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Free radical scavenging activities of differently deacetylated chitosans using an ESR spectrometer

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Abstract

Three kinds of partially deacetylated hetero-chitosans (90% chitosan, 90% deacetylated chitosan; 75% chitosan, 75% deacetylated chitosan; 50% chitosan, 50% deacetylated chitosan), were prepared, and their scavenging activities were investigated against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, alkyl radical, hydroxyl radical and superoxide radical using electron spin resonance spectrometer. Five mg/ml of 90, 75 and 50% chitosan scavenged 38.72, 35.52 and 13.99% of DPPH radical, respectively. The scavenging activities of hetero-chitosans increased from 3 to 69.39% with increasing concentration from 1.25 to 5 mg/ml of alkyl radical. In addition, 90% chitosan with relatively high degree of deacetylation showed the highest radical scavenging effects on the hydroxyl radical and superoxide radical. These results indicate that the radical scavenging activities of hetero-chitosans depend on their degree of deacetylation and concentration.

Keywords: Hetero-chitosans; Free radicals; Scavengers; Electron spin resonance

1. Introduction

Reactive oxygen-mediated modification of DNA, proteins, lipids and small cellular molecules is associated with a number of pathological processes, including atherosclerosis, arthritis, diabetes, cataractogenesis, muscular dystrophy, pulmonary dysfunction, inflammatory disorders, ischemia-reperfusion tissue damage and neurological disorders such as Alzheimer's disease (Frlich & Riederer, 1995). Free radical scavenger is a preventive antioxidant. The term antioxidant is defined as any substance that, when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate (Halliwell & Gutterridge, 1989). Antioxidants can act at different levels in an oxidative sequence. This may be illustrated by considering one of the many mechanisms by which oxidative stress can cause damage by stimulating the free radical chain reaction of lipid peroxidation. Free radical chain reactions within a material could be inhibited by adding chemicals that retard the formation of free radicals, by introducing substances that retard the formation of free radicals or by introducing substances that compete for the existing radicals and remove them from the reaction medium. Many synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, tertiary-butyl-hydroquinone and propyl gallate may be used to retard lipid peroxidation in a lot of fields (Wanita & Lorenz, 1996). However, use of synthetic antioxidants is under strict regulation due to the potential health hazards caused by such compounds (Kim, Kim, Byun, Park, & Ito, 2001; Park, Jung, Nam, Shahidi, & Kim, 2001). Therefore, search for natural antioxidants as alternatives to synthetic ones is of great interest among researchers.

Chitosan, a non-toxic biopolymer, is a partially deacetylated polymer of *N*-acetyl glucosamine, which is obtained after alkaline deacetylation of the chitin derived from the exoskeletons of crustaceans and arthropods. It has received considerable attention for its commercial applications in biomedical and chemical fields because of its biological functions such as antitumor activity (Jeon & Kim, 2002; Suzuki et al., 1986), antimicrobial activity (Hadwiger & Beckman, 1980; Jeon, Park, & Kim, 2001; Kim, Jeon, & Zan, 2000; Uchida, Izume, & Ohtakara, 1989), antimutagenic activity (Shon, Ha, Jeong, Kim, & Nam, 2001) and immuno-enhancing effects (Jeon & Kim, 2001; Suzuki, Watanabe, Mikami, Matsumoto, & Suzuki, 1992).

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However, little information on the antioxidative activity of chitosan is available until now.

In the present study, three kinds of differently deacetylated chitosans were prepared from crab shell chitin, and their antioxidative activities were evaluated on the basis of their abilities to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, hydroxyl radical, superoxide radical and alkyl radical.

2. Materials and methods

2.1. Materials

Chitin prepared from crab shells was donated by Kitto Life Co. (Seoul, Korea). Potassium polyvinylsulfate (PVSK) to determine degree of deacetylation of partially deacetylated chitosans was purchased from Woko Pure Chemical Industries Ltd (Osaka, Japan). Hypoxanthine, xanthine oxidase, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), 1,1-diphenyl-2-picrylhydrazyl (DPPH), α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone (4-POBN), 2,2-azo-bis-(2-amidinopropane)-hydrochloride (AAPH) were purchased from Sigma Chemical Co. (St Louis, USA). All other reagents were of the highest grade available commercially.

2.2. Preparation of hetero-chitosans

Partially deacetylated chitosans were prepared from crab chitin by *N*-deacetylation with 40% (w/w) sodium hydroxide solution at 100 °C for various durations. The average molecular weights of the chitosans were 1.4×10^5–3.1×10^5 as determined by viscosimetry (Roberts & Domszy, 1982). After the reaction, hetero-chitosan samples were washed thoroughly with distilled water and freeze dried.

2.3. Titration method

The degree of deacetylation of hetero-chitosans was carried out by the method of Kina, Tamura, and Ishibashi (1974). Hetero-chitosans (0.5 g) were dissolved in 99.5 g of 5% (v/v) acetic acid. One gram of chitosan/acetic acid solution was mixed with 30 ml distilled water, and then 2–3 drops of 0.1% toluidine blue (indicator) were added in the reaction solution. The solution was titrated with N/400 PVSK, which had been calibrated with acetylpyridinium chloride monohydrate. The degree of deacetylation (DD) was calculated as follows

$$\mathrm{DD}\ (\%) = [X/161/(X/161 + Y/203)] \times 100$$

where

$$X = 1/400 \times 1/1000 \times f \times 161 \times V$$

and

$$Y = 0.5 \times 1/100 - X$$

where f is the factor for N/400 PVSK and V is the titrated volume of PVSK.

2.4. Infrared spectroscopy

Hetero-chitosan samples prepared in the form of potassium bromide (KBr) disks were studied. The KBr disk was prepared according to the method of Sabnis and Block (1997) with slight modifications. Chitosan samples (10 mg) were dried overnight at 60 °C under reduced pressure. The dried hetero-chitosan samples were mechanically blended with 100 mg of KBr. The mixture was compacted using an IR hydraulic press at a pressure of 8 tons for 60 s. They were dried for 24 h at 60 °C under reduced pressure before measuring. The spectra of hetero-chitosan samples in the forms of KBr disk were obtained using an FT-IR spectrometer (Perkin Elmer Spectrum 2000, Beaconsfield Bucks, England) with a frequency range of 4000–400 cm^{-1}. The degree of deacetylation of the hetero-chitosan samples was calculated by the method of Baxter, Dillan, Taylor, and Roberts (1992) as follows

$$\mathrm{DD}\ (\%) = 100 - [A_{1655}/A_{3450}] \times 115$$

where A_{1655} and A_{3450} are the absorbance at the wavelength of 1655 and 3450 cm^{-1}.

2.5. Preparation of chitosan solution

Chitosan solution (1%) was prepared by dispersing 1 g of chitosan in 10 ml of distilled water, while stirring with 5.5 ml of 1 M lactic acid, and making the final volume up to 100 ml with distilled water. The solution was adjusted to pH 5.5 using saturated $NaHCO_3$ solution.

2.6. DPPH radical assay

DPPH radical scavenging activity was measured using the method described by Nanjo et al. (1996). An ethanol solution of 60 μl each sample (or ethanol itself as control) was added to 60 μl of DPPH (60 μmol/l) in ethanol solution. After mixing vigorously for 10 s, the solutions were then transferred into a Teflon capillary tube and fitted into the cavity of the electron spin resonance (ESR) spectrometer. The spin adduct was measured on JES-FA ESR spectrometer (JEOL, Tokyo, Japan) exactly 2 min later. Measurement conditions: central field 3475 G, modulation frequency 100 kHz, microwave frequency 9.44 GHz, modulation amplitude 2 G, microwave power 5 mW, gain 6.3×10^5, temperature 298 K.

2.7. Alkyl radical assay

Alkyl radicals were generated by AAPH. The phosphate-buffered saline (pH 7.4) reaction mixtures containing 10 mmol/l AAPH, 10 mmol/l 4-POBN and indicated concentrations of tested samples, were incubated at 37 °C

in a water bath for 30 min (Hiramoto, Johkoh, Sako, & Kikugawa, 1993), and then transferred to a capillary tube. The spin adduct was recorded on a JES-FA ESR spectrometer. Measurement conditions: central field 3475 G, modulation frequency 100 kHz, microwave frequency 9.44 GHz, modulation amplitude 2 G, microwave power 5 mW, gain 6.3×10^5, temperature 298 K.

2.8. Hydroxyl radical assay

Hydroxyl radicals were generated by the Fenton reaction, and reacted rapidly with nitrone spin trap DMPO: the resultant DMPO-OH adduct was detectable with an ESR spectrometer (Rosen & Rauckman, 1984). The ESR spectrum was recorded 2.5 min after mixing in a phosphate buffer solution (pH 7.4) with 0.3 M DMPO 0.2 ml, 10 mM $FeSO_4$ 0.2 ml and 10 mM H_2O_2 0.2 ml using an ESR spectrometer set at the following conditions: central field 3475 G, modulation frequency 100 kHz, microwave frequency 9.44 GHz, modulation amplitude 2 G, microwave power 5 mW, gain 6.3×10^5, temperature 298 K.

2.9. Superoxide radical assay

Superoxide radicals were generated from hypoxanthine–xanthine oxidase system (Rosen & Rauckman, 1984). A 4 mM hypoxanthine 50 μl was mixed with phosphate buffered saline (PBS) 30 μl, samples 50 μl, 4.5 M DMPO 20 μl and 0.4 U/ml xanthine oxidase 50 μl. The reaction mixture transferred into a 100 μl Teflon capillary tube. After 45 s the ESR spectrum was recorded using an ESR spectrometer. Manganese oxide was used as an internal standard. Experimental conditions as follows: central field 3475 G, modulation frequency 100 kHz, microwave frequency 9.44 GHz, modulation amplitude 2 G, microwave power 5 mW, gain 6.3×10^5, temperature 298 K.

3. Results and discussion

The deacetylation of crab chitin was done by 40% sodium hydroxide treatment at 100 °C for 1, 2, 3, 4, 5, 6 and 7 h durations on a small scale. The degrees of deacetylation measured by titration are shown in Fig. 1. Larger scale production of hetero-chitosans was carried out by 40% sodium hydroxide treatment at 100 °C for time lengths of 290, 370 and 440 min based on the results shown in Fig. 1, and the degree of deacetylation was determined by titration and IR spectroscopy. The FT-IR spectra of hetero-chitosans showed subtle differences in the absorption intensities (Fig. 2). Baxter et al. (1992) reported that there is considerable variation in the value obtained from the same spectrum depending on the particular ratio used and that only the A_{1655}/A_{3450} ratio gives the typical deacetylation curve given by titrimetric analysis although the absolute values are not in agreement. The degree of deacetylation of

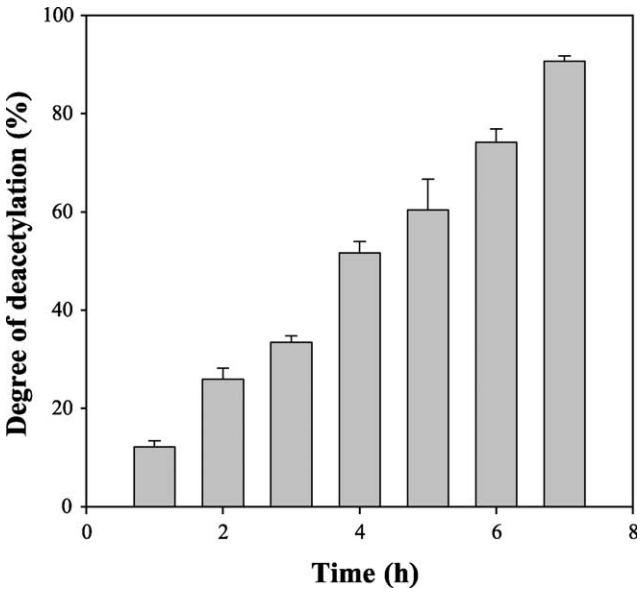

Fig. 1. Degree of deacetylation of hetero-chitosans from chitin by 40% sodium hydroxide solution (w/v) in different incubation times at 100 °C. The deacetylation values were calculated from titration method using potassium polyvinylsulfate.

hetero-chitosans prepared in different time lengths was 51.7, 74.2 and 90.7% by the titration method, and 47.6, 75.1 and 88.3% by FT-IR spectroscopy, respectively. Therefore, three kinds of hetero-chitosans were prepared, and designated as 90, 75 and 50% chitosan.

The IR spectroscopic method, which was initially proposed by Moore and Roberts (1978), is commonly used for the estimation of the degree of deacetylation values of chitosan. It has several advantages because it is relatively fast and does not require dissolution of the chitosan sample in an aqueous solvent (Baxter et al., 1992; Sabnis & Block, 1997).

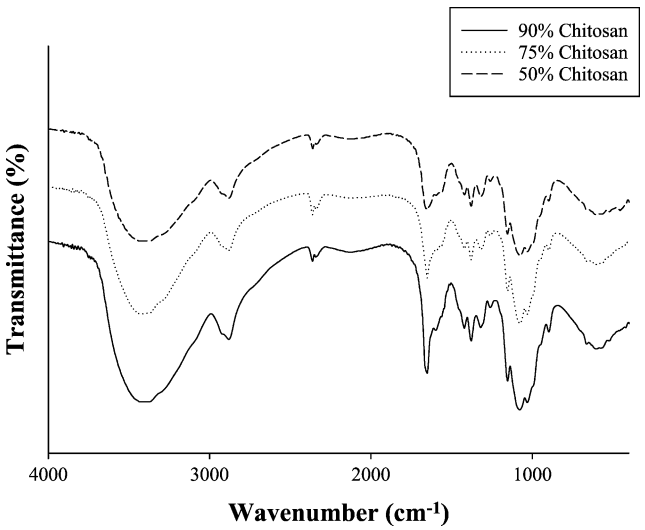

Fig. 2. FT-IR spectra of 90, 75, and 50% deacetylated chitosan.

In this study, scavenging activities were calculated by the following equation

$$\text{Radical scavenging activity } (\%) = (1 - H'/H_0) \times 100$$

where H' and H_0 are the relative peak height of the ESR signal of each sample with and without differently deacetylated chitosans, respectively. The ESR spectrum of DPPH radical is shown in Fig. 3. It was observed that 5 mg/ml of 90, 75 and 50% chitosan scavenged 38.72, 35.52 and 13.99% of DPPH radical, respectively. All the hetero-chitosans examined were found to possess DPPH radical scavenging activity. The alkyl radical spin adduct was observed when AAPH was incubated with the spin trap 4-POBN at 37 °C for 30 min, and decrease of ESR signals was observed with the dose-increment of hetero-chitosans (Fig. 4). Scavenging activity of hetero-chitosans increased from 3 to 69.39% with increasing concentrations from 1.25 to 5 mg/ml. In addition, 5 mg/ml of 50, 75 and 90% chitosan scavenged 43.01, 69.39 and 68.41% of alkyl radicals, respectively.

Hydroxyl radicals generated in Fe^{2+}/H_2O_2 system were trapped by DMPO forming spin adduct which could be detected by ESR spectrometer, and the typical 1:2:2:1 ESR signal of the DMPO-OH adduct was observed (Fig. 5). Hetero-chitosans (0.1 mg/ml) quenched 95% of the hydroxyl radical signals, and the scavenging activity increased with the concentration of the hetero-chitosans. Hetero-chitosans suppressed the signals of the superoxide anion-DMPO adduct on ESR charts, and the manner was dose dependent (Fig. 6). The scavenging activity of superoxide radical was calculated by the following equation

$$\text{Radical scavenging activity } (\%) = (1 - H'/H_0) \times 100$$

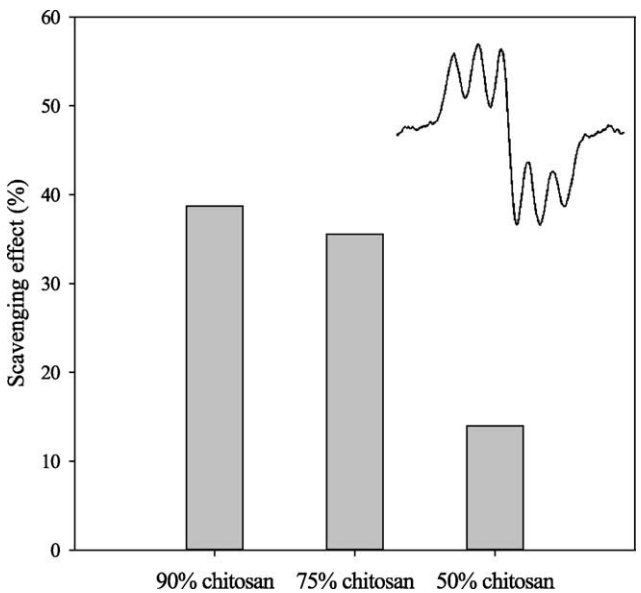

Fig. 3. ESR spectrum of DPPH radical obtained in an ethanol solution of 30 μmol/l DPPH at concentrations of 5 mg/ml chitosan samples. Inset shows ESR spectrum of DPPH radical.

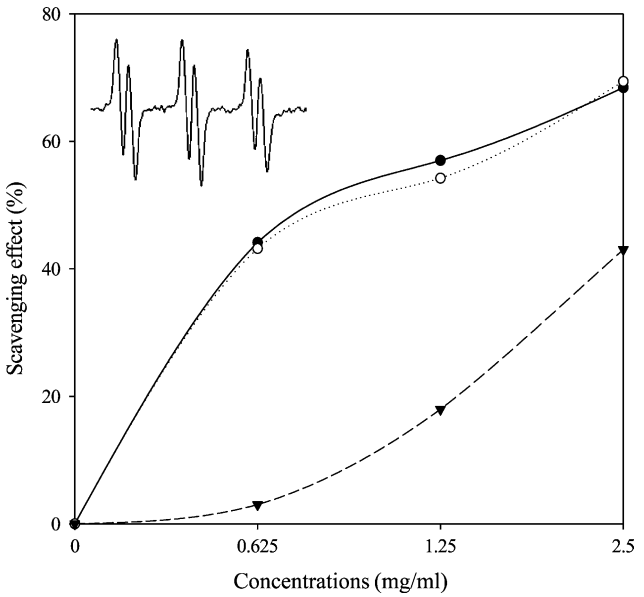

Fig. 4. Scavenging effects of 90% chitosan (●), 75% chitosan (○), and 50% chitosan (▼) on alkyl radical observed during incubation of AAPH with 4-POBN at various concentrations of hetero-chitosans. The incubation was done in a water bath containing 0.05 mol/l PBS, 10 mmol/l AAPH and 0.1 mmol/l 4-POBN. Inset shows ESR spectrum of the spin adduct of 4-POBN/alkyl radical generated from AAPH.

where H' and H_0 are the ESR signal intensity of sample in the presence and absence of hetero-chitosans, respectively. As shown in Fig. 6, it was observed that 0.5 mg/ml of 50, 75 and 90% chitosan scavenged 46.53, 49.66 and 53.24% of superoxide radicals, respectively. Vitamin C was used as the reference for an antioxidant in this study, and the scavenging

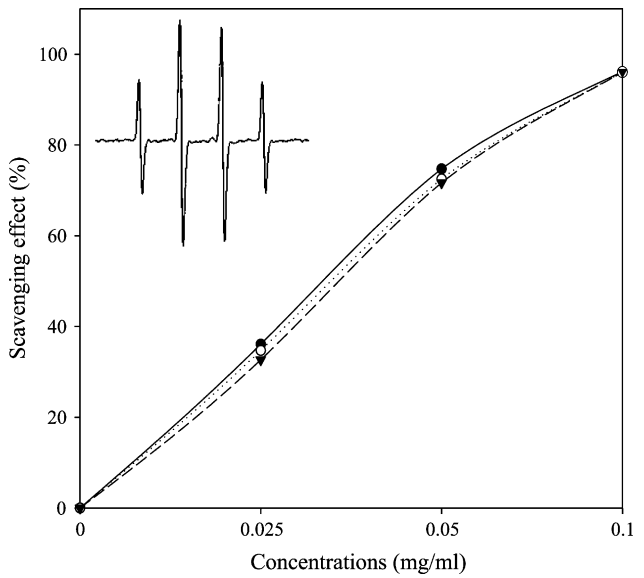

Fig. 5. Scavenging effects of 90% chitosan (●), 75% chitosan (○), and 50% chitosan (▼) on hydroxyl radical obtained in Fenton reaction system at various concentrations of hetero-chitosans. Inset shows ESR spectrum of the spin adduct of DMPO-OH.

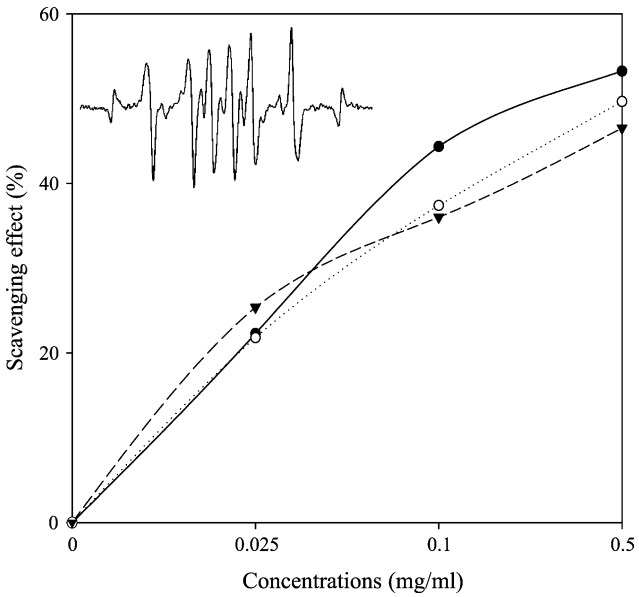

Fig. 6. Scavenging effects of 90% chitosan (●), 75% chitosan (○), and 50% chitosan (▼) on superoxide radical obtained in hypoxanthine–xanthine oxidase system at various concentrations of hetero-chitosans. Inset shows ESR spectrum of superoxide radical.

values of vitamin C on DPPH radical, alkyl radical, hydroxyl radical and superoxide radical were 90.37, 65.21, 83.32 and 41.37% at the concentration of 0.04, 0.3, 0.05 and 0.02 mg/ml, respectively. These results were similar to the scavenging value of vitamin C on hydroxyl radical and lower to the scavenging values of vitamin C on DPPH radical, alkyl radical and superoxide radical, respectively (data not shown).

Chitin consists of 2 acetamido-2 deoxy-(1 → 4)-β-D-glucopyranose residues (*N*-acetyl-D-glucosamine units). Chitosan is a *N*-deacetylated derivative of chitin and consists of 2-amino-2-deoxy-(1 → 4)-β-D-glucopyranose residues (D-glucosamine units). Hetero-chitosans have obvious scavenging activities on DPPH, alkyl, hydroxyl, and superoxide radical. Matsugo et al. (1998) reported that three different water-soluble chitosan derivatives by the acylation of chitosan inhibited thiobarbituric acid reactive substrate formation in *t*-butylhydroperoxide and benzoyl peroxide induced lipid peroxidations. Xie, Xu, and Liu (2001) reported that water-soluble chitosan derivatives were prepared by graft copolymerization of maleic acid sodium onto hydroxypropyl chitosan and carboxymethyl chitosan sodium, and their scavenging activities were observed against hydroxyl radical. In addition, Xie et al. (2001) reported that the scavenging mechanism of chitosan is related to the fact that free radical can react with the residual free amino groups NH_2 to form stable macromolecule radicals, and the NH_2 groups can form ammonium groups NH_3^+ by absorbing a hydrogen ion from the solution. The present study revealed that the scavenging activities on various radicals depended on degree of deacetylation of chitosan; also 90% chitosan with high degree of deacetylation values exhibited the highest scavenging activity. These results suggest that hetero-chitosans eliminate various free radicals by the action of nitrogen on the C-2 position of the chitosan.

Free radicals with the major species of reactive oxygen species (ROS) are unstable and react readily with other groups or substances in the body, resulting in cell damage and hence human disease (Halliwell & Gutterridge, 1989). It is generally considered that the inhibition of lipid peroxidation by an antioxidant may be due to the free radical scavenging activity. Lipids of biological membranes, especially those in the spinal cord and brain containing highly oxidizable polyunsaturated fatty acids, are particularly affected (Braughler & Hall, 1989). Moreover, the brain contains considerable amounts of prooxidant transition metal ions and utilizes a lot of oxygen. These properties set the stage for ROS generation and the subsequent acute cellular injury. Free radicals are implicated in the aging process and in some disease including Alzheimer's, Parkinson, ischemic injury, arthritis, myocardial infarction, arteriosclerosis and cancer (Ames, Shigenaga, & Hagen, 1993; Braughler & Hall, 1989; Ross, 1993). In polysaccharide, some researchers reported that polysaccharide extracts from the alga *Scagassum thunbergii* and the plant *Saussurea involucrate* have free radical scavenging effects (Liu, Ooi, & Chang, 1997).

In summary, free radical scavenging activity of hetero-chitosans was investigated on DPPH radical, alkyl radical, hydroxyl radical and superoxide radical using ESR spectroscopy. Ninety-percent deacetylated chitosan (90% chitosan) with relatively high degree of deacetylation showed the highest radical scavenging effects on various radicals, and the activity depended on their concentrations. Therefore, these results suggest that chitosan might have a potential as a free radical scavenger or an antioxidant.

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