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Enhancement of antioxidant activity of chitosan by irradiation

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Abstract

Chitosan was irradiated in acetic acid solution (1%) with different doses (2–20 kGy) of Co-60 γ rays to investigate the enhancement of antioxidant activity of irradiated chitosan. The structure of irradiation degraded chitosan was characterized by GPC, FT-IR and ^{1}H NMR spectroscopy. The molecular weight of chitosan decreased with increasing irradiation dose. Radical mediated lipid peroxidation inhibition, reducing power, superoxide anion radical and hydroxyl radical quenching assays were used for the evaluation of the antioxidant activity of irradiated chitosan. Chitosan (M_w 2.1×10^3) which had been irradiated at 20 kGy exhibited high reductive capacity and expressed good inhibition of linoleic acid peroxidation. At a concentrtion of 0.1 mg/mL it could scavenge 74.2% of superoxide radical. At 2.5 mg/mL, scavenging percentage of chitosan irradiated for 0, 2, 10, 20 kGy against hydroxyl radical was 16.6%, 41.1%, 47.1% and 63.8%, respectively. The results show that γ ray irradiation, especially for 20 kGy, of chitosan gives enough degradation to increase its antioxidant activity as a result of a change in molecular weight.

Keywords: Chitosan; Irradiation; Antioxidant; Molecular weight

1. Introduction

Chitosan, an amino polysaccharide, has received much attention as a functional biopolymer for many diverse applications in food, pharmaceutical and cosmetics (Kumar, 2000; Shahidi, Arachchi, & Jeon, 1999). In many of these applications, specific molecular weights of polysaccharides are required. Chitosan with an average M_w in the range of 5000–10,000 Da possesses strong bactericidal and superior biological activities (Kittur, Vishu Kumar, & Tharanathan, 2003). Chitosan of 20 kDa prevents progression of diabetes mellitus and exhibits higher affinity for lipopolysaccharides than 140 kDa chitosan (Kondo, Nakatani, Hayashi, & Ito, 2000). Chitooligomers have special antimicrobial activity (Begona & Ruth, 1997; Zheng & Zhu, 2003) and antitumour activity (Qin, Du, Xiao, Li, & Gao, 2002). So it is of increasing interest to degrade chitosan into low molecular weight fragments under appropriate conditions. It has been reported that chitosan can be degraded by acidic hydrolysis or enzymatic treatment. Chemical treatment is an easy, low cost process, but chemical waste and reproducibility are the main problems. Enzymatic hydrolysis is an effective way to achieve specific cleavage to chitosan oligomers. However, it requires multisteps, particularly, enzyme preparation and purification of the product.

Radiation can provide a useful tool for degradation of different polymers. In the reaction, no other chemical reagents are introduced and there is not a need to control the temperature, environment or additives (Charlesby, 1981). Recently, radiation effects on carbohydrates such as chitosan, sodium alginate, carrageenan, cellulose, pectin have been investigated to enhance their bioactivities and to reduce the environmental pollution (Chmielewski, Haji-Saeid, & Ahmed, 2005). Matsuhashi and Kume (1997) reported that chitosan degraded by irradiation could increase its antimicrobial activity as a result of a change

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in molecular weight. Chitosan irradiated at 100 kGy under dry condition inhibited the growth of *Escherichia coli* completely. Czechowska-Biskup, Rokita, Ulanski, and Rosiak (2005) studied the fat-binding capacity of irradiated chitosan and irradiation has proved useful in improving fat-binding properties of chitosan as an active component of dietary food additives – one gram of irradiated chitosan may bind up to 20 g of fat. Tham et al. (2001) showed that irradiated chitosan is effective as a plant growth promoter and heavy metal eliminator in crop production of plants stressed with vanadium.

Recently, the antioxidant activity of chitosan and its derivatives has attracted the attention. Since they exert strong antioxidant activities and their effects are also similar to those of phenolic antioxidants (Park, Je, & Kim, 2004). In this study, chitosan has been modified by irradiation to enhance its antioxidant activity, and the relation between the antioxidant activity and molecular properties of irradiated chitosan are discussed.

2. Materials and methods

2.1. Materials

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

2.2. Irradiation

Chitosan (2 g) was dissolved in 1% (v/v) acetic acid solution (100 mL), and then the solution was irradiated by Co-60 γ rays at doses of 2, 10, 20 kGy.

2.3. Characterization

Weight-average molecular weight (M_w), number-average molecular weight (M_n) and molecular weight dispersion (M_w/M_n) of samples were measured by GPC on connected columns (TSK G5000-PW and TSK G3000-PW), using a refractive index detector. The eluent was 0.2 M CH_3COOH/0.1 M CH_3COONa at a flow rate of 1.0 mL/min. Pullulan standards (Showa Denko, Tokyo, Japan) were used to calibrate the column.

FT-IR spectra were recorded as KBr pellets. Sixteen scans at a resolution of 4 cm^{-1} were averaged and referenced against air.

^1H NMR spectra were recorded in D_2O on an INOVA-600 NMR 600 MHz spectrometer (Varian, Palo Alto, USA).

2.4. Potentiometric determination of the degree of deacetylation (DD)

Chitosan (0.1 g) was dissolved in a known excess of 0.1 M HCl (10 mL). From the pH titration of this solution with 0.1 M NaOH, a curve with two inflexion points was obtained. The amount of the acid consumed between these two points was considered to correspond to the amount of the free amino groups in the solution (Tolaimate et al., 2000).

2.5. Estimation of water-solubility

The pH dependence of water solubility of chitosan was evaluated from turbidimetry of a solution of chitosan (0.1 g) dissolved in 1% w/v acetic acid (100 mL). The transmittance at 600 nm of the solution was recorded with the stepwise addition of concentrated NaOH, on a UV–vis spectrophotometer using a quartz cell with an optical path length of 1 cm.

2.6. In vitro antioxidant assay

2.6.1. Radical mediated lipid peroxidation inhibition assay

Linoleic acid was oxidized in an emulsified model system to measure the antioxidative activity of antioxidants (Takao, Kitatani, Watanabe, Yagi, & Sakata, 1994). Sample solution (1 mL) and 50 mM sodium phosphate buffer (pH 7.0, 4 mL) were added to 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 the dark at 40 °C for 7 days. The degree of radical mediated linoleic acid peroxidation was measured at 24 h intervals by a ferric thiocyanate method. For that, an aliquot (0.1 mL) of the reaction mixture was mixed with 75% v/v ethanol (4.7 mL), 30% w/v ammonium thiocyanate (0.1 mL) and 0.02 M $FeCl_2$ solution (0.1 mL). After 3 min, the degree of color development that represents lipid peroxidation value was measured spectrophotometrically at 500 nm. Solution without added chitosan was used as a sample blank.

2.6.2. Reducing power

The reducing power of chitosan was determined by the method of Yen and Duh (1993). Different concentrations of chitosan sample solutions (1 mL) were mixed with 0.2 M sodium phosphate buffer pH 6.6 (2.5 mL) and 1% w/v potassium ferricyanide (2.5 mL). The mixtures were incubated for 20 min at 50 °C, then 10% w/v trichloroacetic acid (2.5 mL) was added to the mixtures, followed by centrifugation at 1500*g* for 10 min. The supernatant was mixed with distilled water and 0.1% (w/v) ferric chloride solution and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

2.6.3. Assay of superoxide radical scavenging activity

The assay was based on the capacity of chitosan to inhibit the photochemical reduction of nitroblue tetrazolium in a riboflavin-light-NBT system (Beauchamp & Fridovich, 1971). The method used by Dasgupta for determination of antioxidant activity of *Piper betle L.* leaf extract was

used in modified form (Dasgupta & De, 2004). Reagent mixture (3 mL), containing 50 mM sodium phosphate buffer, pH 7.8, 13 mM methionine, 2 μM riboflavin, 100 μM EDTA and 75 μM nitroblue tetrazolium, was mixed with chitosan sample solution (1 mL). 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. Calculations were based on the equation:

$$\% \text{ Inhibition of superoxide radical} = [(A_0 - A_1)/A_0] \times 100.$$

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

2.6.4. Assay of hydroxyl radical scavenging activity

The assay was based on a benzoic acid hydroxylation method, as described by Chung, Osawa, and Kawakishi (1997). In a screw-capped tube, 10 mM sodium benzoate (0.2 mL), 10 mM $FeSO_4$ (0.2 mL) and 10 mM EDTA (0.2 mL) were added into each tube. Then the sample solution (0.2 mL) and 0.1 M sodium phosphate buffer pH 7.4 (1 mL) were added into the tube to give a total volume of 1.8 mL. Finally, 10 mM hydrogen peroxide solution (0.2 mL) was added to the reaction mixture which was then incubated at 37 °C for 2 h. After this, the fluorescence was measured at 407 nm emission with excitation at 305 nm and calculations were based on the equation:

$$\% \text{ Inhibition of hydroxyl radical} = [1 - (F_1 - F_0)/(F_2 - F_0)] \times 100.$$

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.

3. Results and discussion

3.1. Effect of irradiation dose on M_w and DD

In the GPC curves of chitosan and its degraded products (Fig. 1), the peak of the elution curve shifted toward higher elution volumes with increasing irradiation dose. Irradiation induced scissions of 1–4 glycosidic bonds of chitosan polysaccharide that caused a reduction in the molecular weight of the polymer. GPC measurements showed a rapid decrease of molecular weight at the beginning of the degradation process, indicating that the degradation of the backbone mainly occurred in a random fashion. Table 1 shows the molecular parameter of the degraded chitosan, indicating that chitosan maintained its degree of deacetylation. This is one of the attractive features of irradiating chitosan since it is known that chemical and enzymatic hydrolysis result in changes in the degree of deacetylation.

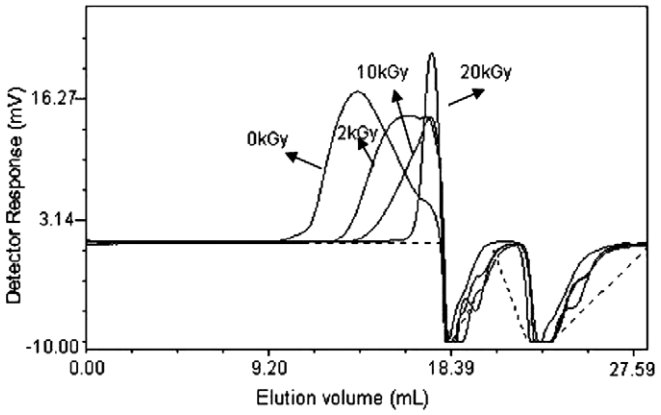

Fig. 1. GPC profilies of chitosan irradiated at 0, 2, 10, 20 kGy.

Table 1
The molecular parameters of irradiated chitosans

Sample reference code	M_w ($\times 10^{-3}$)	DD (%)	M_w/M_n	Irradiation dose (kGy)
CS	210	91.5	7.32	0
CS1	35.2	92.3	4.24	2
CS2	6.3	91.0	2.57	10
CS3	2.1	91.2	1.53	20

3.2. FT-IR spectra

In the FT-IR spectra of chitosan and the irradiated product (Fig. 2), the major peaks of chitosan at 896, 1087, 1598, 1653 and 3430 cm^{-1} belonging to pyranose ring, glucoside, amino, acetamide and hydroxyl groups, respectively, were identifiable. This indicated that the important functional groups were still present after irradiation and that the main polysaccharide chain structure remained during the degradation process. The amide I band had shifted to a lower wave number, it suggested that carbonyl groups had more opportunity to form stronger hydrogen bonds.

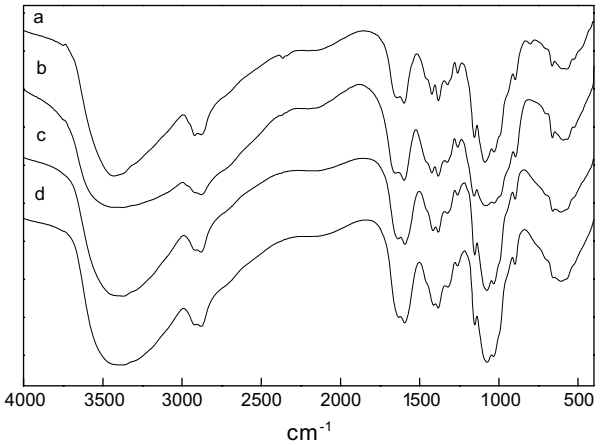

Fig. 2. FT-IR spectra of chitosan irradiated at (a) 0 kGy, (b) 2 kGy, (c) 10 kGy and (d) 20 kGy.

3.3. ^1H NMR spectra

To further confirm the structure of the degraded chitosan, the CS3 was analyzed by ^1H NMR spectroscopy, in D_2O solution at ambient temperature (Fig. 3). The resonance at 1.95 ppm is easily assigned to the three *N*-acetyl protons (Akiyama, Kawazu, & Kobayashi, 1995). The higher field peak at 4.4–4.6 ppm and the peak at 2.6–2.8 ppm correspond to H-1 and H-2 of the units, respectively (Kubota, Tatsumoto, Sano, & Toya, 2000). The weak signal at 5.21 ppm was assigned to H-1(a) of the units, while the signal at 4.61 ppm was assigned to H-1(β) of the units. No attempts were made to identify other peaks, because the spectra of unfractionated partially N-deacetylated chitooligomers are very difficult to interpret. Nevertheless, all signals were the resonances of oligomers of chitin and chitosan, which coincided with the reported data (Qin et al., 2003).

3.4. Solubility of degraded chitosan

The transmittance of the chitosan solutions was used to determine solubility (Fig. 4). In the cases of the lower-molecular-weight chitosan, the water solubility was high and retained over a wide pH range, whereas in the cases of the higher-molecular-weight chitosan, it was high at acidic pH but abruptly decreased at a pH a little over neutrality. Especially, CS3 gave very high solubility but the solubility of CS, CS1 and CS2 decreased with increasing molecular weight in the alkaline region. It seems that the high water solubility of low-molecular-weight chitosan 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 (Kubota et al., 2000). When the chitosans were dissolved in aqueous acetic acid, their solubility at neutral pH appears to be higher than that in pure water. The ionic strength might be a cause for this phenomenon.

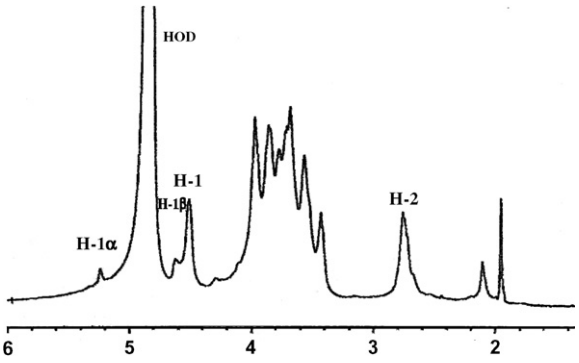

Fig. 3. ^1H NMR spectrum of chitosan irradiated at 20 kGy.

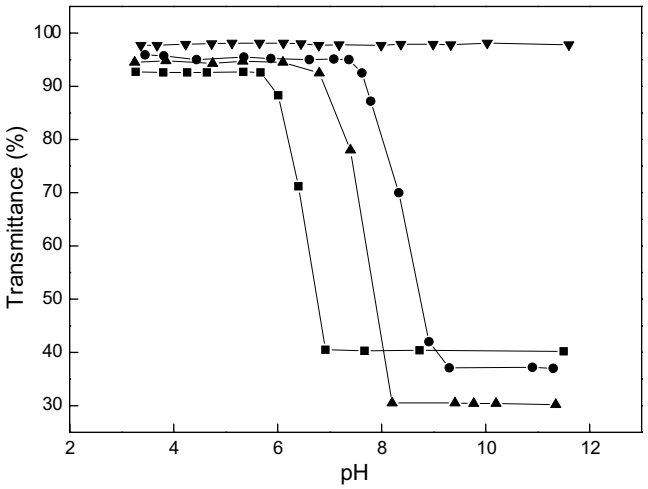

Fig. 4. pH dependence of water solubility of chitosan irradiated at (■) 0 kGy, (▲) 2 kGy, (●) 10 kGy, (▼) 20 kGy.

3.5. In vitro antioxidant activity

3.5.1. Radical mediated lipid peroxidation inhibition assay

Cell membranes are phospholipid bilayers with extrinsic proteins and are the direct target of lipid oxidation (Girotti, 1998). As lipid oxidation of cell membranes increases, the polarity of lipid phase surface charge and formation of protein oligomers increase; and molecular mobility of lipids, number of SH groups, and resistance to thermal denaturation decrease. Malonaldehyde, one of the lipid oxidation products, can react with free amino group of proteins, phospholipid, and nucleic acids leading to structural modification, which induce dysfunction of immune systems.

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. During the linoleic acid oxidation, peroxides are formed. These compounds oxidize Fe^{2+} to Fe^{3+}. The Fe^{3+} ions form a complex with SCN^-, which has a maximum absorbance at 500 nm. Therefore, high absorbance indicates high linoleic acid oxidation. The total antioxidant values of different molecular weights of γ-ray treated chitosan (Fig. 5) show expressed inhibition of linoleic acid peroxidation in the linoleic acid model system. Sample CS3 was found to be a powerful antioxidant.

3.5.2. Reducing power

The reducing power of different molecular weights of γ-ray treated chitosan gas determined by the potassium ferricyanide reduction method, showed that low molecular weight γ-ray treated chitosan exhibited high reducing power, and the reducing power increased with the increase of chitosan concentration (Fig. 6). The reducing capacity is generally associated with the presence of reducing sugars and might be due to hydrogen-donating ability (Shimada, Fujikawa, Yahara, & Nakamura, 1992). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The antioxidant activity of an antioxidant compound has been attributed

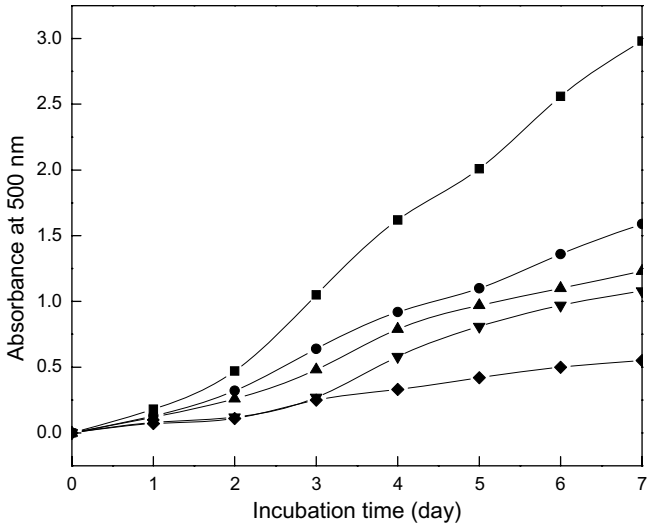

Fig. 5. Antioxidant activity of irradiation degraded chitosan in the linoleic acid emulsion. (■) control, (●) 0 kGy, (▲) 2 kGy (▼) 10 kGy, (◆) 20 kGy.

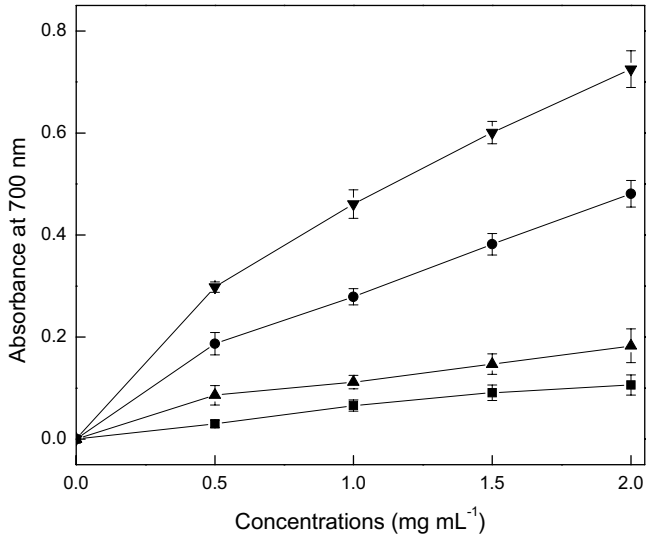

Fig. 6. Reducing power of chitosan irradiated at (▼) 0 kGy, (●) 2 kGy, (▲) 10 kGy, (■) 20 kGy.

to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, reductive capacity and radical scavenging.

3.5.3. Superoxide radical scavenging activity

Superoxide anion is a reduced form of molecular oxygen created by receiving one electron. It is an initial free radical formed from mitochondrial electron transport systems (Bloknina, Virolainen, & Fagerstedt, 2003). Superoxide anion radicals are produced by a number of cellular reactions, including various enzyme systems, such as lipoxygenases, peroxidase, NADPH oxidase and xanthine oxidase (Bloknina et al., 2003). They play an important role in the formation of other cell-damaging free radicals, such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems (Bloknina et al., 2003). In the present study a superoxide radical scavenging assay was based on the capacity of chitosan to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) in a riboflavin-light-nitroblue tetrazolium system. Significant scavenging of superoxide radical was evident at all the tested concentrations of CS3. As shown in Fig. 7, at 0.1 mg/mL, the scavenging percentage of CS3 against superoxide radical was 74.2%. Scavenging activity of low molecular weight chitosan against superoxide radical was more pronounced than that of high molecular weight chitosan. This may be the effect of intramolecular hydrogen bonds (Xing et al., 2005). High molecular weight chitosan has a compact structure and the effect of intramolecular hydrogen bond is stronger. The strong effect of the intramolecular hydrogen bond weakens the activities of the hydroxyl and amino groups, and the chance of exposure of their hydroxyl and amine groups might be restricted which would account for less radical scavenging activity.

3.5.4. Hydroxyl radical scavenging activity

The hydroxyl radical is the most reactive free radical and can be formed from superoxide anion and hydrogen peroxide, in the presence of metal ions, such as copper or iron. It can react with non-selective compounds such as proteins, DNA, unsaturated fatty acids and almost every biological membrane. In the present study, hydroxyl radicals are generated by Fenton's reaction of Fe^{2+} with hydrogen peroxide solution (Gutteridge, 1984). Benzoate is hydroxylated to hydroxybenzoates. Benzoate is weakly fluorescent but, after monohydroxylation, forms highly fluorescent products. Measurement of spectrofluorometric changes in the reaction mixture has been used to detect damage by hydroxyl radical at various concentrations.

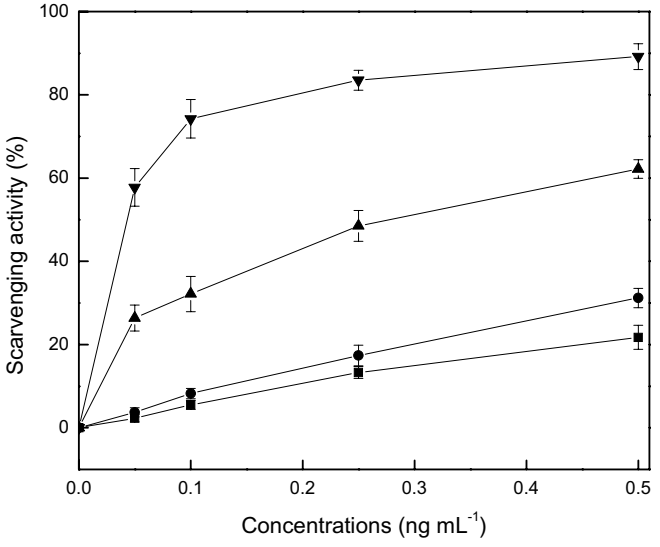

Fig. 7. Superoxide radical scavenging activity of chitosan irradiated at (■) 0 kGy, (●) 2 kGy, (▲) 10 kGy, (▼) 20 kGy.

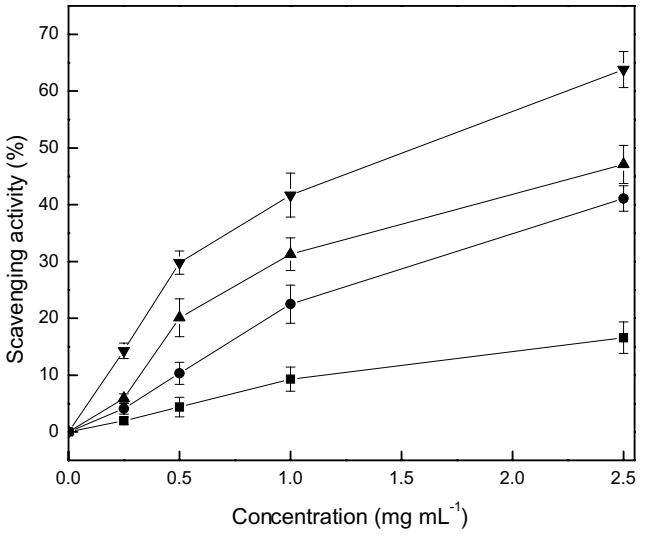

Fig. 8. Hydroxyl radical scavenging activity of (■) 0 kGy, (●) 2 kGy, (▲) 10 kGy, (▼) 20 kGy.

All the samples of different molecular weights of γ-ray treated chitosan exhibited effective scavenging activity against hydroxyl radical (Fig. 8). At 2.5 mg/mL, the scavenging percentages of CS, CS1, CS2 and CS3 against hydroxyl radical are 16.6%, 41.1%, 47.1% and 63.8%, respectively. Obviously, low molecular weight chitosan exhibits higher hydroxyl radical scavenging activity. Hydroxyl radical scavenging activity of chitosan can be partially attributed to its metal chelating ability. 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 (Guzman, Saucedo, Revilla, Navarro, & Guibal, 2003). And Fe^{2+} chelating ability of low molecular weight chitosan was more pronounced than high molecular weight chitosan (Xing et al., 2005). Huang, Mendis, and Kim (2005) added EDTA to the reaction system to release the Fe^{2+} that had become chelated by the chitosan, and then the scavenging activity against hydroxyl radical decreased. EDTA is a more effective chelator for Fe^{2+} than chitosan, and so the released Fe^{2+} can react with H_2O_2 to generate excessive amounts of hydroxyl radicals, which result in the decrease of radical scavenging activity. Therefore, Fe^{2+} chelating directly correlates to the scavenging behavior of chitosan.

4. Conclusions

Chitosan has two hydroxyl groups and one amino group in each of its monosaccharide construction units. The hydroxyl groups in the polysaccharide units can react with free radicals by the typical H-abstraction reaction (Xue, Yu, Hirata, Terao, & Lin, 1998). On the other hand, according to free radical theory, the amino groups in chitosan can react with free radicals to form additional stable macroradicals. Therefore, the active hydroxyl and amino groups in the polymer chains are the origin of the scavenging ability of chitosan.

In this study, the enhancement of the antioxidant activity of chitosan by irradiation was achieved. The characterisation of the structure of the degraded chitosan by FT-IR and ^1H NMR spectrum showed the backbone structure of chitosan was retained after irradiation. Radical mediated lipid peroxidation inhibition assay, reducing power, superoxide radicals and hydroxyl radicals quenching assays showed that low dose irradiation, especially 20 kGy, of chitosan gives enough degradation to increase its antioxidant activity as a result of a change in molecular weight.

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