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Structural factors affecting radical scavenging activity of chitooligosaccharides (COS) and its derivatives

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Abstract

Two chitooligosaccharides (COS) derivatives were synthesised by introducing carboxyl ($-COCH_2CH_2COO^-$) and quaternized amino ($-CH_2CH(OH)CH_2N(CH_3)_3^+$) groups to the amino position of COS with different substitution degrees (CCOS-1–3 and QCOS-1–3) for the purpose of altering total amount of hydrogen atoms capable of reacting with radicals and modifying their metal ion chelating ability. Scavenging of carbon-centered and DPPH radicals was directly affected by the amount of abstractable hydrogen atoms in COS molecules. In contrast, structure-activity relationships revealed that chelation of Fe^{2+} ions indirectly contributed for their observed hydroxyl radical scavenging activity apart from hydrogen abstraction.

Keywords: Chitooligosaccharides (COS); Radical scavenging; Ion chelation; Hydrogen abstraction

1. Introduction

In vivo accumulation of free radicals has long been identified to be harmful since it can affects adversely to the function and stability of cellular components such as lipid, protein and DNA (Andrew, 2001; Benedicta, Gang, & Vernon, 2004; Jacob, Roger, Marijn, Theo, Ebienus and Jan, 2004). The endogenous defense system, which includes vitamins and antioxidative enzymes such as superoxide dismutase, catalase, glutathione peroxidases fights against these radicals and helps to repair the damage (Bernhard, 2005). However, with ageing and due to some physiological imbalances this natural antioxidant defense system becomes far less effective (Andrew, 2001; Rafique, Schapira, & Cooper, 2004). Thus, use of external antioxidants attracts therapeutic importance to maintain a proper internal redox environment. Antioxidant is any substance that significantly delays or prevents oxidation when present at lower concentrations compared to that of the oxidizable substrate (Halliwell, Aeschbach, Loliger, & Aruoma, 1995). Recently, researchers have focused on extracting natural antioxidants from dietary foods including fruits, vegetables and other plants with medicinal importance, since the synthetic antioxidants report to have adverse effects in some cases (Halliwell et al., 1995; Jun, Ben-Zhan, & Balz, 2003). However, most of the above extracts do not contain antioxidant compounds in substantial amounts, and that becomes a major limitation for the use of natural antioxidants.

Chitosan is a naturally abundant β-1,4-linked copolymer of *N*-acetyl-2-amino-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose units. Different chitosan derivatives are reported to possess significant biological activities such as, antitumor or anticancer (Caiqin, Yumin, Ling, Zhan, & Xiaohai, 2002) and antioxidant activities (Kim, Byun, Park, & Shahidi, 2001). Even though, some chitosan derivatives have been studied for their antioxidant activities (Je, Park, & Kim, 2004; Park, Je, & Kim, 2004; Ronge, Song, Huahua, Weiwei, Quanbin and Zhien, 2005; Xie, Xu, & Liu, 2001), no clear explanation is available for the observed radical scavenging abilities in relation to their structural properties. However, Xie et al. (2001) presumed

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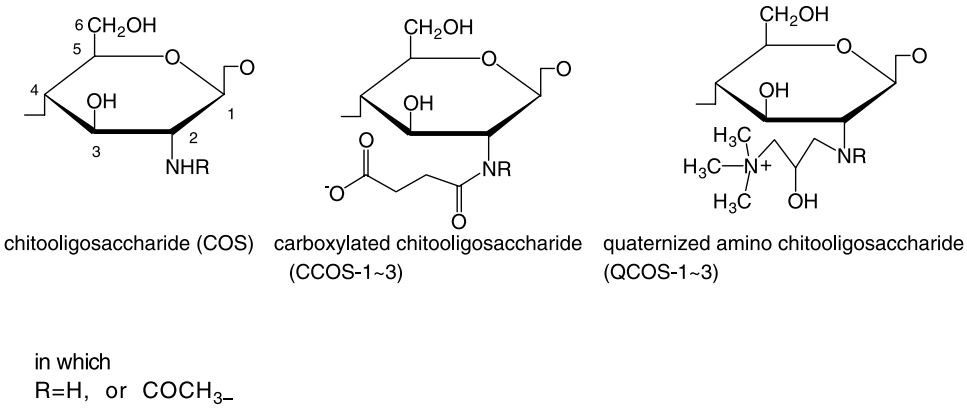

Fig. 1. Schematic illustration of chitooligosaccharides (COS) and their synthesized derivatives with different substitution groups.

that the hydrogen abstraction ability of chitosan plays a vital role for its radical scavenging. In this study, we synthesized two COS derivatives with different substitution degrees to alter total amount of hydrogen atoms capable of reacting with free radicals and modifying their metal ion chelating ability (Fig. 1). Further, their antioxidant activities were studied by assessing scavenging behaviors on different free radicals and observed that metal ion chelation ability was also important for the observed activities.

2. Materials and methods

2.1. Chemicals and instruments

Chitooligosaccharides (COS) were kindly donated by Kitto Life Co. (Seoul, South Korea) with 23.46% acetylation degree (determined by elemental analysis: C%, 43.39%; N%, 7.825%; H, 6.815%) and 6.0–7.0$\times 10^3$ Da molecular weight range (determined by MALDI-TOF mass spectrometry (David, 1999)). Reagents including succinic anhydride, 2,3-epoxypropyl chloride and trimethyl amine, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 5,5-dimethyl-pyrrokine *N*-oxide (DMPO), 2,2-azo-*bis*-(2-amidinopropane)-hydrochloride (AAPH), and α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone (4-POBN) were obtained from Sigma® Chemical Co. (St Louis, MO).

Micro acilyzer model G3 (Asashi Kasei Inc., Kanagawa, Japan) equipped with an AC-110 dialysis membrane was used to purify COS and its derivatives. FT-IR Spectra were measured on a Spectrum 2000 spectrometer (Perkin–Elmer, USA). Proton NMR (^{1}H NMR) and carbon NMR (^{13}C NMR) spectra were recorded in D_2O on a JNM-ECP-400 (400 MHz) spectrometer (JEOL, Japan). Elemental analysis (C, N, and H%) was performed on an elementar analyse system (Elementar Vario EL, USA) and were within ± 0.4% of theoretical values. Bio-Gel P-3000 gel permeation chromatography (GPC) column connected with a Shodex RI-71 refractive index detector was used to determine the molecular weight of COS derivatives and UV-160A Varian spectrometer (Shimadzu, Japan) was used to detect optical density. All the radical scavenging assays were performed using a JES-FA ESR spectrometer (JEOL, Tokyo, Japan).

2.2. Synthesis of COS derivatives

COS derivatives were prepared with different substitution degrees and characterized according to previously reported methods (Ronghua, Yumin, & Jianhong, 2003a; Ronghua et al., 2003a). Molecular weights of COS derivates were determined in acetate buffer (0.2 M CH_3COOH/0.1 M CH_3COONa) against standard Superhdex® Pullullan molecular weight markers (Caiqin et al., 2002). From elution volumes no obvious differences were observed among molecular weights of COS and its derivatives. Chemical structures of synthesized derivatives were determined by ^{1}H NMR and ^{13}C NMR data and the degree of substitution of different functional groups was assessed by elemental analysis or ^{1}H NMR integral calculus.

2.2.1. Carboxylated chitooligosaccharides (CCOS-1–3)

FT-IR (KBr, ν, cm^{-1}): 3408 (s, O–H), 2931 and 2860 (w, C–H), 1723 (w, ester C=O, only in CCOS-3), 1653 (m, amide C=O), 1565 (m, carboxyl C=O), 1408 (m, carboxyl C=O), 1112, 1068, 1030 (s, pyranose ring); (Ronghua et al., 2003a; Lingyun, Yumin, & Ronghua, 2003) ^{13}C NMR (400 MHz, D_2O, δ, ppm): 22 (N–CH_3), 32 (CH_2CH_2 close to carboxyl group), 56 (C-2), 61 (C-6), 69, 73 and 76, 78 (C-3, 4, 5), 102 (C-1), 174 (amide C=O), 176 and 180 (C=O); ^{1}H NMR (400 MHz, D_2O, δ, ppm): 1.9 (CH_3), 2.6, 3.3–3.6, and 4.5 (C-1–6), 2.4 (–CH_2CH_2–), (Rafique et al., 2004) Elemental analysis: CCOS-1, C% (43.20), N% (6.351), H% (5.63); CCOS-2, C% (39.87), N% (5.020), H% (4.250); CCOS-3, C% (39.77), N% (4.610), H% (4.924). According to C/N ratio the degree of substitution of carboxyl group per pyranose unit in CCOS-1, CCOS-2 and CCOS-3 was 0.3666, 0.6992 and 0. 8989, respectively.

2.2.2. Quaternized amino chitooligosaccharides (QCOS-1–3)

FT-IR (KBr, ν, cm^{-1}): 3400 (s, O–H), 2927 and 2860 (w, C–H), 1647 (m, amide C=O), 1480 (s, CH_3 of quaternary groups), 1144, 1071, 1030 (s, pyranose); (Jiyoung, Jongkeun, Taekseung, & Wonho, 2003; Ronghua, Yumin, Jianhong, & Lihong, 2003b) ^{13}C NMR (400 MHz, D_2O, δ, ppm): 22 (N–CH_3), 52 (C-2), 54 (N–CH_3), 56.9 (OCH_2CH_2O), 61.8 (N–CH_2), 61 (C-6), 64, 65 (N–CH_2CH–OH), 69, 70 and 76 (C-3, 4, 5), 102 (C-1), 176 (C=O); ^1H NMR (400 MHz, D_2O, δ, ppm): 1.9 (CH_3), 2.6, 3.3–3.6, and 4.5 (C-1–6), 3.2 (N–CH_3), 3.5 (N–CH_2CH–O) (Ronghua, et al., 2003b) Elemental analysis: QCOS-1, C% (43.81), N% (7.829), H% (6.780); QCOS-2, C% (45.89), N% (7.798), H% (7.644); QCOS-3, C% (44.91), N% (7.880), H% (8.260). C/N ratios of derivatives could not be used for analyzing substitution degree of quaternized amino groups due to non-significant differences between calculated and analyzed values. Therefore, the integral calculus of peaks at 3.3–3.6, and 4.5 ppm (C-1–6), 3.2 ppm (N–CH_3), 3.5 ppm (N–CH_2CH–O) in ^1H NMR spectra were compared for the substitution degree calculation. The substitution degree per pyranose unit of QCOS-1–3 obtained from ^1H NMR spectra were 0.2624, 0.5378, and 0.7674, respectively (Fig. 2).

2.3. Ferrous ion chelating activity assay

Ferrous ion-chelating ability of COS and its derivatives was evaluated according to Ronge et al. (2005), with minor modifications. Briefly, different concentrations of sample solution (1 ml) was mixed with 0.1 ml of $FeCl_2$ (2 mmol/l) and 0.2 ml of ferrozine (5 mmol/l) and adjusted to 5 ml with deionized-distilled water, then incubated at room temperature for 10 min. Optical density of colored ferrous iron-ferrozine complex formed in the presence or absence of sample was spectrometrically measured at 562 nm. EDTA is used as a positive control to compare the activity of samples. The ability of samples to chelate ferrous ion was calculated according to the following equation, where A and A_0 were the optical density at 562 nm with and without samples, respectively.

$$\text{Ferrous ion chelating activity (\%)} = \left(\frac{1-A}{A_0}\right) \times 100$$

2.4. Determination of antioxidant activity

2.4.1. DPPH radical scavenging assay

DPPH radical scavenging activity was measured according to Nanjo, Goto, Seto, Suzuki, Sakai and Hara (1996). Sixty micro liters of DPPH in methanol was added to 60 μl of sample solution (or ethanol itself as a control) and vortexed for 10 s. After 2 min, reaction mixture was transferred to a sealed capillary tube and DPPH radical signal was recorded using ESR spectrometer. Recording conditions: central field, 336 mT; modulation frequency, 9.44 GHz; sweeping time, 4×30 s, modulation amplitude, CH1 = 1000.0, CH2 = 2.0; microwave power, 5.0 mW; temperature, 298 K. DPPH radical scavenging ability was calculated following equation in which H and H_0 were relative peak height of radical signals with and without sample, respectively.

$$\text{Radical scanning activity (\%)} = \left(\frac{1-H}{H_0}\right) \times 100$$

2.4.2. Carbon-centered radicals scavenging assay

To generate carbon-centered radicals, 20 μl of phosphate buffered-saline (PBS pH 7.4), AAPH (40 mM), 4-POBN (40 mM) and sample solution were mixed vigorously and incubated in a water bath at 37 °C for 30 min (Hiramoto,

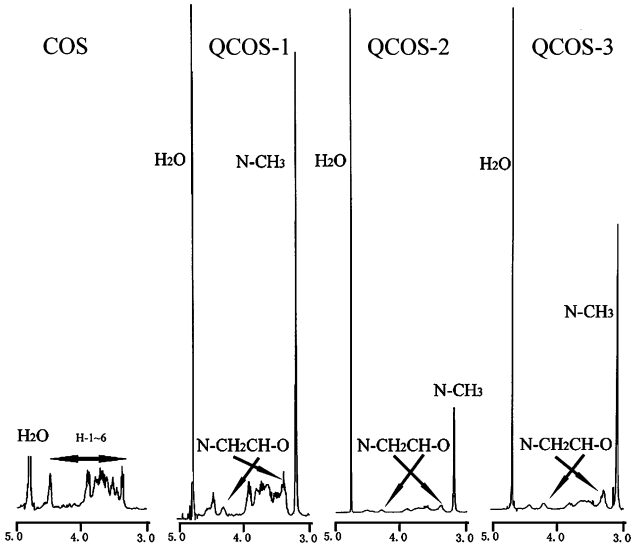

Fig. 2. ^1H NMR spectra of COS and its quaternized amino derivatives with different substitution degrees. Substitution degrees per pyranose unit of QCOS-1–3 (0.2624, 0.5378, and 0.7674, respectively), were obtained by comparing the integral calculus of chemical shifts assinged to C-1–6 (3.3–3.6, and 4.5 ppm), N–CH3 (3.2 ppm), N–CH2CH–O (3.5 ppm).

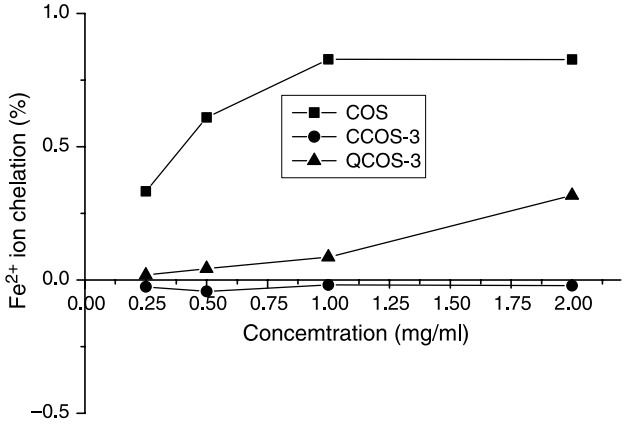

Fig. 3. Ferrous ion chelation activity of COS and its derivatives with higher substitution degree of carboxyl or quaternized amino groups. Chelation ability of EDTA against ferrous ions was 52.88% at 0.1 mg/ml concentration.

Table 1
Scavenging percentages of COS and its differentially substituted derivatives on DPPH radicals

Sample	COS	QCOS-1	CCOS-1	QCOS-2	CCOS-2	QCOS-3	CCOS-3
Substitution degree (mg/ml)	0	0.2624	0.3666	0.5378	0.6992	0.7674	0.8989
0.125	63.22	51.78	46.10	45.79	34.54	21.74	14.65
0.25	85.09	82.71	52.32	68.43	45.18	47.31	39.33
0.5	85.69	81.53	84.75	83.41	67.53	79.85	47.88
1.0	86.11	83.18	84.66	92.13	82.84	–	81.57
2.5	92.37	89.85	83.14	87.92	–	–	86.50

Johkoh, Sako, & Kikugawa, 1993). After the reaction, solution was transferred to a sealed capillary tube and spin adduct was recorded using ESR spectrometer. Carbon-centered radicals scavenging ability was calculated according to the above equation. Recording conditions: central field, 336 mT; modulation frequency, 9.44 GHz; sweeping time, 3×30 s, modulation amplitude, CH1 = 2500.0, CH2 = 2.0; microwave power, 5 mW; temperature, 298 K.

2.4.3. Hydroxyl radical scavenging assay

Hydroxyl radicals were generated using Fenton reaction and a standard spin trapping agent, DMPO was used to trap hydroxyl radicals (Burton and Ingold, 1986). Different concentrations of COS derivatives (50 μl) or same volume of phosphate buffer (pH 7.4) as a control was added to 50 μl of 0.3 M DMPO, 50 μl of 10 mM $FeSO_4$ and, reaction was initiated by adding 50 μl of 10 mM H_2O_2. After 2.5 min reaction mixture was transferred to a capillary tube and DMPO-OH adduct was detected using ESR spectrometer. Recording conditions: central field, 336 mT; modulation frequency, 9.44 GHz; sweeping time, 2×30 s, modulation amplitude, CH1 = 200.0, CH2 = 2.0; microwave power, 1 mW; temperature, 298 K. Hydroxyl radical scavenging ability of samples was calculated according to the above equation.

2.5. Statistics

Results of radical scavenging or chelating assays were presented as means of triplicates and Student's *t*-test was used to determine the level of significance.

3. Results and discussion

3.1. Structural features of COS and its derivatives in relation to antioxidant activity

Hydroxyl groups at C-3 and C-6 positions as well as amino group at C-2 position of pyranose ring are believed to be beneficial in antioxidant activity for their abstractable hydrogen atoms (Xie et al., 2001; Ronge et al., 2005; Park et al., 2004; Je et al., 2004). In this research we synthesized two different chitosan derivatives to alter abstractable hydrogen atoms, by introducing functional groups to amino position (Fig. 1). Those substitutions were confirmed by FT IR or NMR spectra. For examples in FT-IR spectra, amide absorption (1640 cm^{-1}) of CCOS-1 and CCOS-2, or ester carbonyl absorption (>1700 cm^{-1}) of CCOS-3, and (1480 cm^{-1}) QCOS-1–3 confirm the substitution as in Section 2. In NMR spectra (Section 2), the new chemical shifts can also be assigned for the introduced groups –$COCH_2CH_2COO^-$ and (–$CH_2CH(OH)CH_2N(CH_3)_3^+$). Thus, above two derivatives made it possible to study the influence of abstractable hydrogen atoms at hydroxyl and amino groups of COS on antioxidant activity.

Other than scavenging of existing radicals, chelation of some transition metal ions can also be an effective way to decrease the generation of radicals. It has been reported that, chelation of ferrous ions can greatly retard the generation of hydroxyl radicals in Fenton reaction (Halliwell et al., 1995; Andres and Arthur, 2004). According to our results, introduced functional groups (except in CCOS-3) could

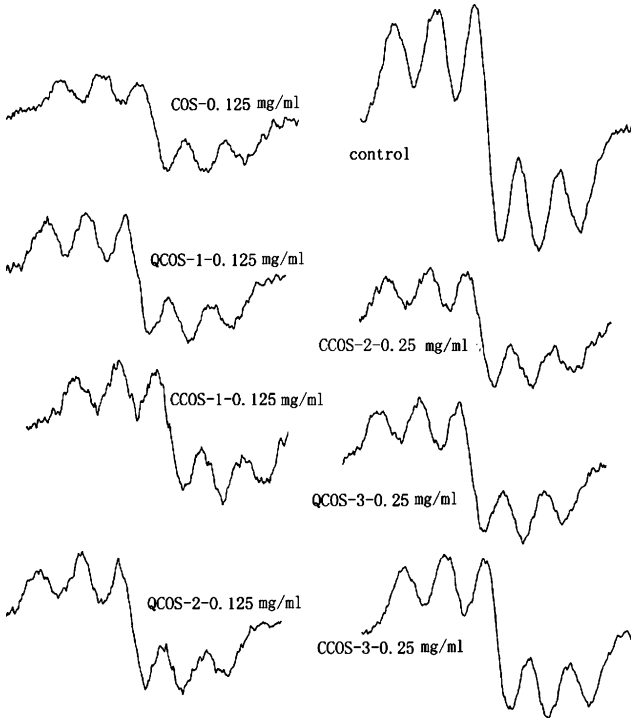

Fig. 4. ESR spectra of DPPH radicals in the presence of COS and its derivatives with different substitution degrees. ESR assay conditions were maintained for all measurement as described in the text.

Table 2
Scavenging percentages of COS and its differentially substituted derivatives on carbon-centered radicals

Sample	COS	QCOS-1	CCOS-1	QCOS-2	CCOS-2	QCOS-3	CCOS-3
Substitution degree (mg/ml)	0	0.2624	0.3666	0.5378	0.6992	0.7674	0.8989
0.0625	65.45	62.05	60.57	57.52	40.23	42.31	26.45
0.125	67.45	66.44	60.22	62.01	65.97	34.45	32.25
0.25	69.03	67.25	61.49	64.25	65.80	65.77	50.82
0.5	68.24	68.66	62.23	65.19	65.67	72.36	69.13
1.25	75.03	68.46	–	–	–	76.89	74.42

influence the chelation ability of metal ions (Fig. 3). Therefore, it can be expected that, differential ion chelation activities of COS derivatives can contribute differently for their antioxidant activities.

3.2. Radical scavenging activities of COS and its derivatives

As shown in Table 1, COS exhibited a considerably high DPPH radical scavenging ability (85.69%, even higher than α-tocopherol (69.43%) at 0.5 mg/ml). Interestingly, at low concentrations of COS derivatives exhibited an inverse DPPH radical scavenging activity regardless to the structure of groups substituted. Moreover, their scavenging activities were gradually decreased as substitution degree increases (Table 1). In contrast, no clear difference was observed for substitution degree and scavenging activity at higher concentrations. At 1.0 mg/ml, all the scavenging activities were ranged from 80 to 90% and were not orderly arranged. Signal intensities of ESR spectra of DPPH radicals exhibited a direct correlation with scavenging activity, in which intensity of signals increased in the order of QCOS-1, CCOS-1, QCOS-2 (at 0.125 mg/ml), and CCOS-2, QCOS-3, CCOS-3 (0.25 mg/ml), following increment in substitution degree (Fig. 4). Similar to DPPH radicals, scavenging of carbon-centered radicals also did not exhibit significant ($P < 0.05$) effect at higher concentrations (above 0.5 mg/ml) for both CCOS and QCOS (Table 2). Further, their scavenging activity decreased with the increment of substitution degree, which was also confirmed by increased intensities of ESR signals (COS, QCOS-1, CCOS-1 (at 0.0625 mg/ml), QCOS-2, CCOS-2 (at 0.125 mg/ml) and QCOS-3, CCOS-3 (at 0.5 mg/ml)) as shown in Fig. 5.

Hydroxyl radicals are accepted as one of the most reactive radical species and COS exhibited a high scavenging activity of 67.40% at a low concentration of 0.0625 mg/ml (Table 3). Interestingly, in contrast to the results of DPPH and carbon-centered radicals, scavenging effects of CCOS-2 and CCOS-3 on hydroxyl radicals were reduced to negative values (−22.14 and −30.03% for CCOS-2 and CCOS-3 at 0.5 mg/ml). Higher ESR signal intensities compared to the control, clearly indicate negative influence of CCOS-2 and CCOS-3 for hydroxyl radical scavenging (Fig. 6). Hydroxyl radical scavenging of all quaternized amino derivatives (QCOS-1–3), was similar to that of DPPH and carbon-centered radicals. Therefore, it can be suggested that, structural properties of substituted groups in CCOS and QCOS derivatives play a considerable influence on scavenging of hydroxyl radicals apart from their substitution degree.

3.3. Structural factors affecting radical scavenging behavior of COS and its derivatives

As mentioned above, structural features of COS were chemically modified to decrease the total content of abstractable hydrogen atoms from hydroxyl groups at C-3 and C-6 positions or from free amino group at C-2 position. Further, their chelating ability against ferrous ions was also changed according to the type or degree of substitution. Thus, the structure-scavenging activity relationships of above derivatives can be explained depending on their abstractable hydrogen atom content and ion chelation ability.

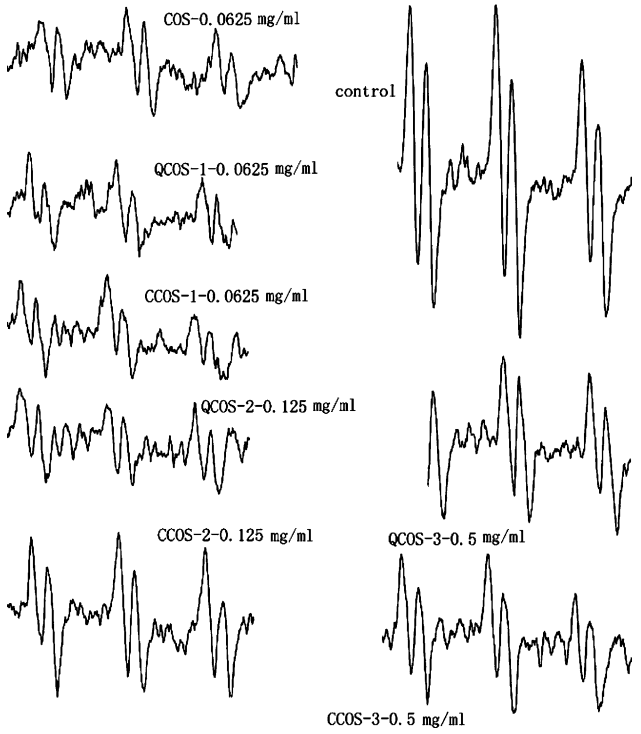

Fig. 5. ESR spectra of carbon-centered radicals in the presence of COS and its derivatives with different substitution degrees. ESR assay conditions were maintained for all measurement as described in the text.

Table 3
Scavenging percentages of COS and its differentially substituted derivatives on hydroxyl radicals

Sample	COS	QCOS-1	CCOS-1	QCOS-2	CCOS-2	QCOS-3	CCOS-3
Substitution degree (mg/ml)	0	0.2624	0.3666	0.5378	0.6992	0.7674	0.8989
0.0625	67.40	67.03	–	66.54	–	63.16	–
0.125	80.68	79.23	–	76.26	–	77.78	–
0.25	81.43	82.32	–	80.76	–	80.83	–
0.5	87.12	84.44	63.23	83.89	−22.14	80.20	−30.03
1.25	85.71	87.38	77.45	–	−25.01	81.13	−34.80
2.5	89.77	–	82.43	–	−9.75	79.88	−14.29
5	89.69	–	87.76	–	23.45	83.85	6.77

One significant phenomenon of scavenging was that, at lower concentrations both CCOS and QCOS derivatives exhibited a decreased scavenging activity on DPPH and carbon-centered radicals with increased substitution degree regardless of structural specificities, and remained uncharge at higher concentrations. DPPH and carbon-centered radicals are usually scavenged by hydrogen abstraction mechanism, and COS and its derivatives have followed the same mechanism to scavenge those radicals. As well known, hydrogen abstraction mechanism is a consumptive one, where the antioxidant reagents react with radicals in a non-recyclable manner. Therefore, in a radical scavenging assay, if antioxidants get depleted before all the radicals are scavenged and cannot be reproduced through another mechanism, generation of radicals will be continued with time (Halliwell et al., 1995). This phenomenon was clearly observed in time dependent carbon-centered radicals scavenging assay in the presence of COS (Fig. 7). In this study, the content of abstractable hydrogen atoms at hydroxyl or amino groups decreased as the substitution degree increased. Since, DPPH and carbon-centered radicals are mainly scavenged via hydrogen donation mechanism, reduction of abstractable hydrogen atoms in CCOS and QCOS was presumed to the observed decreased scavenging potencies at low concentrations. In contrast, at high concentrations of all derivatives, where hydrogen atoms were adequate to scavenge all radicals, no significant difference was observed for scavenging activities.

Another possible consideration in hydroxyl radical scavenging is that, not only substitution degree, but also structural specifics of the compound affect considerably to the scavenging potency. According to our observations, CCOS enhanced the generation of hydroxyl radicals. Further, it was found that this observation directly agreed with the results of their Fe^{2+} ion chelation abilities. As shown in Fig. 3, with higher substitution degrees of carboxyl groups (CCOS-3), no Fe^{2+} ion chelation ability was observed. This implied that, ion chelating can interferes the actual radical scavenging ability of COS derivatives.

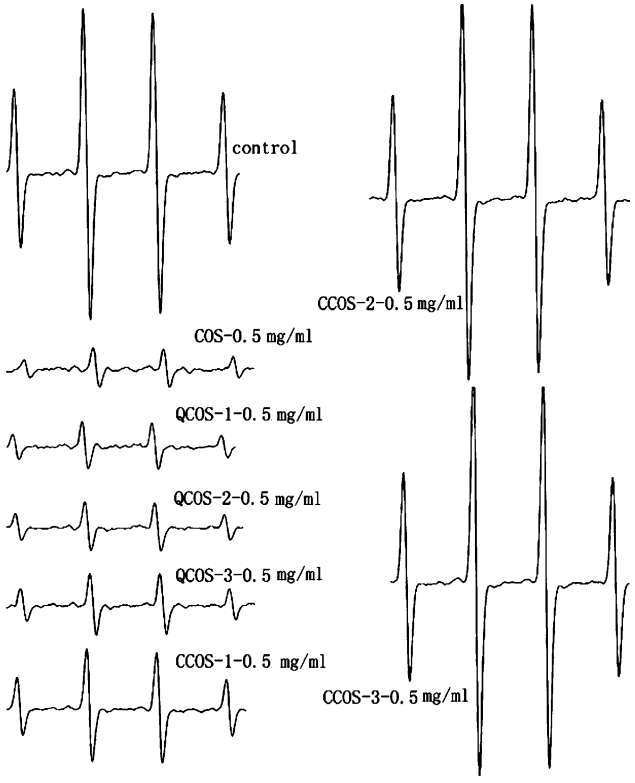

Fig. 6. ESR spectra of hydroxyl radicals in the presence of COS and its derivatives with different substitution degrees. ESR assay conditions were maintained for all measurement as described in the text.

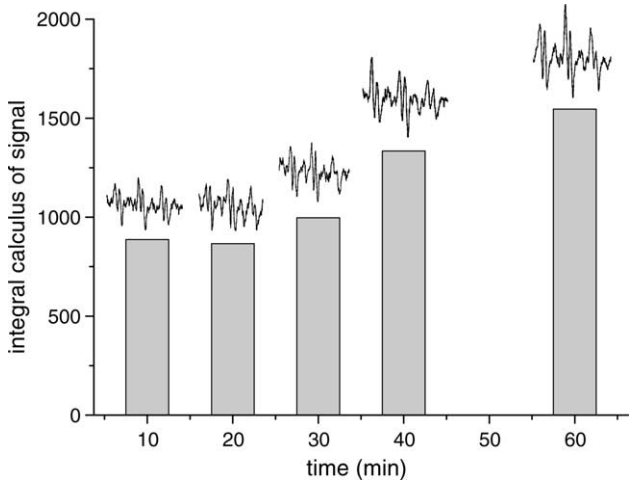

Fig. 7. Time dependent carbon-centered radical scavenging ability of COS. Concentration of COS was 0.03125 mg/ml and after 30 min of reaction control assay integral calculus was1870.

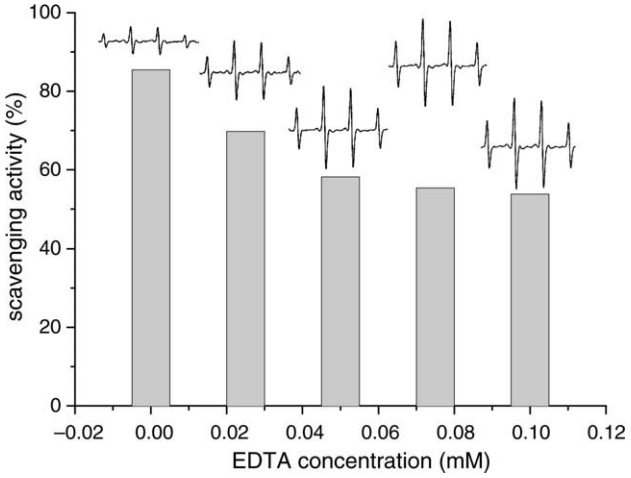

Fig. 8. Hydroxyl radical scavenging ability of COS influenced by EDTA. The concentration of COS was maintained at 0.25 mg/ml. Higher EDTA concentrations lowered the scavenging ability.

As previously reported, generation of radicals can be retarded by chelation of ferrous ions, that behaves as a catalyst in Fenton reaction (Halliwell et al., 1995). Even though chitosan and their derivatives are reported as significant chelators of ferrous ions (Vladimir, Lidia, & Ygor, 1996), no researches have been directed to investigate the contribution of ion chelation on antioxidant activity. To investigate the relationship between hydroxyl radical scavenging and ion chelation property of COS and its derivatives, Fenton reaction conditions were modified by adding a strong ion chelator, EDTA to control chelation of Fe^{2+} ions by COS and subsequent inhibition of hydroxyl radical generation. As depicted in Fig. 8, signal intensity of hydroxyl radicals increased with the increment of EDTA at a fixed concentration (0.25 mg/ml) of COS indicating loss of radical scavenging potency. EDTA is an effective chelator for ferrous ions, but can remain its activity in catalyzing the hydroxyl radical generation in Fenton reaction. When higher concentration of EDTA was added, more and more COS were released from its complex with ferrous ions and act as a weak radical scavenger. According to this mechanism, CCOS with higher degree of substitution (CCOS-2 and CCOS-3) lost their chelating ability against Fe^{2+} ions and subsequently exhibited no hydroxyl radical scavenging ability as shown in Table 3 and Fig. 6. This confirmed that, chelation of Fe^{2+} ions considerably accounts for scavenging hydroxyl radical.

4. Conclusions

Chitooligosaccharides and its derivatives can exert different mechanisms to scavenge different free radicals. With the structure-activity relationships obtained from the present research, we suggest that hydrogen donation is dominant for scavenging of DPPH and carbon-centered radicals. In contrast, chelation of Fe^{2+} ions indirectly contributed for their observed hydroxyl radical scavenging activity apart from hydrogen abstraction.

Acknowledgements

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