroxyl radicals. Its IC_{50} was 0.012 and 3.269 mg/mL, respectively. Moreover, HCTS had obviously reducing power and slight chelating activity. It was a potential antioxidant.

Acknowledgements

The work was supported by the Qingdao Bureau of Science and Technology, China (Grant 02-1-KJ-SHN-24) and the Innovational Foundation of Chinese Academy of Sciences (KZCX3-SW-215).

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