Carbohydrate Polymers 78 (2009) 439–443

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

The influence of the cation of quaternized chitosans on antioxidant activity

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ARTICLE INFO

Article history:
Received 31 March 2009
Received in revised form 27 April 2009
Accepted 27 April 2009
Available online 12 May 2009

Keywords:
Quaternized chitosans
Antioxidant activity
Group electronegativity

ABSTRACT

Various quaternized chitosans (QCSs) were synthesized according to previous method. Their reducing power and antioxidant potency against hydroxyl radicals (·OH) and hydrogen peroxide (H_2O_2) were explored by the established systems *in vitro*. The QCSs exhibited markedly antioxidant activity, especially TCEDMCS, whose IC_{50} on hydroxyl radicals was 0.235 mg/mL. They showed 65–80% scavenging effect on hydrogen peroxide at a dose of 0.5 mg/mL. Generally, the antioxidant activity decreased in the order TCEDMCS > TBEDMCS > EDMCS > PDMCS > IBDMCS > Chitosan. Furthermore, the order of their ·OH and H_2O_2 scavenging activity was consistent with the electronegativity of different substituted groups in the QCSs. The QCSs showed much stronger antioxidant activity than that of chitosan may be due to the positive charge density of the nitrogen atoms in QCSs strengthened by the substituted groups.

1. Introduction

Reactive oxygen species (ROS), including superoxide anion ($O_2^{\cdot}$), hydroxyl radicals (·OH) and hydrogen peroxide (H_2O_2), are generated by normal metabolic process or from exogenous factors and agents, such as ultraviolet radiation. ROS have extensive pathological effects and can cause many diseases, such as cancer, cardiovascular diseases, diabetes, as well as atherosclerosis (Cacciuttolo, Trinh, Lumpkin, & Rao, 1993; Cerutti, 1994; Halliwell & Gutteridge, 1990; Willett, 1990). Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates by scavenging or keeping from free radical and ROS. Synthetic antioxidants, such as butylated hydroxyanisole, butylated hydroxytoluene, *t*-butylhydroquinone, and propyl gallate, play an important role in the elimination of free radical and protect the cells against toxic affects of free radical (Winata & Lorenz, 1996). The role of antioxidants has received increased attention during the past decade. However, the use of such antioxidants has potential health hazards (Park, Jung, Nam, Shahidi, & Kim, 2001). Therefore, in recent years, interests have been developed for searching effective natural antioxidants, since they can protect human body from free radical and retard the progress of many chronic diseases.

Chitosan [poly(1,4-β-D-glucopyranosamine)], the deacetylated derivative of chitin, is one of the most abundant natural resource found on the earth. As a natural renewable resource, it has attracted people's attention for a number of unique properties, such as antimicrobial activity, nontoxicity, biodegradability, as well as its antioxidant activity (Lim & Hudson, 2004; Krajewska, 2005; Li, Liu, Tian, Liu, & Fan, 2007; Kim & Thomas, 2007). Anraku et al. reported that the low molecular weight chitosan could function as an effective protein antioxidant due to its scavenging activity on ·OH and H_2O_2 (Anraku et al., 2008). Esumi et al. reported that ·OH scavenging activity of the gold/chitosan nanocomposites was about 80 times that of ascorbic acid (Esumi, Takei, & Yoshimura, 2003).

Many studies had proved that the antioxidant activity of chitosan and its derivatives was attributed to the degree of deacetylation (Park, Je, & Kim, 2004), molecular weight (Xing et al., 2005), contents of hydroxyl and amino groups and different substituting groups (Xie, Xu, & Liu, 2001), and so on. However, our previous work had proved that the antioxidant activity of chitosan and its derivatives should be related to the different forms of nitrogen atom in the molecules of chitosan, such as primary amine ($-NH_2$, chitosan), imine (>C=N—, Schiff bases of chitosan), secondary amine ($-NHR'$, N-substituted chitosan), and quaternary ammonium ($-N^+RR'R''$, QCSs), in these compounds. And the QCSs had the best antioxidant activity. The increased activity should be attributed to the positive charge density of the nitrogen atom at C-2 in the molecules of QCSs strengthened after being quaternized (Guo, Liu, Chen, Ji, & Li, 2006). In order to further investigate the relationship between antioxidant activity and the charge density

Abbreviations: EDMCS, *N*-ethyl-*N*, *N*-dimethyl chitosan; PDMCS, *N*-propyl-*N*,*N*-dimethyl chito san; IBDMCS, *N*-isobutyl-*N*,*N*-dimethyl chitosan; TBEDMCS, *N*-tribromoethyl-*N*,*N*-dimethyl chitosan; TCEDMCS, *N*-trichloroethyl-*N*,*N*-dimethyl chitosan.

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doi:10.1016/j.carbpol.2009.04.030

of the cation in QCSs, in this paper, two more powerful electronegative groups, the —CBr_3 and —CCl_3 groups (Scheme 1), were introduced into the chitosan to synthesize two new kinds of QCSs (TBEDMCS and TCEDMCS), and their reduction power, antioxidant activity against ·OH and H_2O_2 were assessed *in vitro*, respectively. TBEDMCS and TCEDMCS were expected to have the best activity due to the strongest electronegative activity of their substituted electronegative groups.

2. Materials and methods

2.1. Materials

Chitosan was purchased from Qingdao Baicheng Biochemical Corp. (China). Its degree of deacetylation was 97%, and the viscosity-average molecular weight was 7.0×10^3. Acetaldehyde, propionaldehyde, isobutyraldehyde, tribromoacetaldehyde, trichloroacetaldehyde, sodium iodide (NaI), sodium borohydride ($NaBH_4$), and iodomethane (CH_3I) were purchased from the Sigma–Aldrich Chemical Co. The other reagents were all analytical grades and were used without further purification. The IR spectra were measured on a Jasco-4100 FT-IR spectrometer with KBr disks. The average viscometric molecular weight of chitosan was estimated from the intrinsic viscosity determined in the solvent 0.1 M CH_3COOH/0.2 M NaCl using the Marke–Houwink parameter $\alpha = 0.96$, $K\eta = 1.424$ at 25 °C when the intrinsic viscosity is expressed in mL g^{-1}.

2.2. Preparation of QCSs

QCSs were synthesized as follows (Jia, Shen, & Xu, 2001): 3 g chitosan was dissolved in 100 mL of water, and various aldehydes (3-fold excess to mole mass of chitosan) were added, respectively, with stirring at room temperature. After 2 h, 10% $NaBH_4$ (1.5-fold excess relative to mole mass of aldehyde) was added, and the solutions were reacted for 2 h. The mixture was precipitated in acetone and filtered. The *N*-alkyl chitosans were obtained after drying at 60 °C in vacuum for 12 h. *N*-alkyl chitosan (1 g) was dispersed in 50 mL of *N*-methyl-2-pyrrolidone (NMP) for 12 h at room temperature. Then, 0.5 mL NaOH (1 M), 1 g NaI and 4 mL CH_3I were added. Each reaction was carried out with stirring at 50 °C for 20 h. The product was obtained by precipitation with excess acetone, and the QCSs were obtained by drying at 60 °C in vacuum for 12 h (Scheme 1).

2.3. Hydroxyl radicals scavenging activity assay

The ·OH scavenging activity estimation was carried out according to Guo's method (Guo et al., 2006). The reaction mixture, total volume 4.5 mL, containing the samples of chitosan and QCSs (EDMCS, PDMCS, IBDMCS, TBEDMCS and TCEDMCS), were incubated with EDTA–Fe^{2+} (220 μM), safranine O (0.23 μM), H_2O_2 (60 μM) within potassium phosphate buffer (150 mM, pH 7.4) for 30 min at 37 °C. The absorbance of the mixture was measured at 520 nm. The ·OH bleached the safranine O, so decreased absorbance of the reaction mixture indicated decreased ·OH scavenging ability and the capability of scavenging ·OH was calculated using the follow equation:

$$\text{Scavenging effect } (\%) = \frac{A_{\text{sample 520 nm}} - A_{\text{blank 520 nm}}}{A_{\text{control 520 nm}} - A_{\text{blank 520 nm}}} \times 100$$

where $A_{\text{blank 520nm}}$ was the absorbance of the blank (distilled water instead of the samples), $A_{\text{control 520nm}}$ was the absorbance of the control (distilled water instead of H_2O_2).

2.4. Measurement of reducing power

The reducing power was quantified by the method described earlier by Yen and Chen (Yen & Chen, 1995). Briefly, 1 mL of reaction mixture, containing different concentration of chitosan and QCSs 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 trichloroacetic acid 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.

2.5. Hydrogen peroxide scavenging activity

The ability of QCSs and chitosan to scavenge hydrogen peroxide was determined according to the method of Ruch (Ruch, Cheng, & Klauning, 1989). A solution (10 mM) of hydrogen peroxide was prepared in phosphate-buffered saline (pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230 nm. Chitosan and QCSs with various concentrations and 10 mM hydrogen peroxide were mixed with 0.1 M phosphate buffer (pH 6.0) incubated at 37 °C for 10 min. Absorbance of hydrogen peroxide at 230 nm was determined 10 min later against a blank solution containing QCSs and chitosan in PBS without hydrogen peroxide. The capability of scavenging H_2O_2 was calculated using the follow equation:

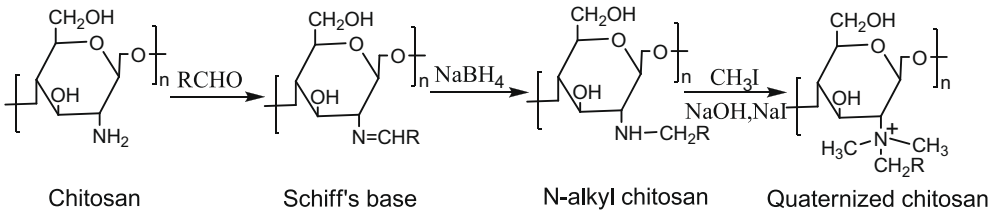
$$\text{Scavenging effect } (\%) = \left[1 - \frac{A_{\text{sample 520 nm}} - A_{\text{control 520 nm}}}{A_{\text{blank 520 nm}}}\right] \times 100$$

where $A_{\text{blank 520nm}}$ was the absorbance of the blank (distilled water instead of the samples), $A_{\text{control 520nm}}$ was the absorbance of the control (distilled water instead of H_2O_2).

3. Results and discussion

3.1. Characterization of QCSs

In the FT-IR spectra of chitosan and the QCSs (Fig. 1), chitosan was typically characterized by absorption regions as follows (Britto



EDMCS: R=-CH_3; PDMCS: R=-CH_2CH_3; IBDMCS: R=-CH $(CH_3)_2$;

TBEDMCS: R=-CBr_3; TCEDMCS: R=-CCl_3

Scheme 1. Synthetic pathway of the QCSs.

& Assis, 2007): the major peaks of chitosan at about 896, 1087, 1590 and 3420 cm^{-1} belonging to pyranose ring, glucoside, amine and hydroxyl groups, respectively, were identifiable. After quaternization, new peaks appeared at about 1660 cm^{-1}, which were assigned to the quaternary ammonium salt. There were peaks at about 1415–1430 cm^{-1}, which were ascribed to the characteristic absorb of N—CH_3 (Guo et al., 2007). Moreover, the peaks at about 550 and 700 cm^{-1} were assigned to C—Br and C—Cl for TBEDMCS and TCEDMCS, respectively. The spectrum data indicated that the QCSs were obtained.

3.2. Hydroxyl radicals scavenging activity of QCSs

Hydroxyl radicals were generated by direct addition of Fe^{2+} to a reaction mixture containing phosphate buffer under normal conditions (Halliwell & Gutteridge, 1990). Among the ROS, hydroxyl radicals showed the strongest chemical activity, which can easily react with amino acids, DNA and membrane components. Fig. 2 shows the ·OH scavenging activity of chitosan and QCSs at various concentrations. All the samples exhibited concentration-dependent ·OH elimination effect. The IC_{50} value, which means the antioxidant concentration to reduce the ·OH by 50%, is a good parameter to evaluate the antioxidant ability. The IC_{50} of QCSs was 0.392, 0.599, 0.270 and 0.235 mg/mL for EDMCS, PDMCS, TBEDMCS and TCEDMCS, respectively. However, the IC_{50} of IBDMCS and chitosan could not be read for the determined concentrations. The IC_{50} of high-molecular weight and high-sulfate-content chitosan was 3.269 mg/mL (Xing et al., 2005); Carboxymethyl chitosan had slight scavenging activity against ·OH, with a scavenging index of 9.3% at a concentration of 1.4 mg/mL (Guo, Xing, Liu, Zhong, & Li, 2008). And the IC_{50} of mannitol, and benzoic acid were 2.50, and 3.50 mg/mL, respectively (Xie et al., 2001). The QCSs showed more notable ·OH scavenging activity than that of chitosan, some chitosan derivatives and some chemical antioxidants. Obviously, TCEDMCS exhibited the strongest ·OH scavenging activity.

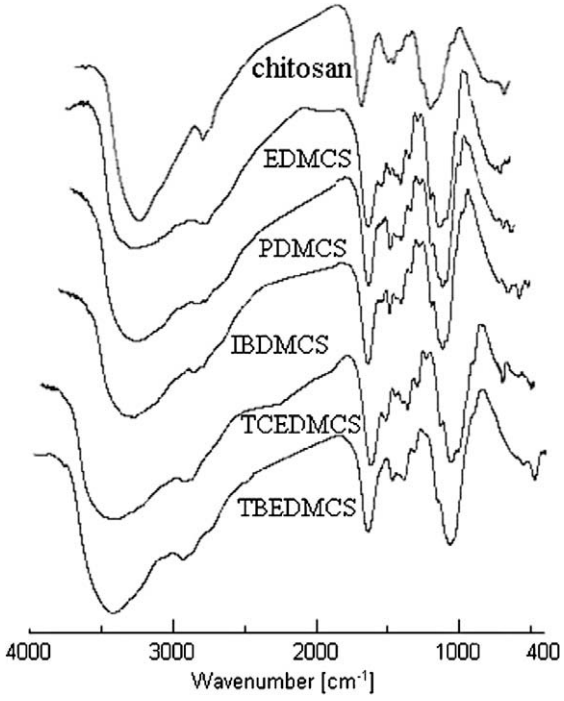


Fig. 1. FT-IR spectra of chitosan and QCSs.

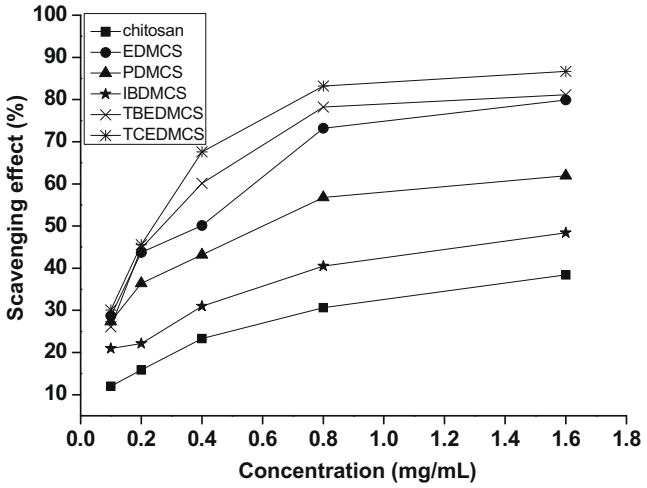


Fig. 2. Scavenging effects of chitosan and QCSs on hydroxyl radical.

3.3. Reducing power of chitosan and QCSs

It was reported that the antioxidant activity is concomitant with the reducing power, which may serve as a significant indicator of the potential antioxidant activity for a compound (Tanaka, Kuie, Nagashima, & Taguchi, 1988). Fig. 3 depicts the reducing power of chitosan and all kinds of QCSs. The reducing power of all the compounds correlated well with increasing concentration, which were 0.51–1.07 at 0.15 mg/mL. It seemed that of all kinds of QCSs showed stronger reducing power than that of BHA, a-tocopherol and ascorbic acid, which showed reducing powers of 0.96, 0.45 and 0.68 at 0.10 mg/mL, respectively (Yen, Yang, & Mau, 2008). In addition, as shown in Fig. 3, the reducing power of TCEDMCS was higher than the other compounds. Generally speaking, the reducing properties are associated with the presence of reductones. It was believed that the reductones can break the free radicals' chain by donating a hydrogen atom (Duh, Du, & Yen, 1999). Our data on the reducing power of all kinds of QCSs suggested that the substituted groups were likely to contribute significantly toward the observed antioxidant effect.

3.4. Scavenging activity of QCSs on hydrogen peroxide

Hydrogen peroxide, a reactive non-radical, is very important as it can penetrate biological membranes. Although H_2O_2 itself is not

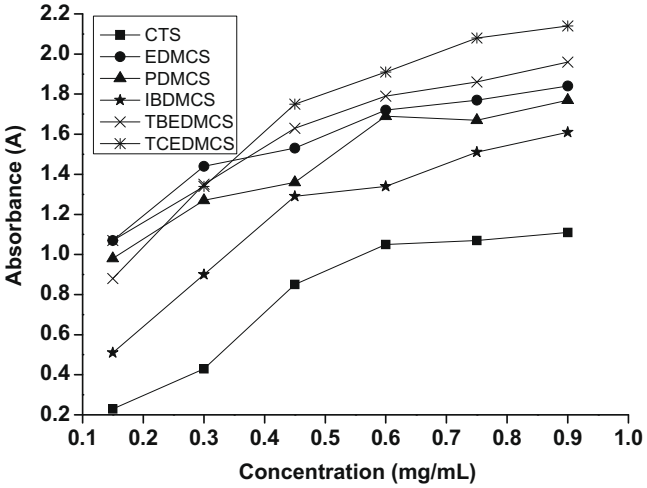


Fig. 3. Reducing power of chitosan and QCSs.

very reactive, it may cause tumors by converting into more reactive species such as singlet oxygen and hydroxyl radicals. Hydrogen peroxide promotes tumors in mouse skin (Slaga, Klein-Szanto, Triplett, Yotti, & Trosko, 1981). Hydrogen peroxide, which was induced by several tumor promoters, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA), 12-*O*-retinoylphorbol 13-acetate (RPA), and mezerein, can cause DNA damage (Frenkel & Chrzan, 1987). Hydrogen peroxide scavenging activity of chitosan and its derivatives are shown in Fig. 4. Be similar to the scavenging pattern of hydroxyl radicals, the scavenging effect was in the order of TCEDMCS > TBEDMCS > EDMCS > PDMCS > IBDMCS > Chitosan and the activities were dose-dependent. They showed 65–80% scavenging effect on hydrogen peroxide at a dose of 0.5 mg/mL. It appeared that there was some a direct correlation between ·OH and hydrogen peroxide scavenging activity of chitosan and QCSs.

3.5. The cation of QCSs influencing the scavenging activity on ·OH and H_2O_2

Recently, the antioxidant activity of chtiosan and its derivatives has been attracting the most attention. However, the mechanism for the antioxidant activities is still unclear. Chitosan is unique for its polycationic characteristic with free —NH_2 groups, which can be converted into —NH_3^+ forms in acid medium. Thus, chitosan can be as proton-donator. With the result that, the scavenging mechanism may be related to the fact that free radical can react with hydroxyl and free amino groups in chitosan to form most stable macromolecules by typical H-abstraction reaction or through addition reaction (Xie et al., 2001). In this study, concerning about chitosan and QCSs with the same molecular weight, yet the antioxidant activities of QCSs on hydroxyl radicals and hydrogen peroxide were more pronounced than that of chitosan. Different scavenging activities of the QCSs on ·OH and H_2O_2 should be attributed to their different structures. Scheme 1 shows the nitrogen atoms in QCSs have different positive charges because of different R groups in their molecules. Different R groups have their own electronegativity (Table 1). Group electronegativity is the electrophilic characteristic of a group and it is a very significant parameter for studying the structure and properties of organic compounds. After being quaternized, the positive charge density of the nitrogen atoms in the QCSs was strengthened because of the electronegativity of different R groups. As Table 1 shows, the electronegativity of different R groups in the QCSs was found to be in the order of —CCl_3 > —CBr_3 > —CH_3 > —CH_2CH_3 > —$CH(CH_3)_2$. Thus, the positive charge density of the QCSs should be as follows:

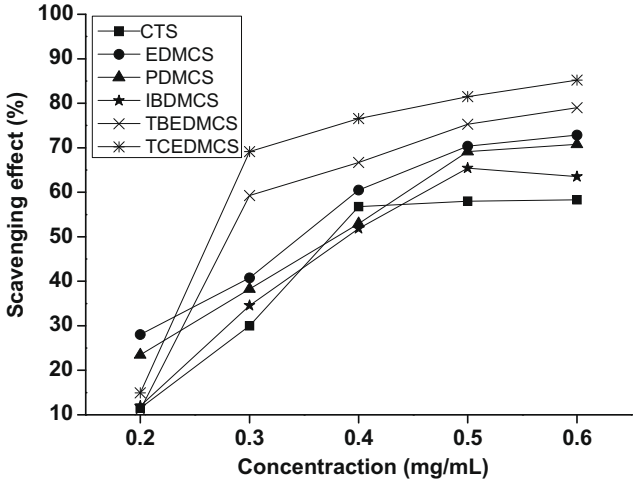


Fig. 4. Scavenging effects of Chitosan and QCSs on hydrogen peroxide.

Table 1
The electronegativity of different R groups in QCSs.

R groups	—CH_3	—CH_2CH_3	—$CH(CH_3)_2$	—CBr_3	—CCl_3
Electronegative activity	2.47[a]	2.29[b]	2.26[c]	2.86[c]	2.98[b]

[a] Taken from Ref. Inamoto and Masuda (1982).
[b] Taken from Ref. Bratsch (1985).
[c] Taken from Ref. Nie (2000).

TCEDMCS > TB EDMCS > EDMCS > PDMCS > IBDMCS, which was consistent with the order of their ·OH and H_2O_2 scavenging activity as Figs. 2 and 4 show. Stronger electronegativity substituted groups would draw more electrons. Thus, the positive charge density of the nitrogen atoms in QCSs should be relatively strengthened. The QCSs were more prone to react with free radical to form most stable macromolecules. As a result, they would have stronger antioxidant activities. TBEDMCS and TCEDMCS had the most intensive positive charge density in their molecules due to the strongest electronegativity of —CBr_3 and —CCl_3 groups. Therefore, as we expected, TBEDMCS and TCEDMCS had better ·OH and H_2O_2 scavenging activity than the other QCSs.

4. Conclusions

In summary, *N*-alkyl chitosan derivatives were prepared by introducing alkyl groups into the amine groups of chitosan via Schiff's base intermediates. And then the QCSs were successfully synthesized through quaternization of the *N*-alkyl chitosan derivatives carried out using methyl iodide. Their antioxidant effects were investigated in a number of model systems. The data obtained in vitro models clearly establish the antioxidant potency of all kinds of QCSs. The mechanism for their antioxidant activities was also discussed in this paper. The antioxidant activities should be influenced by the positive charges of nitrogen atoms in the QCSs. The charge density of the QCSs was strengthened by the electronegativity of the substituted groups. Thus, the QCSs with stronger electronegativity groups, they would have stronger antioxidant activities. The QCSs, especially TBEDMCS and TCEDMCS, showed much stronger ·OH and H_2O_2 scavenging activity than that of chitosan, which suggested that the QCSs should be further explored as practical antioxidant for the pharmaceutical and food industries. However, comprehensive studies need to be carried out to ascertain the safety of QCSs in experimental animal models.

Acknowledgements

This work was supported by key technology research and development programme of Shandong Province (2007GG2QT06020).

Thanks for the financial support by the foundation of Special Prize of President Scholarship for Postgraduate Students of Chinese Academy of Sciences.

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