Carbohydrate Polymers 85 (2011) 832–837

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Biochemical activities of N,O-carboxymethyl chitosan from squid cartilage

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ARTICLE INFO

Article history:
Received 5 February 2011
Received in revised form 1 April 2011
Accepted 1 April 2011
Available online 12 April 2011

Keywords:
Carboxymethylated chitosan
Degree of substitution
Squid cartilage
Bile acid
Antioxidant

ABSTRACT

Chitosan was prepared by alkaline *N*-deacetylation of squid cartilage β-chitin and carboxymethylated derivatives with different degrees of substitution (DS) were synthesized. The DS of the derivatives calculated by pH titration were 0.64, 0.81, 1.0, 1.33 and 1.59. The antioxidant properties and bile acid binding capacity of the derivatives were studied *in vitro*. The carboxymethylation of chitosan caused enhancement of bile acid binding capacity. At 1 mg/mL, carboxymethyl chitosan (CMCS) showed a stronger scavenging effect than chitosan towards DPPH radicals. The CMCS scavenging effect on superoxide radicals was stronger than that of chitosan, and EC_{50} values were below 5.6 mg/mL. The effectiveness of reducing power correlated with the DS of CMCS. At 1.2 mg/mL, the ability of CMCS to chelate ferrous iron was 100%, whereas that of chitosan was only 22.34%. This suggested that carboxymethylation is a possible approach to obtain chitosan derivatives with desirable biological properties.

1. Introduction

Chitin, a linear homopolysaccharide composed of 2-acetamide-2-deoxy-D-glucopyranose units linked by β-(1 → 4) bonds, occurs abundantly in the biomass (Mathur and Narang, 1990; Muzzarelli, 1988; Roberts, 1992). Chitosan (poly-β-1,4-linked glucosamine) is a cationic polysaccharide made from alkaline *N*-deacetylation of chitin. α-Chitin, the most abundant in nature, has a structure of antiparallel chains and is found in the crab, shrimp and lobster, whereas β-chitin, found in squid cartilage, has parallel chains joined through intrasheet hydrogen bonding (Minke and Blackwell, 1978). β-Chitin is characterized by weak intermolecular forces (Rudall, 1963) and has been confirmed to exhibit higher reactivity under various modification conditions as well as higher affinity for solvents than α-chitin (Kurita et al., 1993; Kurita, Ishii, Tomita, Nishimura, & Shimoda, 1994). It is noteworthy that chitosan prepared from β-chitin also exhibited higher reactivity compared to that of α-chitin (Kurita et al., 1993). These results suggest that chitosan derived from β-chitin may have potential as a novel functional biopolymer. Because of its excellent biological properties including the fact that it is readily biodegradable, acts as antioxidant and antibacterial, chitosan has been proposed for use in the biomedical, food, agriculture, biotechnology and pharmaceutical fields (Kofuji et al., 2005). However, the poor solubility of chitosan has limited its applications.

To extend the utilization of chitosan, various chemical modifications such as sulfonation (Holme and Perlin, 1997), quaternization (Britto and Assis, 2006; Jia, Shen, & Xu, 2001) and PEG-grafting (Ouchi, Nishizawa, & Ohya, 1998), have been widely carried out and the derivatives have been intensively studied for industrial as well as scientific interest. In particular carboxymethylation has often been applied to impart better water solubility to polysaccharides (Wang, Yu, & Mao, 2009). Based on the different substitution positions of the carboxymethyl group, carboxymethyl chitosan can be divided into several types: O-carboxymethyl chitosan, N-carboxymethyl chitosan, N,N-dicarboxymethyl chitosan and N,O-carboxymethyl chitosan (Mattioli-Belmonte et al., 1999; Muzzarelli, 1988). The substitution positions and the degree of carboxymethyl groups in the polymer chain will directly affect the properties of carboxymethyl chitosan (Muzzarelli, Tanfani, Emanuelli, & Mariotti, 1982).

Recently, the antioxidant activity of chitosan and its derivatives have attracted attention because of their nontoxic nature and natural abundance (Castagnino et al., 2008; Feng, Du, Li, Wei, & Yao, 2007). However, few studies on the antioxidant properties of carboxymethyl chitosan prepared from squid cartilage are available. In this paper, chitosan was prepared by alkaline *N*-deacetylation of squid cartilage β-chitin and five N,O-carboxymethyl chitosans (CMCS) with different degrees of substitution (DS) of carboxymethyls were synthesized in order to investigate their antioxidant activities and bile acid binding capacities. Antioxidant activity was investigated by scavenging DPPH, hydroxyl and superoxide anion radicals. As well, their reducing power, chelating ability against ferrous ions and

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doi:10.1016/j.carbpol.2011.04.007

Table 1
The reaction conditions, degrees of substitution, solubility, yield and the bile acid binding capacity of carboxymethyl chitosan.

Product	50% NaOH (ml)	Chloroacetic acid (g)	Temperature (°C)	Time (h)	Color	DS	Yield (%)	Bile acid binding capacity (mg/g)
Chitosan	–	–	–	–	White	–	–	14.02
CMCS1	2	3	40	2	White	0.64	75.64	52.05
CMCS2	4	4.5	40	4	White	0.81	95.85	43.37
CMCS3	4	3	60	5	White	1.00	114.54	58.95
CMCS4	5	3	70	3	Pale yellow	1.33	161.33	20.51
CMCS5	5	4.5	60	4	Pale yellow	1.59	183.69	18.14

the *in vitro* bile acid binding capacity of the derivatives were evaluated.

2. Materials and methods

2.1. Chemicals

Chitosan, degree of deacetylation 90%, was prepared from squid cartilage with a viscosity average-molecular weight of 6.52×10^5. Chloroacetic acid, isopropanol, bile acid (derived from taurocholate), furfural, 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide (H_2O_2), potassium ferricyanide ($K_3Fe(CN)_6$), trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), ferrozine, ethylene diamine tetra-acetic acid (EDTA), nitro blue tetrazolium (NBT), phenazine methsulfate (PMS), nicotinamide adenine dinucletide-reduced (NADH), ferrous sulfate ($FeSO_4$), and ascorbic acid were purchased from Aladdin-reagent Co., Ltd. (Shanghai, China). All other chemicals were analytical grade and used without further purification. All water used in extraction and analysis had been distilled and deionized.

2.2. Preparation of carboxymethyl chitosan

Carboxymethyl chitosan (CMCS) was prepared by the method of Liu, Guan, Yang, Li, and Yao (2001). Chitosan (1.5 g) was suspended in 15 mL solvent (water/isopropanol, 1:4, v/v) for 30 min, and then alkalization was carried out by adding 2–5 mL 50% NaOH at 40–70 °C for 1 h. Chloroacetic acid was dissolved in isopropanol, added into the reaction mixture dropwise over 30 min and the system was subjected to a continuous reaction for 2–5 h. The reaction was stopped by adding 70% ethanol and the pH was adjusted to neutrality with acetic acid. The reaction product was filtered, rinsed in 70–90% ethanol to desalt, and dried under vacuum at 60 °C. The products were N,O-carboxymethyl chitosans and the DS of the products are shown in Table 1.

2.3. FT/IR spectroscopy

A Nicolet FTIR spectrometer (Magna-IR 760 ESP, Nicolet Instrument Corp., Madison, WI) was employed to characterize the infrared spectra of chitosan and carboxymethyl chitosan.

2.4. Bile acid binding capacity assay

Based on the method of Muzzarelli et al. (2006) and Wang et al. (2009) with minor modifications, the effect of CMCS on the bile acid binding capacity was investigated *in vitro*. Sample (0.05 g) was mixed with 2 mL of 5 mg/mL bile acid, and the mixtures were adjusted to a total volume of 25 mL with distilled water. The mixtures were incubated for 2 h at 37 °C, and then filtered. The resulting samples (1.0 mL) were mixed with 1 mL 1% furfural and 45% sulfuric acid, and then the mixtures were incubated for 20 min at 70 °C, and the absorbance was measured at 605 nm.

2.5. DPPH radical scavenging ability

The effect of carboxymethyl chitosan on DPPH radical was measured using the modified method of Yamaguchi, Takamura, Matoba, & Terao (1998). A total of 5.0 mL of the ethanol solution of DPPH (50 mg/L) was incubated with 5.0 mL carboxymethyl chitosan samples at different concentrations (0.5–1.0 mg/mL). The reaction mixture was shaken thoroughly and incubated for 30 min at 33 °C, and the absorbance was measured at 517 nm against a blank. Ascorbic acid was used as the reference standard. The EC_{50} value (mg/mL) is the effective concentration at which DPPH radicals were scavenged by 50%. The radical scavenging activity was calculated using the following equation:

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

where A_{control} is the absorbance of the control (distilled water instead of carboxymethyl chitosan).

2.6. Hydroxyl radical scavenging ability

The scavenging ability on hydroxyl radicals was determined according to the method of Lee et al. (2005). The reaction mixture contained $FeSO_4$ (9 mM), salicylic acid (9 mM), carboxymethyl chitosan samples at different concentrations (1–6 mg/mL) and H_2O_2 (10 mM). The reaction mixture was shaken thoroughly and incubated for 1 h at 37 °C. The absorbance of the mixtures was measured at 510 nm against a blank. Ascorbic acid was used for comparison. The EC_{50} value (mg/mL) is the effective concentration at which hydroxyl radicals were scavenged by 50%.

2.7. Measurement of reducing power

The reducing power of carboxymethyl chitosan samples was measured by the method described by Oyaizu (1986). The reaction mixture contained different concentrations of CMCS samples (1 mL), 0.2 M sodium phosphate buffer pH 6.6 (2.5 mL) and 1% (w/v) potassium ferricyanide (2.5 mL). The mixtures were incubated for 20 min at 50 °C, and then 10% (w/v) TCA (2.5 mL) was added to the mixtures and then centrifuged at 4000 rpm for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and 0.1% (w/v) ferric chloride solution (0.5 mL). The absorbance values of the reaction mixtures were determined at 700 nm. The absorption indicated the intensity of reducing ability, and increased absorbance of the reaction mixture indicated increased reducing power.

2.8. Metal ion chelating assay

The chelating ability of carboxymethyl chitosan was determined according to the method of Carter (1971) with minor modifications. Each sample (0.2–2.0 mg/mL, 2.5 mL) was mixed with 9.25 mL of methanol, 0.25 mL of 1.51 mM $FeSO_4$ and 0.5 mL of 2.4 mM ferrozine. After 20 min at 37 °C, the absorbance of the mixtures was determined at 562 nm against a blank. A lower absorbance indicates

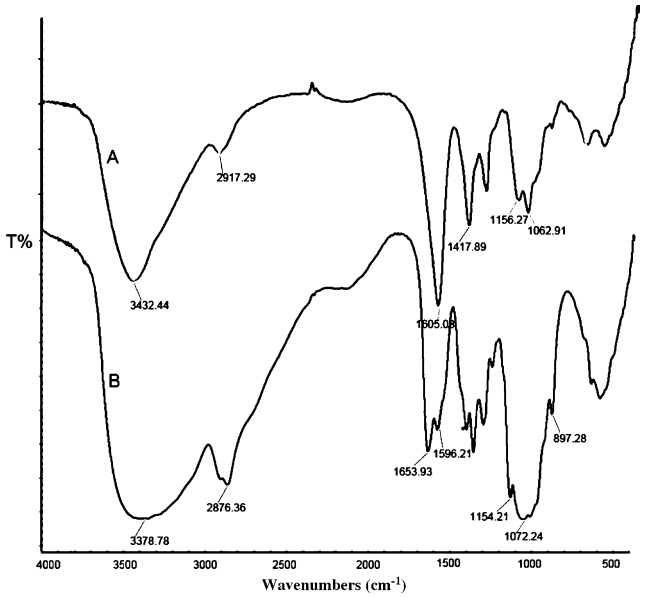

Fig. 1. IR spectra of carboxymethyl chitosan (CMCS1, degree of substitution 0.64) (A) and chitosan (B).

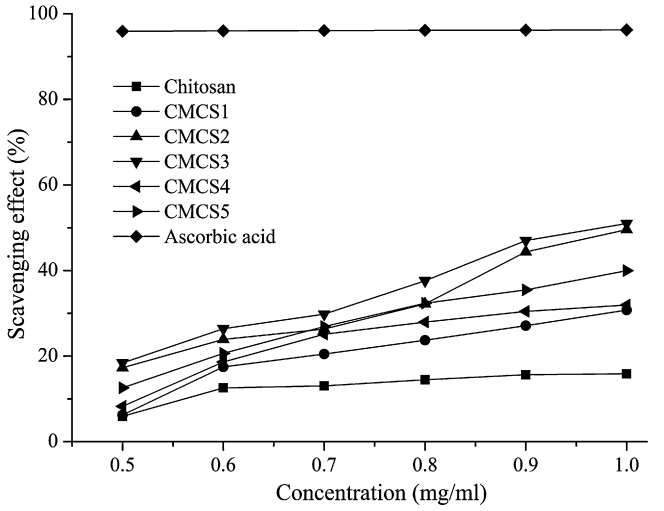

Fig. 2. Scavenging effect of chitosan and carboxymethyl chitosan (CMCS) with different degrees of substitution (DS) towards DPPH radicals. DS of CMCS1, CMCS2, CMCS3, CMCS4 and CMCS5 were 0.64, 0.81, 1.0, 1.33 and 1.59, respectively.

a higher chelating ability. EDTA was used for comparison. The ability of carboxymethyl chitosan to chelate ferrous ion was calculated using the following equation:

$$\text{chelating ability}(\%) = \left(1 - \frac{A_1}{A_0}\right) \times 100$$

where A_0 and A_1 were the optical density at 562 nm without and with samples, respectively.

2.9. Superoxide radical scavenging assay

The superoxide scavenging ability of carboxymethyl chitosan was assessed by the method of Li, Zheng, Liu, & Jia (1992) with some modifications. Each sample (1–5 mg/mL, 2.5 mL) were mixed separately with 2.5 mL of PMS (163 μM), 2.5 mL NADH (471.6 μM), and 2.5 mL of NBT (342.5 μM) in Tris–HCl buffer (0.05 M pH8.0). After 5 min at room temperature, the absorbance was measured and the capability of scavenging superoxide radical was calculated. The EC_{50} value (mg/mL) is the effective concentration at which superoxide radicals were scavenged by 50%.

2.10. Statistical analysis

All of the analyses were performed at least in triplicate. Each experimental data point represents the mean from three independent experiments. The deviation from the mean at the 95% significance level was used to determine the differences in biological activity.

3. Results and discussion

3.1. Infrared spectra analyses

Infrared spectroscopy has been used to determine the structure of chitin and chitosan (Muzzarelli, 1988). Fig. 1 displays the FT-IR spectrum of CMCS and chitosan. As shown in Fig. 1, the main absorption bands of chitosan were 3378.78 (O–H stretch), 2876.36 (C–H stretch), 1596.21 (N–H bend), 1154.21 (bridge O stretch) and 1072.24 cm^{-1} (C–O stretch). The peaks of chitosan at 898.07, 1072.24 and 1653.93 cm^{-1} belonging to the pyranose ring, glucoside and acetamide groups, respectively, were identifiable. After carboxymethylation, two new bands at 1605.03 cm^{-1} and 1417.89 cm^{-1} were observed, which were characteristic peaks of the stretching vibration of asymmetric and symmetric –COO- groups, respectively (Liu et al., 2001; Muzzarelli & Tanfani, 1982; Wang et al., 2009). Therefore, the FT-IR showed the existence of carboxymethyl groups in the derivative.

3.2. In vitro bile acid binding capacity

The *in vitro* bile acid binding capacities of chitosan and CMCS are shown in Table 1. All five CMCS derivatives had significantly higher bile acid-binding capacities than chitosan, implying more cholesterol-lowering effects. The binding capacity of CMCS1, CMCS2, CMCS3, CMCS4, and CMCS5 against bile acid were 52.05 mg/g, 43.37 mg/g, 58.95 mg/g, 20.51 mg/g and 18.14 mg/g, which were 3.7-fold, 3.1-fold, 4.2-fold, 1.5-fold, and 1.3-fold higher than that of chitosan, respectively. These data suggested the possible application of carboxymethyl chitosan as a cholesterol-lowering adjuvant.

As shown in Table 1, the bile acid-binding capacities of CMCS were higher than that of chitosan. Wu, Wang, & Ma (2004) reported that the bile acid-binding capacity of chitosan mainly depended on its cationic structure. Compared with chitosan, the inner structure of CMCS was severely disrupted by the introduction of carboxymethyl groups. The ability to form hydrogen bonds declined sharply, that is, the amino groups were activated, and this is helpful to its bile acid-binding capacity. Furthermore, factors such as ionic and hydroxyl group interactions and trapping in the polymer matrix have been suggested to contribute to the overall bile acid-binding capacity of polymers (Chang, Lee, Yoo, & Lee, 2006; Liu et al., 2010; Shin, Lee, Lee, & Lee, 2005). Thus, the mechanisms involved in the bile acid-binding capacity of chitosan are complex and need to be researched further.

3.3. Scavenging effect on DPPH radicals

The effect of carboxymethylation on the DPPH radical-scavenging activity of chitosan was investigated. As shown in Fig. 2, the scavenging ability of chitosan and CMCS increased from 0.5 to 1 mg/mL, and EC_{50} values (mg/mL) of chitosan, CMCS1, CMCS2, CMCS3, CMCS4 and CMCS5 were 4.66, 1.41, 1.02, 0.98, 1.34, and 1.16, respectively. At 0.5 mg/mL, the scavenging ability of ascorbic

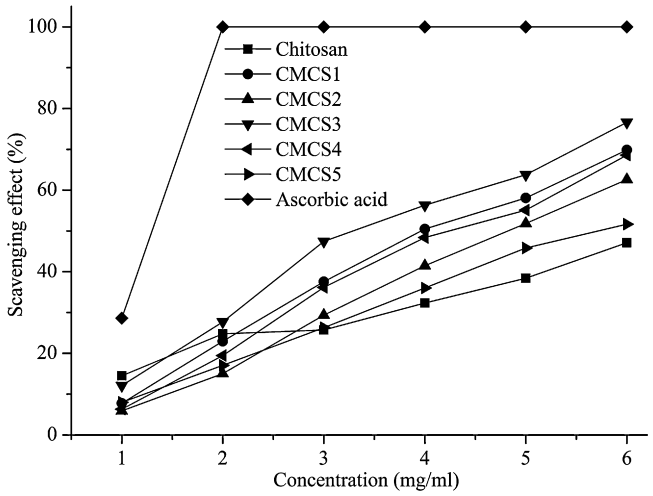

Fig. 3. Scavenging effect of chitosan and carboxymethyl chitosan (CMCS) with different degrees of substitution (DS) towards hydroxyl radicals. DS of CMCS1, CMCS2, CMCS3, CMCS4 and CMCS5 were 0.64, 0.81, 1.0, 1.33 and 1.59, respectively.

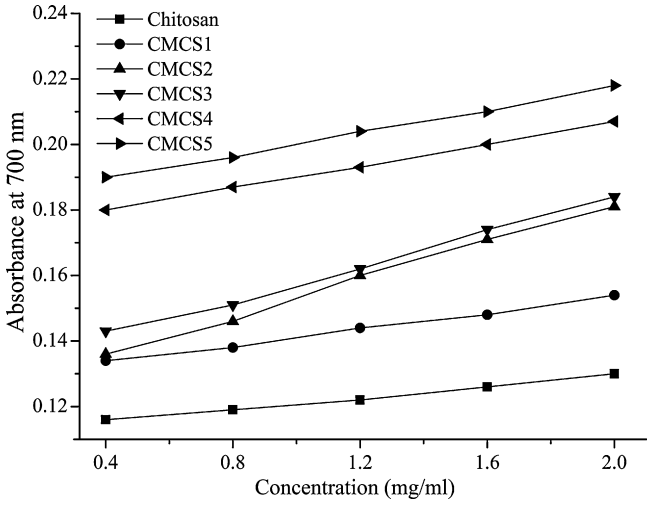

Fig. 4. Reducing power of chitosan and carboxymethyl chitosan (CMCS) with different degrees of substitution of CMCS1, CMCS2, CMCS3, CMCS4 and CMCS5 were 0.64, 0.81, 1.0, 1.33 and 1.59, respectively.

acid on DPPH radicals was 100%. The scavenging ability of CMCS on DPPH was stronger than chitosan and weaker than ascorbic acid. Compared with ascorbic acid, chitosan and CMCS were not effective scavengers for DPPH radicals. It is well accepted that DPPH free radical scavenging by antioxidants is due to their hydrogen-donating ability (Chen & Ho, 1995). The introduction of carboxymethyl groups on the chitosan may improve its hydrogen-donating ability.

3.4. Hydroxyl radical scavenging activity

The hydroxyl radical generated by the Fenton reaction were scavenged by chitosan and CMCS. Fig. 3 shows that the scavenging ability towards hydroxyl radicals of chitosan and CMCS improved with increases in their concentrations, and EC_{50} values (mg/mL) of chitosan, CMCS1, CMCS2, CMCS3, CMCS4, and CMCS5 were 6.74, 4.23, 4.85, 3.71, 4.39 and 5.64, respectively. The scavenging ability of ascorbic acid increased as its concentration increased, and then appeared to reach a plateau of 100% at 2 mg/mL. Typically, polysaccharides which are capable of scavenging hydroxyl radicals all have one or more alcohol or phenolic hydroxyl groups and their scavenging ability is directly related to the number of active hydroxyl groups in the molecule (Ji et al., 2007). The introduction of hydrophilic carboxymethyl groups on chitosan decreased the intramolecular and intermolecular hydrogen bonds resulting in the exposure of more hydroxyl groups (Li, Jiang, Xue, & Chen, 2002), thus the scavenging abilities of CMCS towards hydroxyl radicals are better than that of chitosan. As the DS increased, the number of hydroxyl groups decreased and thus the scavenging abilities of CMCS with a higher DS are poorer than that of CMCS with a lower DS.

3.5. Reducing power

The reducing power assay has also been used to evaluate the ability of natural antioxidants to donate electrons. Fig. 4 depicts the reducing power of chitosan and CMCS. The reducing power of CMCS with different DS correlated well with increasing concentration (Fig. 4). At 2 mg/mL, the absorbance of chitosan, CMCS1, CMCS2, CMCS3, CMCS4 and CMCS5 were 0.13, 0.154, 0.181, 0.184, 0.207 and 0.218, respectively. The data indicated that the addition of the carboxymethyl groups to chitosan improved the reducing power of CMCS. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity and is generally associated with the presence of reductones which have been shown to exert antioxidant action by breaking the free radicals' chain by donating hydrogen atom (Duh, Du, & Yen, 1999). The antioxidant activity of an antioxidant compound has been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, reductive capacity, and radical scavenging (Feng et al., 2007). The data on the reducing power of chitosan and carboxymethyl chitosan suggested that it was likely to significantly contribute towards the observed antioxidant effect.

3.6. Chelating effect on ferrous ions

Ferrous ion chelating effects of chitosan and CMCS were determined by measuring the decrease in the absorbance at 562 nm of the iron (II)-ferrozine complex. Chelating abilities of all CMCS on ferrous ions reached 100% at 1.2 mg/mL, but the ferrous ion-chelating effect of chitosan was only 33.7% at 2 mg/mL (Fig. 5). This might be due to the fact that the binding of the carboxyl group on the chitosan improved the ferrous ion-chelating effect. However, EDTA showed a chelating ability of 100% at a concentration as low as 0.2 mg/mL. Factors affecting the ion-chelating effect of CMCS

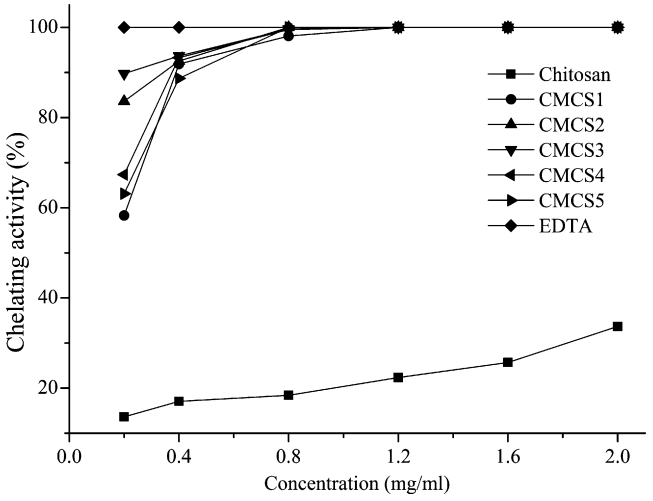

Fig. 5. Chelating effect of chitosan and carboxymethyl chitosan (CMCS) with different degrees of substitution (DS) on ferrous ions. DS of CMCS1, CMCS2, CMCS3, CMCS4 and CMCS5 were 0.64, 0.81, 1.0, 1.33 and 1.59, respectively.

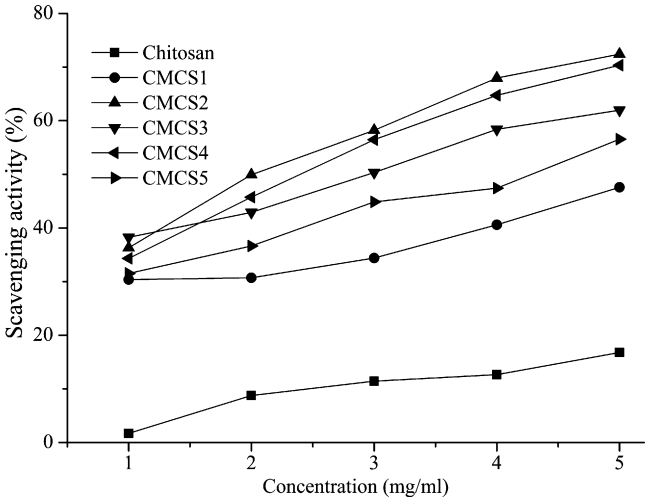

Fig. 6. Scavenging effect of chitosan and carboxymethyl chitosan (CMCS) with different degrees of substitution (DS) towards superoxide radicals. DS of CMCS1, CMCS2, CMCS3, CMCS4 and CMCS5 were 0.64, 0.81, 1.0, 1.33 and 1.59, respectively.

and chitosan are rather complex. Ferrous ions are the most effective pro-oxidants (Yamaguchi, Tatsumi, Kato, & Yoshimitsu, 1988) and they are commonly found in food systems where they can initiate lipid peroxidation and start a chain reaction that leads to the deterioration of flavor and taste in food (Duh et al., 1999).

3.7. Superoxide radical scavenging activity.

The scavenging ability of the chitosan and CMCS is shown in Fig. 6. The inhibitory effect of all forms of CMCS towards superoxide radical correlated well with their concentrations. At a concentration of 5 mg/mL, the scavenging effect on superoxide radicals of chitosan, CMCS1, CMCS2, CMCS3, CMCS4 and CMCS5 were 16.79%, 47.59%, 72.4%, 61.96%, 70.34% and 56.55%, respectively (Fig. 6) and the EC_{50} values (mg/mL) were 14.58, 5.56, 2.23, 2.94, 2.53 and 4.08, respectively (Fig. 6). This result showed that CMCS was more active towards the elimination of superoxide radicals than chitosan. This is likely because of the formation of strong intermolecular and intramolecular hydrogen bonds in chitosan that inhibited the reaction of superoxide anion with the active hydroxyl and amino groups in the polymer chains (Ji et al., 2007; Sun, Xie, & Xu, 2004). The differences in the scavenging activity of the forms of CMCSs are likely attributed to the variations in the content of active hydroxyl and amine groups. After etherification, the substituting carboxymethyl group with strong electron-withstanding ability was introduced into the chitosan chains. These substitutions were very helpful for enhancing the scavenging effect towards the superoxide radical.

4. Conclusion

A variety of N,O-carboxymethyl chitosan (CMCS) samples were prepared from squid cartilage β-chitin, and their bile acid binding capacities and antioxidant activities were assessed *in vitro*. CMCS exhibited higher bile acid binding capacity than that of chitosan. The highest bile acid binding capacity reached 58.95 mg/g compared to a previously reported value for chitosan of 7.75 mg/g (Liu, Xia, & Zhang, 2008). CMCS was a better antioxidant than chitosan, especially in terms of its reducing power, scavenging ability towards DPPH and superoxide radicals, and chelating ability of ferrous ions. On the basis of the results obtained, CMCS with presumed antioxidant properties and bile acid binding capacities may be used as a source of antioxidants, and a possible food supplement or ingredient in the pharmaceutical industry.

Acknowledgments

This work was financially supported by the grants from the National Natural Science Foundation of China (NO. 20876143 and 30970638), and the Science and Technology Department of Zhejiang Province of the People's Republic of China (NO. 2008C14067).

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