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ORIGINAL PAPER

Antioxidant activity of half *N*-acetylated water-soluble chitosan in vitro

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Abstract Different molecular weight water-soluble chitosan (half *N*-acetylated chitosan) was prepared and the structure of water-soluble chitosan was characterized by FT-IR. The pH dependence of water solubility of water-soluble chitosan was evaluated from turbidity. Total antioxidant activity, reducing power, superoxide anion radical and hydroxyl radical quenching assay, metal chelating activity, and H_2O_2 scavenging activity were used for the evaluation of different molecular weight half *N*-acetylated chitosan in vitro. Low-molecular weight water-soluble chitosan (WSC4) exhibited high reductive capacity and expressed good inhibition of linoleic acid peroxidation in the linoleic acid model system. WSC4 (0.25 mg/mL) scavenged 78.8% of superoxide radical. At 5 mg/mL, scavenging percentage of WSC1, WSC2, WSC3, and WSC4 against hydroxyl radical was 49.3%, 66.8%, 77.1%, and 83.7%, respectively. These results indicate that water-soluble chitosan is an ideally natural antioxidant, and its antioxidant activity depends on its molecular weight.

Keywords Chitoan · Water soluble · Half *N*-acetylated · Antioxidant · Molecular weight

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Introduction

Reactive oxygen species (ROS), including superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide etc., are often generated by oxidation product of biological reactions or exogenous factors [1]. ROS can readily react with most biomolecules including lipids, proteins, amines, lipoproteins, carbohydrates, and DNA [2]. Excessive generation of ROS, induced by various stimulating factors such as certain pollutants, tobacco smoke, and which exceed the antioxidant capacity of the organism will lead to aging, cancer, and other many diseases [3]. Various exogenous antioxidants play an important role in the elimination of ROS and protect the cells against toxic affects of ROS [4]. The most commonly used antioxidants at the present time are butylated hydroxyanisole, butylated hydroxytoluene, *t*-butylhydroquinone, and propyl gallate [5]. However, the use of synthetic antioxidants is under strict regulation due to the potential health hazards caused by such compounds [6]. Therefore, during past few decades, interest had been developed to search for effective natural antioxidants from different sources for use in foods or medicinal materials to replace synthetic antioxidants.

Chitosan, an amino polysaccharide, has received much attention for a wide range of unique applications in food, including bioconversion for the production of value-added food products, preservation of food from microbial deterioration, purification of water and clarification, and deacidification of fruit juices [7]. Recently, the antioxidant activity of chitosan and its derivatives has attracted the most attention. The results of these studies have shown that they exert stronger activities and their effects are also similar to those of phenolic antioxidants [8, 9].

Considering multifunction and low toxicity of chitosan, research on antioxidant activity of chitosan and its derivatives will be helpful to expand their application in foods and medicinal materials. However, its insolubility at neutral or high pH often limits the application of chitosan and its derivatives. Therefore, this study was performed to prepare half *N*-acetylated water-soluble chitosan and to assess its antioxidant activity in vitro. Total antioxidant activity, reducing power, superoxide radicals and hydroxyl radicals quenching assay, metal chelating activity and H_2O_2 scavenging activity were used for the evaluation of its antioxidant activity in vitro.

Materials and methods

Materials

Chitosan, as initial material from shrimp shells, was obtained from Yuhuan Biochemical Co. (Zhejiang, China). Linoleic acid, nitroblue tetrazolium and ferrozine were purchased from Sigma Chemical Co. All other chemicals and reagents used were of analytical grade.

Preparation and characterization of different molecular weight water-soluble chitosan

Chtiosan (20 g) was dissolved in 500 mL 2.8% acetic acid. Then 500 mL of ethanol containing 4.5 mL acetic anhydride was added with stirring at room temperature. The reaction mixture was precipitated by addition of concentrated KOH solution after 1 h. The product was filtered off and then washed neutral. The half *N*-acetylated chitosan was dried at 50 °C.

Half *N*-acetylated chitosan (16 g) was completely dissolved in 400 mL 1% acetic acid, and the solution was neutralized to pH 5.6. The solution in the reaction vessel was placed in a water bath at 50 °C and 20 mL of cellulase solution was added in order to initiate reaction. At various intervals, 50 mL of the reaction mixture was taken out for the preparation of different molecular weight samples.

Weight-average molecular weight (M_w), number-average molecular weight (M_n), and molecular weight dispersion (M_w/M_n) of sample were measured by GPC. The degree of deacetylation (DD) of water-soluble chitosan was determined by potentiometry. The molecular parameters of the samples were listed in Table 1.

The pH dependence of water solubility of water-soluble chitosan was evaluated from turbidity using 0.5% chitosan solution. FT-IR spectra were recorded with KBr pellets on a Nicolet FT-IR 360 spectrophotometer. Sixteen scans at a resolution of 4 cm^{-1} were averaged and referenced against air.

Table 1 The molecular parameter of samples

Sample	M_w ($\times 10^{-3}$)	DD (%)	M_w/M_n
WSC1	281	53.5	6.71
WSC2	97.2	51.1	5.21
WSC3	17.5	49.8	2.89
WSC4	1.7	47.9	1.52

Total antioxidant activity

Total antioxidant activity of different molecular weight water-soluble chitosan was measured in a linoleic acid emulsion system [10]. Simply, 5 mL sample solution (2 mg/mL) in 50 mM phosphate buffer (pH 7.0) was added into a mixture of 99.5% ethanol (5 mL) and linoleic acid (0.065 mL), and then the volume of the mixture was adjusted to 12.5 mL with distilled water. The mixture was incubated in dark (40 °C) for 7 days. The peroxide value was determined by reading the absorbance at 500 nm after reaction with $FeCl_2$ and ammonium thiocyanate at every 24 h interval during incubation. The solution without added chitosan used as blank samples [11]. In a separate experiment, sample was replaced with α-tocopherol.

Reducing power

The reducing power of water-soluble chitosan was determined by the method of Yen and Duh [12]. Different concentrations of chitosan samples were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixtures were incubated for 20 min at 50 °C. After incubation, 2.5 mL of 10% trichloroacetic acid were added to the mixtures, followed by centrifugation at 3000 rpm for 10 min. The supernatant was mixed with distilled water and 0.1% ferric chloride solution and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Assay of superoxide radical scavenging activity

The assay was based on the capacity of chitosan to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) in the riboflavin–light–NBT system [13]. The method used by Dasgupta for determination of the antioxidant activity of *Piper betle L.* leaf extract in vitro [14]. Each 3 mL reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, NBT (75 μM), and 1 mL chitosan sample solution. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after a 10 min illumination from a fluorescent lamp. The entire reaction assembly was

enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture were kept in the dark and served as blanks.

$$\%\text{Inhibition} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100$$

where A_0 was the absorbance of the control and A_1 was the absorbance of samples.

Assay of hydroxyl radical scavenging activity

The assay was based on benzoic acid hydroxylation method, as described by Chung et al. [15]. In a screw-capped tube, 0.2 mL sodium benzoate (10 mM) and 0.2 mL of $FeSO_4{\cdot}7H_2O$ (10 mM), and EDTA (10 mM) were added. Then the sample solution and a phosphate buffer (pH 7.4, 0.1 mol) were added to give a total volume of 1.8 mL. Finally, 0.2 mL of a H_2O_2 solution (10 mM) was added. The reaction mixture was then incubated at 37 °C for 2 h. After this, the fluorescence was measured at 407 nm emission with excitation at 305 nm.

$$\%\text{Inhibition} = \left[1 - \frac{(F_1 - F_0)}{(F_2 - F_0)}\right] \times 100$$

where F_0 is fluorescence intensity with no treatment, F_2 is fluorescence intensity of treated control, F_1 is fluorescence intensity of treated sample.

Metal chelating activity [16, 17]

Briefly, the samples (1 mg/mL) were added to a solution of 2 mM $FeCl_2$. The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL). After the mixture had reached equilibrium, the absorbance of the solution was then measured spectrophotometrically at 562 nm. The ability of chitosan to chelate ferrous ion was calculated using the following equation where A_0 and A_1 were the optical density at 562 nm without and with samples, respectively.

$$\text{Chelating ability}\,(\%) = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100$$

Scavenging of hydrogen peroxide

A sample solution (1 mg/mL) was adjusted to 3.4 mL with 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 then at every 10 min. For each sample, a separate blank sample was used for background subtraction [18].

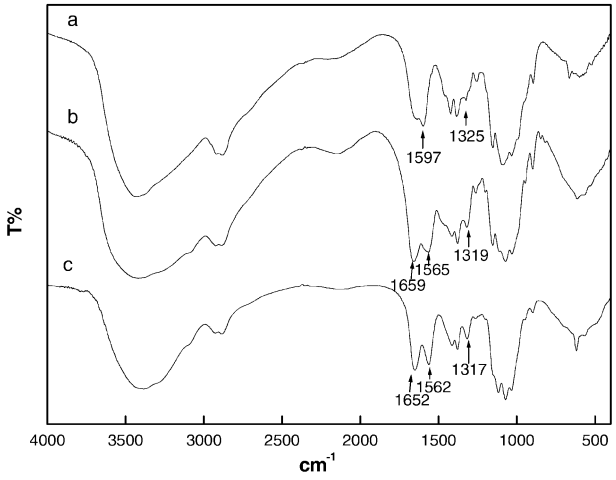

Fig. 1 FT-IR spectra of chitosan (**a**), half *N*-acetylated chitosan WSC1 (**b**), and degraded half *N*-acetylated chitosan WSC4 (**c**)

Results and discussion

FT-IR spectra

FT-IR spectroscopy has been shown to be a powerful tool for the study of the physicochemical properties of polysaccharides. Curves a, b, and c in Fig. 1 show the IR spectra of initial chitosan CS and *N*-acetylated chitosan WSC1 and WSC4. The absorption bands at 1659, 1597, 1325 cm^{-1} in CS are attributed to the amide **I** band, N–H bending mode of $-NH_2$, and amide **III** band, respectively [19]. And in the IR spectra of WSC1, a new absorption bands at 1565 cm^{-1} appears, which is referred to as amide **II** and the amide **III** band shifts to low wave number (1319 cm^{-1}). It suggests that the DD of WSC1 decreased in comparison to that of the initial chitosan [19]. The spectrum of WSC4 is similar to that of WSC1, but the amide **I** and the amide **III** band all shift to low wave number, the former suggested that carbonyl groups had more opportunity to form stronger hydrogen bonds in that the scission of polymer chains led to the increasing mobility of molecules and with the decrease of the molecular weight of chitosan, the degree of deacetylation of the main hydrolysis products decreased. All these data coincided well with the data of potentiometric determination of DD.

Water solubility

All the samples showed good water solubility. And when the *N*-acetylated chitosan was dissolved in aqueous acetic acid, its solubility at neutral pH appears to be higher than that in pure water. The ionic strength might be a cause for this phenomenon. Figure 2 shows the pH dependence of the transmittance of the half *N*-acetylated chitosan solution (0.5%).

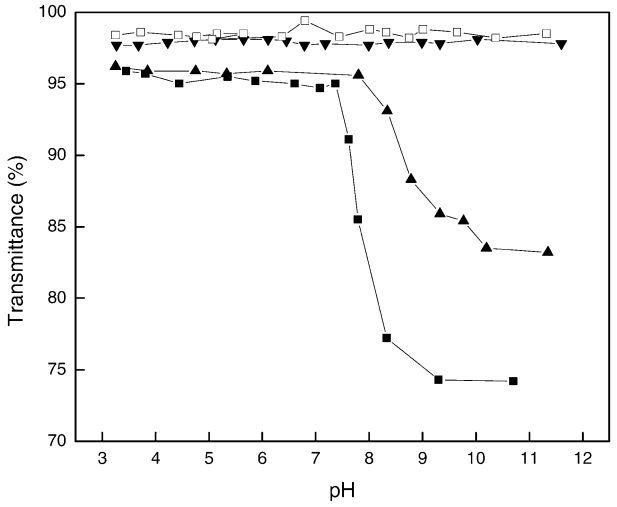

Fig. 2 pH dependence of water solubility of chitosan and half *N*-acetylated chitosan with different molecular weights. (■)WSC1, (▲)WSC2, (▼)WSC3, (□)WSC4

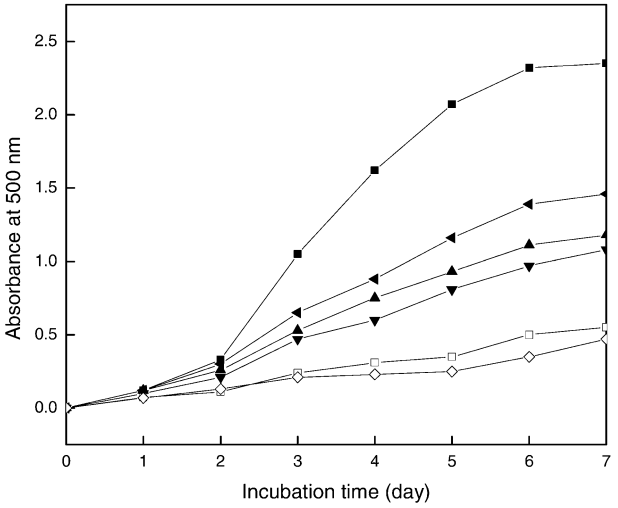

Fig. 3 Antioxidant activity of different molecular weight half *N*-acetylated chitosan and α-tocopherol in the linoleic acid emulsion was determined by thiocyanate method. (■)control, (◀)WSC1, (▲)WSC2, (▼)WSC3, (□) WSC4, (◇) α(-tocopherol

In the cases of the lower molecular weight half *N*-acetylated chitosan, the water solubility was high and retained over a wide pH range, whereas in the cases of the higher molecular weight half *N*-acetylated chitosan, it was high at acidic pH but abruptly decreased at a pH a little over neutrality. Especially, the sample with $M_w < 1.8 \times 10^4$ gave very high solubility but the solubility of rest decreased with increasing molecular weight in the alkaline region. It seems that the high water solubility of the half *N*-acetylated chitosan of low molecular weight is attributed to the decrease of intermolecular interactions, such as van der Waals forces; the lower the molecular weight, the lower the intermolecular attraction forces [20].

Total antioxidant activity

Total antioxidant activity of different molecular weight half *N*-acetylated chitosan was determined in a linoleic acid emulsion system. Linoleic acid, an unsaturated fatty acid is usually used as a model compound in lipid oxidation and antioxidation-related assays in which carbon-centered, peroxyl radicals and hydroperoxides, etc., are involved in the oxidation process [21]. During the linoleic acid oxidation, peroxides formed. These compounds oxidize Fe^{2+} to Fe^{3+}. The later Fe^{3+} ions form complex with SCN^-, which had maximum absorbance at 500 nm. Therefore, high absorbance indicates high linoleic acid oxidation. Figure 3 shows the total antioxidant of different molecular weight half *N*-acetylated chitosan. All the samples expressed inhibition of linoleic acid peroxidation in the linoleic acid model system. And WSC4 was found to be a powerful antioxidant. Its total antioxidant activity was similar to that of α(-tocopherol.

Reducing power

Figure 4 depicts the reducing power of different molecular weight half *N*-acetylated chitosan using the potassium ferricyanide reduction method. At 2 mg/mL concentration, WSC1, WSC2, WSC3, and WSC4 showed absorbances of 0.104, 0.144, 0.301, and 0.653, respectively. Thus, low-molecular weight half *N*-acetylated chitosan exhibited high reducing power. Moreover, the reducing power of the samples correlated well with increasing concentration. Figure 4 shows the reducing power increased with the increasing of chitosan concentration. The reducing capacity is generally associated with the presence of reductones [22], and might be due to hydrogen-donating ability [23]. The reducing capacity

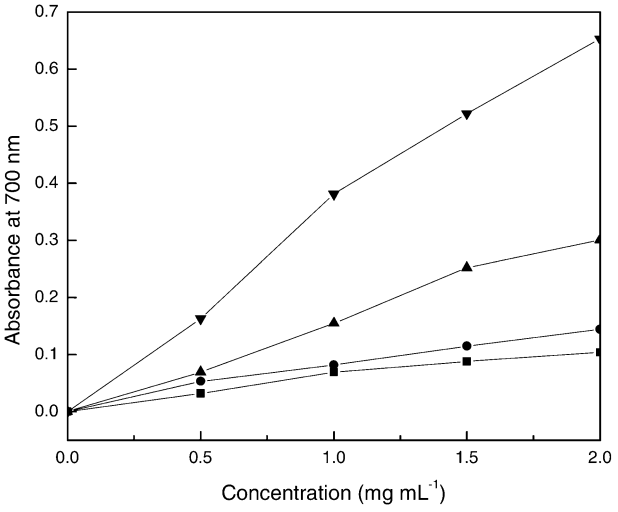

Fig. 4 Reducing power of different molecular weight half *N*-acetylated chitosan. (■)WSC1, (●)WSC2, (▲)WSC3, (▼) WSC4

of a compound may serve as a significant indicator of its potential antioxidant activity. Duh et al. [24] have observed a direct correlation between antioxidant activities and reducing power of certain plant extracts. The antioxidant activity of an antioxidant compound have 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.

Superoxide radical scavenging activity

Chtiosan has two hydroxyl groups and one amino group in its construction unit. Polysaccharides with scavenging effect on superoxide anion have the same structural feature that they all have one or more alcohol or phenolic hydroxyl groups. And the scavenging effect was related to the number of active hydroxyl groups in the molecular [25]. On the other hand, according to the free-radical theory the amino groups in chitosan can react with free radicals to form more stable macroradicals. Therefore, the active hydroxyl and amino groups in the polymer chains are the origin of the scavenging ability of chitosan.

Photochemical reduction of flavins generates superoxide anion which reduces NBT, resulting in the formation of blue formazan in the riboflavin–light–NBT system. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. Figure 5 shows the superoxide radical scavenging activity of different molecular weight half *N*-acetylated chitosan at various concentrations. Significant scavenging of superoxide radical was evident at all the tested concentration of WSC4. As shown in Fig. 5, at 0.25 mg/mL, scavenging percentage of WSC4 against superoxide radical was 78.8%. Using the same concentration, WSC1, WSC2, and WSC3 resulted 15.1, 30.3, and 35.5% of scavenging against superoxide radical. Scavenging activity of low-molecular weight half *N*-acetylated chitosan against superoxide radical was more pronounced than that of high-molecular weight half *N*-acetylated chitosan. This may be the effect of intramolecular hydrogen bond [17]. High-molecular weight chitosan has compact structure and the effect of intramolecular hydrogen bond is stronger. On the contrary, low-molecular weight chitosan owns incompact structure. It means to be the soft effect intramolecular hydrogen bond. Moreover, low-molecular weight chitosan has more free hydroxyl and amino groups than high-molecular weight chitosan, its scavenging activity against superoxide radical is more pronounced than that of high-molecular weight chiosan.

Hydroxyl radical scavenging activity

Hydroxyl radicals are generated by direct addition of Fe^{2+} to a reaction mixture containing phosphate buffer [26]. Benzoate is hydroxylated to hydroxybenzoates. Benzoate is weakly fluorescent but, after monohydroxylation, forms highly fluorescent products. Measurement of spectrofluorometric changes has been used to detect damage by hydroxyl radical. Figure 6 shows the hydroxyl radical scavenging activity of different molecular weight half *N*-acetylated chitosan at various concentrations. All the samples exhibit effective scavenging activity against hydroxyl radical. At 5 mg/mL, scavenging percentage of WSC1, WSC2, WSC3, and WSC4 against hydroxyl radical was 49.3%, 66.8%, 77.1%, and 83.7%, respectively. Obviously, low-molecular weight chitosan exhibits higher hydroxyl radical scavenging activity. As we know that hydroxyl radical is derived

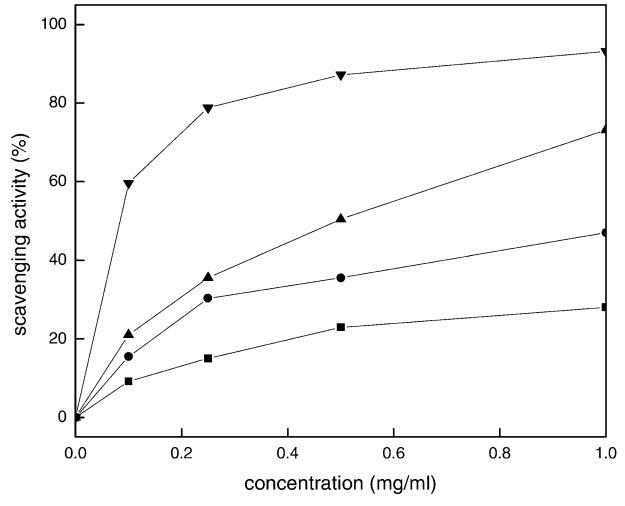

Fig. 5 Superoxide radical scavenging activity of different molecular weight half *N*-acetylated chitosan. (■)WSC1, (●)WSC2, (▲)WSC3, (▼) WSC4

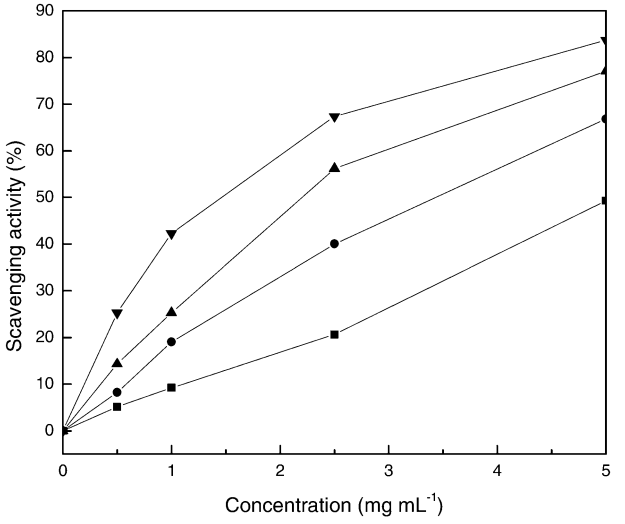

Fig. 6 Hydroxyl radical scavenging activity of different molecular weight half *N*-acetylated chitosan. (■)WSC1, (●)WSC2, (▲)WSC3, (▼) WSC4

Table 2 Metal chelating activity and H_2O_2 scavenging activity of different molecular weight half *N*-acetylated chitosan

Sample	%Chelating activity ± SD ($n = 3$)	% H_2O_2 scavenging ± SD ($n = 3$)
WSC1	14.5 ± 1.32	13.7 ± 3.25
WSC2	17.3 ± 2.01	19.1 ± 5.13
WSC3	26.2 ± 1.93	19.8 ± 5.87
WSC4	41.8 ± 3.21	28.6 ± 7.59

from Fenton reaction, which is caused by the reaction of Fe^{2+} and H_2O_2. Hydroxyl radical scavenging activity of chitosan can be partially attributed to its metal chelating ability. Table 2 shows the metal chelating ability of different molecular weight half *N*-acetylated chitosan. The Fe^{2+} chelating ability of chitosan mainly comes from the presence of amino groups, which contain lone electron pairs that help to form chitosan-Fe^{2+} complexes [27]. More free amino groups of low-molecular weight chitosan resulted in high metal chelating activity. Huang et al. [28] added EDTA to the reaction system to release the Fe^{2+} which were chelated by chitosan, the scavenging activity against hydroxyl radical decreased. Therefore, Fe^{2+} chelating directly correlates to the scavenging behavior of chitosan. The removing of hydrogen peroxide also resulted in the decrease of hydroxyl radical. Table 2 shows the hydrogen peroxide scavenging activity of different molecular weight half *N*-acetylated chitosan. The scavenging activity of WSC4 is superior to that of others.

Conclusion

This study was performed to prepare half *N*-acetylated water-soluble chitosan and to assess its antioxidant activity in vitro. Total antioxidant activity, reducing power, superoxide radicals and hydroxyl radicals quenching assay, metal chelating activity and H_2O_2 scavenging activity were used for the evaluation of its antioxidant activity. Half *N*-acetylated water-soluble chitosan expressed high antioxidant activity and the activity depended on its molecular weight and concentration.

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