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Synthesis and characteristics of chitin and chitosan with the (2-hydroxy-3-trimethylammonium)propyl functionality, and evaluation of their antioxidant activity in vitro

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ABSTRACT

Quaternary amino groups were introduced into chitin and chitosan to obtain *O*-(2-hydroxy-3-trimethylammonium)propyl chitin (OHT-chitin) and *N*-(2-hydroxy-3-trimethylammonium)propyl chitosan (NHT-chitosan). They were characterized by FTIR spectra, and GPC. The molecular weight M_w of OHT-chitin and NHT-chitosan were 8986 and 9723 with polydispersity of 1.01 and 1.02, respectively. Their antioxidant activities *in vitro* were further studied. It was found that β-carotene-linoleic acid values of OHT-chitin and NHT-chitosan at 0.8 mg/mL were up to 91% and 96%, while that of chitosan was 40%. Based on photobleaching of α,α-diphenyl-β-picrylhydrazyl (DPPH) at 326 nm, the DPPH inhibitory activity of OHT-chitin and NHT-chitosan was 30.9% and 31.9% at 5 mg/mL, whereas chitosan only gave 4.8%. It was also exhibited that OHT-chitin and NHT-chitosan had better antioxidant activity than chitosan according to the reducing power as well as H_2O_2 scavenging activity.

1. Introduction

Oxygen free radicals or reactive oxygen species (ROS) including superoxide anion radicals, as well as hydrogen peroxide, have played a dual role as both deleterious and beneficial species. Beneficial effects of ROS only occur at low/moderate concentrations. Overproduction of ROS results in membrane lipid peroxidation, DNA alteration, and enzyme inactivation (Regoli & Winston, 1999; Valko et al., 2007). It also leads to aging, cancer and other human disease (Arouma, 1994). So far, many synthetic exogenous antioxidants, such as butylated hydroxyanisole, butylated hydroxytoluene, *t*-butylhydroquinone and propyl gallate, have been used to eliminate ROS and protect the cells against toxic effects of ROS. However, a number of reports indicated that these synthetic antioxidants may cause weak cancerogenic effect after a long-term and high-level ingestion (Shahidi & Zhong, 2010; Yvonne, Dawn, & Meshell, 2005). Therefore, recent efforts have been focused on the extraction, identification, modification and application of natural antioxidants in foods or medical materials to avoid pathogenic risk of synthetic antioxidants (Wu et al., 2011). Among the natural antioxidants, chitosan and chitosan derivatives have attracted a great deal of attention not only because of their characteristic of anti-oxidative and radical scavenging activities but also due to their extensive availability (Castagnino et al., 2008).

Chitosan and chitosan derivatives are commonly produced by deacetylation of chitin and further derivatization. After cellulose, chitin is the most ubiquitous natural polysaccharide, which is composed of β(1→4)-linked 2-acetamido-2-deoxy-β-D-glucose (*N*-acetylglucosamine). The bicentennial of the discovery of chitin has been celebrated with a review article in this journal (Muzzarelli et al., 2012).

Nowadays, chitin and chitosan have been shown to be useful in various fields, such as food, photography, cosmetics, water engineering, medicine and biomedical materials, owing to their biodegradable, nontoxic, biocompatible, broad antimicrobial, and nutritional characteristics (Alishahi, Mirvaghefi, & Tehrani, 2011; Moreira, Pereda, Marcovich, & Roura, 2011; Muzzarelli, 2009). They also clearly point to an immense potential future development in antioxidant (Jayakumar, Menon, Manzoor, Nair, & Tamura, 2010). However, chitin is only soluble in few dilute organic acid solutions and inorganic acid because it forms strong inter- and intra-molecular hydrogen bonds (Wu, Sasaki, Irie, & Sakurai, 2008).

N-(2-hydroxy-3-trimethylammonium)propyl chitosan chloride (NHT-chitosan) is one of the water-soluble quaternary ammonium salts of chitosan and easily prepared through chitosan coupled with glycidyl trimethyl ammonium chloride (GTMAC) (Qin et al., 2004). Although it has been demonstrated to have a good moisture-retention capacity and antimicrobial activity, there is relatively little work reported on its antioxidant activity. To our knowledge, there is no report on the synthesis of *O*-(2-hydroxy-3-trimethylammonium)propyl chitin (OHT-chitin) and its antioxidant activity in vitro.

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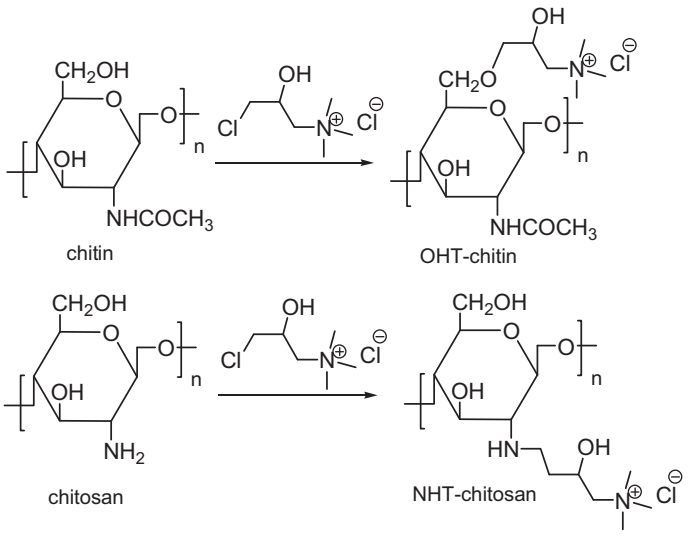

Scheme 1. Synthesis of OHT-chitin and NHT-chitosan.

Herein, the goal of the present study is to prepare two water soluble chitin and chitosan derivatives, OHT-chitin and *N*-(2-hydroxy-3-trimethylammonium)propyl chitosan (NHT-chitosan), and assess their antioxidant activity *in vitro*. Based on the data of β-carotene-linoleic acid activity, α,α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging activity, reducing power and scavenging activity of hydrogen peroxide, OHT-chitin and NHT-chitosan exhibit to be potential antioxidants.

2. Materials and methods

2.1. Chemicals

Chitin was obtained from shrimp shell according to the literature (Shahidi, Arachchi, & Jeon, 1999). Chitosan with cp viscosity of 100 MPa s (1%, 20 °C) and a DS deacetylation of value 80% was purchased from Qingdao Honghai Bio-technology Company (Shandong, China). β-Carotene, linoleic acid, Tween 20, hydrogen peroxide (H_2O_2), ascorbic acid, α,α-diphenyl-β-picrylhydrazyl (DPPH) radical, deoxyribose, iron(III) chloride ($FeCl_3$), ethylenediaminetetraacetic acid disodium salt (EDTA), dipotassium hydrogen phosphate, potassium dihydrogen phosphate, thiobarbituric acid, trichloroacetic acid, hydrochloric acid (37%, w/w) and potassium ferricyanide were obtained from Aladin Reagent Company (Shanghai, China). 3-Chloro-2-hydroxypropyltrimethylammonium chloride (CTA) was obtained from Tokyo Chemical Industry Company (Japan).

2.2. General procedure

The water-soluble quaternized chitin and chitosan were prepared according to a similar method described by Chen et al. (2010) with minor modification (Scheme 1).

Chitin or chitosan (2.00 g) was dispersed in 2-propanol (20 mL) with being stirred for 0.5 h at room temperature, and then aq. NaOH (5.00 g, 40 wt %) was added. After it was further stirred for 2 h, CTA (10.74 g) was added into the mixture and stirred further 6 h at 40 °C. Then the mixture was washed with 95% ethanol to get a white ropy solid. After filtration, the solid was dissolved in water (100 mL), filtered to remove insoluble gel-like particles. Finally, the filtrate was purified by the addition of anhydrous ether. The precipitated white ropy solid was collected and dried overnight under vacuum at 80 °C.

2.3. Structural characterization of OHT-chitin and NHT-chitosan

FT-IR spectra were recorded with KBr pellets on a Nicolet Nexus 870 FT-IR spectrophotometer.

Molecular weight distributions were measured on a conventional gel permeation chromatography (GPC) system equipped with a Waters 1525 Isocratic HPLC pump, a Waters 2414 refractive index detector, and a set of Waters Styragel columns (HR1, HR2 and HR4, 7.8 mm × 300 mm). GPC measurements were carried out at 35 °C using a DMF salt solution (5.5 g $NaNO_3$/L) as the eluent with a flow rate of 1.0 mL/min. The system was calibrated with linear polystyrene standards.

2.4. Antioxidant activity

2.4.1. β-Carotene-linoleic acid activity

The measurement of percent inhibition of peroxidation in a linoleic acid system was evaluated by using β-carotene bleaching test (Amin, Zamaliah, & Chin, 2004; Curcio et al., 2009; Lee, Kim, & Lee, 2010). Briefly, 1 mL of β-carotene solution (0.2 mg/mL in chloroform) was added to linoleic acid (0.02 mL) and Tween 20 (0.2 mL) mixture. After removal of chloroform with a rotary evaporator at 40 °C, 100 mL of distilled water was immediately added dropwise to the mixture with vigorous stirring to form an emulsion at room temperature. 5 mL of sample solution with different concentrations ranging from 0 to 0.8 mg/mL (chitosan was dissolved in 1% (v/v) acetic acid while OHT-chitin and NHT-chitosan were dissolved in distilled water) were transferred to different test tubes containing 5 mL of emulsion. 5 mL of emulsion with 5 mL of distilled water was used as control. Tubes were then gently shaken and placed in a water bath at 50 °C for 2 h. The absorbance of samples and control was measured at 470 nm and recorded at 30 min intervals (Shimadzu, UV-1800).

Antioxidant activity was measured in terms of successful bleaching of β-carotene using Eq. (1):

$$\text{Aox A} = 1 - \frac{A_0 - A_{120}}{A'_0 - A'_{120}} \quad (1)$$

where A_0 and A'_0 are the initial absorbance of sample and control, respectively, whereas A_{120} and A'_{120} are the absorbance of sample and control after 120 min, respectively. All samples were assayed in triplicate and data were mean values ± SEM (standard error of the mean).

2.4.2. DPPH radical scavenging activity

The scavenging effect of OHT-chitin and NHT-chitosan on a stable free radical DPPH was evaluated according to the literature (Lee et al., 2010). For this purpose, 1 mL of each polymer solution was put in a volumetric flask (25 mL), and then 4 mL of ethanol and 5 mL of ethanol solution of DPPH (200 μM) were added to afford a solution of DPPH with a final concentration of 100 μM. The mixtures were incubated in a water bath at 25 °C for 30 min. Then the absorbance of the resulting solution was measured from 200 nm to 600 nm with a spectrophotometer (Shimadzu UV-1800).

The DPPH radical scavenging activity was expressed as a percentage of inhibition activity according to Eq. (2):

$$\text{Inhibition\%} = 1 - \frac{A_x}{A_0} \quad (2)$$

where A_0 is the absorbance measured for control, whereas A_x is the absorbance measured in sample at different concentrations. All samples were assayed in triplicate and data were mean values ± SEM.

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2.4.3. Reducing power

The reducing power of quaternized chitin and chitosan derivatives was determined according to the literature (Yen & Duh, 1993). 1 mL of polymer sample with different concentrations ranging from 0 to 5 mg/mL was mixed with 0.2 mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of 1% potassium ferricyanide. The mixtures were incubated at 50 °C for 20 min. After incubation, 1 mL of 10% trichloroacetic acid was added, and then the mixtures were centrifuged at 6000 rpm for 10 min (Sigma 3–30 K). 2 mL of supernatant was mixed with 0.35 mL of 3% ferric chloride solution and 1 mL of distilled water, and then the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. All samples were assayed in triplicate and data were mean values ± SEM.

2.4.4. Scavenging of hydrogen peroxide

Hydrogen peroxide scavenging activity of quaternized chitin and chitosan was evaluated according to the literature (Feng, Du, Li, Wei, & Yao, 2007; Sahgal et al., 2009) with slight modification. A sample solution (5 mg/mL) was added to 2.4 mL of 0.1 M phosphate buffer (pH 7.4), and mixed with 0.6 mL of a 43 mM solution of hydrogen peroxide. The absorbance was recorded from 0 to 40 min and at every 10 min interval until 240 min at 230 nm. For each sample, a separate blank without addition of hydrogen peroxide was used for background subtraction.

The antioxidant activity was expressed as a percentage of scavenging activity on hydrogen peroxide according to Eq. (3):

$$\text{Inhibition}\% = 1 - \frac{A_0 - A_x}{A'_0 - A'_x} \quad (3)$$

where A_0 and A'_0 are the absorbance measured at the initial incubation time for sample and control, respectively, whereas A_x and A'_x are the absorbance of sample and control measured at successive time, respectively. All samples were assayed in triplicate and data were mean values ± SEM.

3. Results and discussion

3.1. FT-IR spectra

The FTIR spectra of four polymers are shown in Fig. 1. Comparing the spectra of chitosan (b) and quaternized chitosan (c), the amine scissoring band at 1659 cm^{-1} and 1567 cm^{-1} of chitosan disappear, implying that the epoxide groups of CTA have coupled with the NH_2 groups rather than the OH groups of chitosan (Nam, Kim, & Ko, 1999). Also, a new sharp peak at 1453 cm^{-1} appeared is the characteristic of the methyl groups stretching of $N(CH_3)_3$ of NHT-chitosan. Furthermore, the band due to N—H bending vibration, which is observed at 1659 cm^{-1} in chitosan film, was lowered to 1579 cm^{-1} because —CH_2CH(OH)— of CTA which coupled with —NH is electron-withdrawing group.

Compared with FTIR spectra of chitin (a) and OHT-chitin (d), the characteristic peaks at (a), 1661 cm^{-1} is assigned to the stretching vibration of C=O group (amide I), while 1559 cm^{-1} (N—H, amide II), 1377 cm^{-1} (C—H, CH_3), 1315 cm^{-1} (C—N and N—H, amide III), 1158 cm^{-1}, 1072 cm^{-1}, and 1022 cm^{-1} are assigned to the C—O—C and C—O stretching vibrations modes. All of those peaks are in accordance with chitin (Cortizo, Berghoff, & Alessandrini, 2008). As to the FTIR spectra of OHT-chitin (d), the C=O stretching frequency

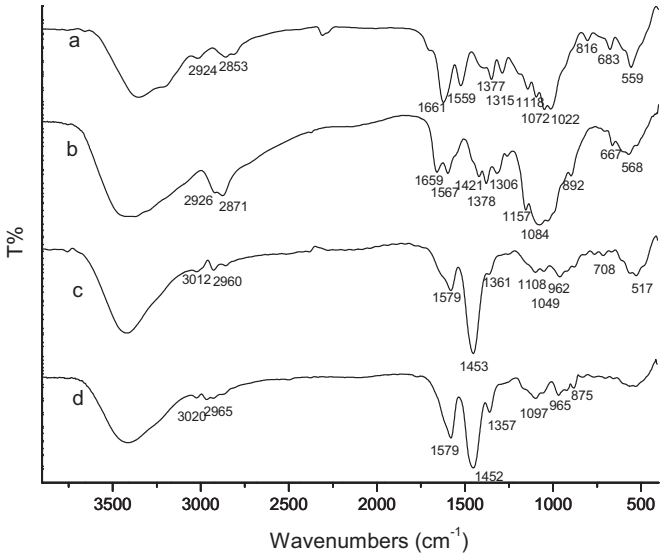

Fig. 1. FT-IR spectra of chitin (a), chitosan (b), NHT-chitosan (c) and OHT-chitin (d).

of amide is found to be lowered to 1579 cm^{-1} with an increased intensity. That might indicate some acetamido groups of chitin were deacetylated under harsh alkaline reaction condition. New intense peak 1452 cm^{-1} is probably due to the C—H stretching of —$N^+(CH_3)_3$ of OHT-chitin.

3.2. GPC

Molecular weights of chitin and chitosan derivatives are very important for industrial and scientific uses since different molecular weight polymers have different functions (Aranaz et al., 2009). To estimate the molecular weight distribution of each fraction, its weight-average molecular weight (M_w) and number-average molecular weight (M_n) were measured by GPC, as summarized in Table 1.

The polydispersity (PD) is determined from the ratio of M_w to M_n to be 1.01 and 1.02 for NHT-chitosan and OHT-chitin, respectively. The data in Table 1 also suggest that a series of samples fractions have narrow dispersity with similar molecular weights distribution.

3.3. Antioxidant activity

3.3.1. β-Carotene-linoleic acid activity

In the β-carotene-linoleic acid model system, free radicals generating by the oxidation of linoleic acid can attack the highly unsaturated β-carotene, and as a consequence, the characteristic orange color disappears. The presence of antioxidant can avoid the destruction of the β-carotene conjugate system to keep the orange color. Antioxidant activity was determined by measuring the inhibition of conjugated diene hydroperoxides arising from linoleic acid oxidation.

As shown in Fig. 2, antioxidant activity of OHT-chitin and NHT-chitosan increase progressively along with the increase of concentrations ranging from 0 to 0.4 mg/mL. Inhibitory activity of NHT-chitosan and OHT-chitin reach 86% and 80%, respectively.

Table 1
GPC results of OHT-chitin and NHT-chitosan.

Sample name	M_n	M_w	M_p	M_z	M_{z+1}	Polydispersity	M_z/M_w	M_{z+1}/Mw
NHT-chitosan	9641	9723	9273	9807	9893	1.009	1.009	1.017
OHT-chitin	8852	8986	9164	9122	9260	1.015	1.015	1.030

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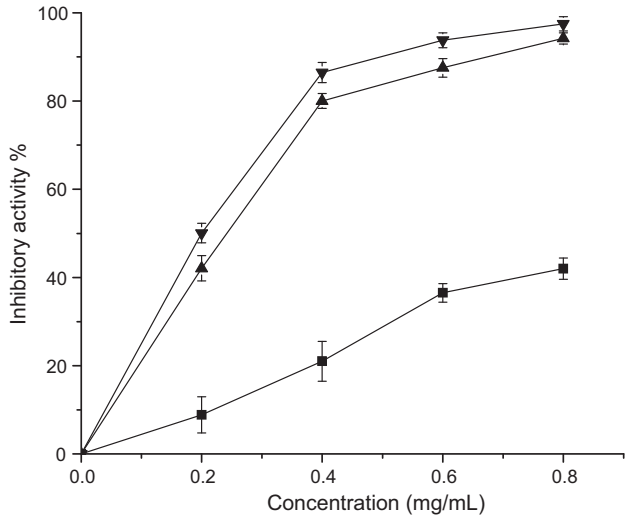

Fig. 2. β-Carotene-linoleic acid activity of (■) chitosan, (▲) OHT-chitin, (▼) NHT-chitosan.

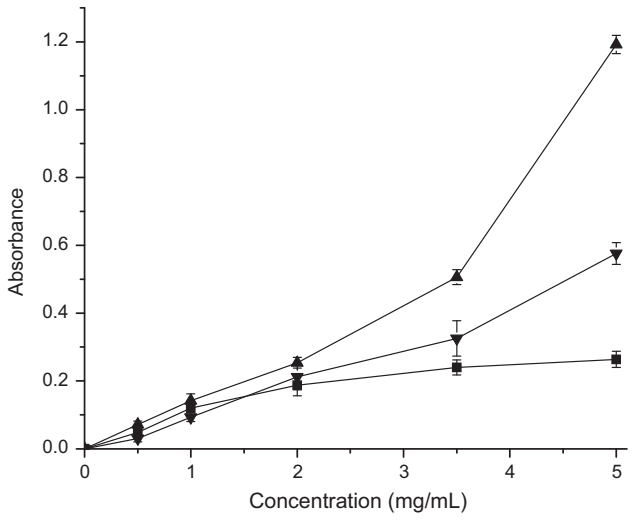

Fig. 4. Reducing power of (■) chitosan, (▲) OHT-chitin, (▼) NHT-chitosan.

However, inhibitory activity of chitosan is 21% at 0.4 mg/mL. Beyond that concentration, the inhibitory activity of both OHT-chitin and NHT-chitosan increase smoothly. When polymers concentration is 0.8 mg/mL, inhibitory activity of OHT-chitin and NHT-chitosan is up to 91% and 96%, respectively, whereas that of chitosan is only 40%.

3.3.2. Determination of scavenging effect on DPPH radicals

DPPH is a semi-stable free radical, acting as an electron acceptor from antioxidants and can be characterized using UV-vis (Fig. 3). It is a useful reagent for evaluating antioxidant activity of compounds based on photobleaching of DPPH at λ_{max} = 517 nm (Curcio et al., 2009). In this study, no significant difference in the DPPH decay rate is observed in ethanol solution system. A set of UV-vis spectra measured upon irradiation of DPPH in polymer/ethanol suspensions are shown in Fig. 3. The absorbance decrease of DPPH absorption bands at 326 nm and 517 nm is accompanied by the concurrent absorption band at 433 nm which corresponds to the formation of DPPH photoproducts. This photobleaching phenomenon of DPPH at 326 nm is similar as previous report (Brezov, Dvoranov, & Stasko, 2007). Herein, DPPH radical scavenging activities based on photobleaching at both 326 nm and 517 nm are recorded. All samples demonstrate a concentration-dependent DPPH radical scavenging activity at both 326 nm and 517 nm (Table 2). However, there is no obvious improvement of the inhibitory activity after 5 mg/mL (data not shown).

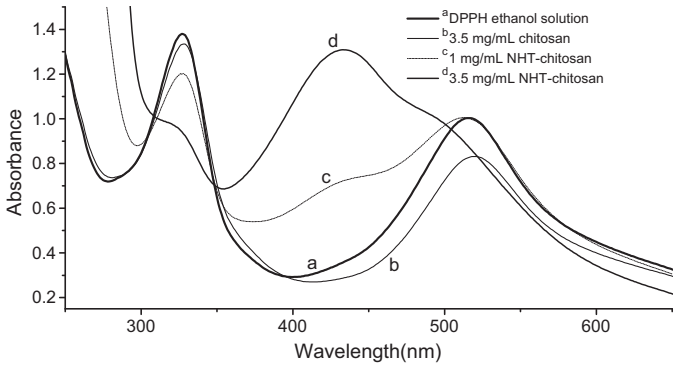

Fig. 3. UV-vis spectra of the mixture of chitin derivatives with various concentrations and DPPH ethanol solution.

As shown in Table 2, OHT-chitin and NHT-chitosan have better DPPH radical scavenging activities than chitosan, the inhibitory activity of OHT-chitin and NHT-chitosan reach 30.9% and 31.9% at 5 mg/mL, respectively, whereas that of chitosan is only 4.8%. Although the inhibitory activity of chitosan is 23.2% which is slight higher than those of OHT-chitin and NHT-chitosan (14.8% and 14.0%, respectively) based on photobleaching of DPPH (λ_{max} = 517 nm), there is no clear difference in DPPH radical scavenging activity based on photobleaching at 326 nm even at 5 mg/mL. This result suggests that the mechanism of DPPH radical scavenging activity of OHT-chitin and NHT-chitosan is different from that of chitosan, which is worthy to be studied in the future.

3.3.3. Reducing power

The reducing capacity is generally associated with the presence of reducing sugars due to their hydrogen-donating ability (Shimada, Fujikawa, Yahara, & Nakamura, 1992). As shown in Fig. 4, the reducing power increases along with the increase of concentration of polymer samples. That of OHT-chitin improved dramatically when the concentration is higher than 3.5 mg/mL. The reducing power of OHT-chitin and NHT-chitosan are up to 1.192 and 0.576 at 5 mg/mL, whereas that of chitosan is 0.263. Furthermore, OHT-chitin exhibits excellent reducing capacity comparing with NHT-chitosan and chitosan. Those results maybe because: (1) chitosan has two hydroxyl groups and one amino group in its structure, while OHT-chitin and NHT-chitosan have more active hydroxyl groups than chitosan, thus, they have more sites to bind metal ions; (2) the function group is connected to 6-OH in the structure of OHT-chitin, which leads to more steric effect to bind metal ions than that of NHT-chitosan, in which the function group is connected to 2-NH_2.

3.3.4. Scavenging of hydrogen peroxide

Hydrogen peroxide can be generated in biological and food systems. It can attack several cellular energy-producing systems when it keeps in high level (Lin & Chou, 2004). H_2O_2-scavenging activity of polymers increases as their concentration increases up to 5 mg/mL (data not shown). As shown in Fig. 5, different kinds of polymers exhibit different extents of H_2O_2-scavenging activities. It is also found that the H_2O_2-scavenging activity of polymers increases along with the time. The scavenging activity of OHT-chitin, NHT-chitosan and chitosan bumps up to 19%, 27% and 18% after 20 min, and reaches to 56%, 59% and 21%, at 240 min, respectively.

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Table 2
Inhibition percentages of DPPH radicals by chitosan, NHT-chitosan, and OHT-chitin with different concentrations at 326 nm and 517 nm.

Wavelength (nm)	Inhibition%				
	Sample	Concentration (mg/mL)			
		0.5	1	3.5	5
326	Chitosan	2.95 ± 2.53	3.67 ± 2.44	4.03 ± 1.74	4.75 ± 1.47
	NHT-chitosan	10.31 ± 1.62	16.36 ± 1.31	31.65 ± 1.05	31.86 ± 1.73
	OHT-chitin	6.63 ± 1.71	13.33 ± 1.97	30.28 ± 1.18	30.93 ± 1.06
517	Chitosan	5.33 ± 2.07	10.06 ± 1.92	13.55 ± 2.58	23.27 ± 2.11
	NHT-chitosan	1.28 ± 2.32	7.40 ± 1.36	13.40 ± 1.71	17.05 ± 1.51
	OHT-chitin	1.18 ± 2.46	5.13 ± 2.32	11.28 ± 1.87	14.77 ± 1.63

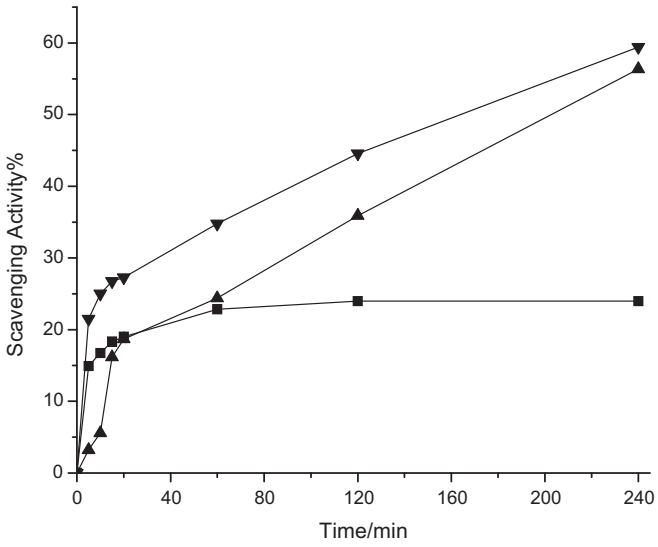

Fig. 5. Hydrogen peroxide scavenging activity of (■) chitosan, (▲) OHT-chitin, (▼) NHT-chitosan.

It was previously reported that hydroxyl radicals generated from H_2O_2 can destruct the structure of chitosan. It was also demonstrated that chitosan derivatives can affect H_2O_2-scavenging activity, so that the amount of H_2O_2 in the reaction system was reduced (Lin & Chou, 2004). Herein, the H_2O_2-scavenging activity of our polymer samples correlates well with these results.

3.3.5. The hydroxyl groups from CTA influencing the antioxidant activities

The antioxidant activity of chitosan may be because its hydroxyl and free amino groups being as proton-donators that can react with oxygen free radicals or reactive oxygen species to form most stable macromolecules (Liu et al., 2009). In this study, the antioxidant activities of OHT-chitin and NHT-chitosan on DPPH and H_2O_2 are much better than those of chitin and chitosan mostly because they have more active hydroxyl groups in alkyl chain coming from CTA. With the result of that, OHT-chitin and NHT-chitosan donate more protons to free radicals which are electron-deficient species by typical H-abstraction reaction or through addition reaction.

4. Conclusion

Quaternary amino groups were introduced into chitin and chitosan biopolymers for the purpose of soluble characteristic, altering the amount of hydroxyls that are capable of reacting with radicals, and modifying the chelating capacity. It is demonstrated that the modified polysaccharides, OHT-chitin and NHT-chitosan in our case, can behave as effective radical scavengers, and their scavenging activities are concentration-dependant. The present results are useful for further development of functional biomaterials which can be used as a source of natural antioxidants, as a possible food supplement or in the pharmaceutical industry.

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