

Effect of molecular weight of chitosans on their antioxidative activities in apple juice

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Abstract

This work studies how chitosans with low molecular weight (LMWC, $M_w = 12$ kDa), medium molecular weight (MMWC, $M_w = 95$ kDa) and high molecular weight (HMWC, $M_w = 318$ kDa) affect antioxidant activity in an aqueous system and in apple juice. Antioxidant activity was determined, including that of DPPH radicals, hydrogen peroxide and superoxide anion radicals, as well as metal ion chelating capacity, ABTS radicals of chitosans with different molecular weights (DMWCs) in apple juice.

LMWC exhibited stronger scavenging activity toward DPPH radicals, superoxide anion radicals and hydrogen peroxide, compared to either MMWC or HMWC. At a concentration of 0.8 mg/ml, the LMWC in apple juice exhibited 88.2%, 99.8% and 93.0% scavenging activities toward DPPH radicals, hydrogen peroxide and superoxide anion radicals, respectively. At a concentration of 1.0 mg/ml the LMWC in apple juice exhibited 70.0% ferrous ion chelating activity. The TEAC (Trolox Equivalent Antioxidant Capacity) values of LMWC (3.24 ± 0.21) were markedly higher than those of HMWC (1.75 ± 0.12) in apple juice. The data obtained *in vitro* models clearly establish the antioxidant potency of DMWCs. These *in vitro* results suggested that LMWC can increase antioxidant activity in apple juice. However, comprehensive studies need to be conducted to ascertain the *in vivo* safety of LMWC in experimental animal models. © 2006 Elsevier Ltd. All rights reserved.

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1. Introduction

Chitosan, a cationic polysaccharide with a high molecular weight, is a linear polymer that comprises β -1,4-linked glucosamine (GlcN) with various amounts of *N*-acetylated GlcN residues. It is typically obtained by the alkaline deacetylation of chitin extracted from an abundant source of shellfish exoskeletons or the cell walls of some microorganisms and fungi (Hirano, Ohe, & One, 1976). It has attracted marked interest as a biomedical material, because it has unique biological activities, such as antitumour (Saiki, Murata, Nakajima, Tokura, & Azuma, 1990), immunostimulatory (Maeda, Murakami, Ohta, & Tajima,

1992) and antibacterial (Kobayashi, Watanabe, Suzuki, & Suzuki, 1990; Tokoro et al., 1989) activities. In addition, the molecular weights of chitosans also affect their biological activities. For instance, chitosans with molecular weight within the range 5–20 kDa, exhibit greater biological activities than total chitosan (Muzzarelli & Muzzarelli, 2002). Low molecular weight chitosan (LMWC) with a weight of 20 kDa prevents the progression of diabetes mellitus and has a higher affinity for lipopolysaccharide than 140 kDa chitosan (Kondo, Nakatani, Hayashi, & Ito, 2000). LMWC with a molecular weight of 5–10 kDa has potential for use in DNA delivery systems (Jeon, Park, & Kim, 2001). Richardson, Kolbe, and Duncan (1999) showed that LMWC exhibits the highest bactericidal activity towards pathogenic bacteria.

In recent years, various investigators have observed the antioxidant activity of chitosan derivatives. For example,

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Xie, Xu, and Liu (2001) showed that hexanoyl chitosan and *N*-benzoylhexanoyl chitosan can trap peroxide radicals in an organic solvent when 2,2'-azobis (2,4-dimethylvaleronitrile) initiates the radical chain reaction. Matsugo et al. (1998) obtained chitosan derivatives, *via* the acylation of chitosan using acid anhydride; they observed that these derivatives inhibited the formation of thiobarbituric acid reactive substrate in *t*-butylhydroperoxide and that benzoyl peroxide induced lipid peroxidation. Xue, Yu, Hirata, Terao, and Lin (1998) found that the chitosan derivatives prepared by graft copolymerization of maleic acid sodium on hydroxypropyl chitosan and carboxymethyl chitosan sodium exhibited scavenging activities against hydroxyl radicals. Lin and Chou (2004) demonstrated that disaccharide chitosan derivatives display various antioxidative activities.

Recent evidence indicates that the biological activities of low molecular weight LMWC vary markedly from each other. For instance, Yin, Lin, Zhang, and Yang (2002) reported scavenging activity of LMWC on superoxide radicals was more pronounced than that of high molecular weight chitosan (HMWC). It also indicated that LMWC can scavenge superoxide radicals, and its scavenging activity was 80.3% at 0.5 mg/ml (Esumi, Takei, & Yoshimura, 2003). Yin et al. (2002) showed that gold-chitosan nanocomposites can suppress the activity of hydroxyl radicals. Xing et al. (2004) determined the effects of molecular weight and/or substitution degree of sulfated polysaccharides on their antioxidant activity. Low molecular weight sulfated chitosan had stronger scavenging activity on superoxide/hydroxyl radicals than that of high molecular weight sulfated chitosan. However, very few attempts have been made to evaluate the antioxidant activity of chitosan in food.

Apple juice is important because it is nutritional and inhibits the oxidation of low-density lipoprotein in humans. It is widely consumed in most countries (Pearson, Tan, German, Davis, & Gershwin, 1999). Roller and Covill (1999) identified the antifungal properties of chitosan against yeasts and moulds in apple juice.

This work compared the effects of different molecular weight chitosans (DMWC) on antioxidant activity and the possible antioxidant effects of DMWC in apple juice. Antioxidant activities were evaluated using various *in vitro* assay systems, including DPPH, superoxide, hydroxyl radicals, ABTS and metal ion chelating assays.

2. Materials and methods

2.1. Chitosan and chemicals

All chitosan samples had undergone 98.50% *N*-deacetylation and were obtained in powder form from VA & G Bioscience Inc (Taoyuan, Taiwan). The HMWC ($M_w = 318$ kDa) was acid-soluble; medium molecular weight chitosans (MMWC) ($M_w = 95$ kDa) and LMWC ($M_w = 12$ kDa) were water-soluble. These compounds

were placed in separate plastic bottles and stored at ambient temperature throughout the experiments. All other chemicals were obtained from commercial sources and were of analytical grade. Horseradish peroxidase (HRPase), nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), hydrogen peroxide (H_2O_2), thiobarbituric acid (TBA), ethylenediaminetetraacetic acid (EDTA), ferrozine, nicotinamide adenine dinucleotide-reduced (NADH), trichloroacetic acid (TCA), potassium ferricyanide and ferric chloride were purchased from Sigma Chemicals Co; (St. Louis, MO, USA).

The antioxidant activities of individual chitosans with different molecular weights were evaluated in both an aqueous system and apple juice.

2.2. Preparing chitosan solutions

In the preparation of 1.0 l of 0.2–1.0% chitosan solutions, 0.2, 0.4, 0.8 and 1.0 g of chitosan were dispersed in 900 ml of distilled water, to which 50 ml of glacial acetic acid was added to dissolve the chitosan. The pH of each solution was adjusted to pH 5.0 by adding 0.1M NaOH and each solution was made up to 1.0 l. An acid solution without chitosan, pH5.0, was used as a control.

2.3. Preparing apple juice containing DMWC

Clear, UHT-treated, shelf-stable apple juice, that contained no added preservatives and was packed in laminate, was purchased from a local retailer. To 45 ml of this apple juice in a 250 ml Erlenmeyer flask was added 5 ml of DMWC solution. To the control flask was added 5 ml of water, rather than chitosan solution. The chitosan concentration used ranged from 0.2 to 1.0 g/l of juice.

2.4. Scavenging of DPPH radical

The effect of chitosans on DPPH radicals was studied, using the modified method of Shimada, Fujikawa, Yahara, and Nakamura (1992). Briefly, 100 μ M DPPH solution in methanol was prepared and 1.0 ml of this solution was added to 4.0 ml test samples at different concentrations. The reaction mixture was shaken well and incubated for 30 min at room temperature; the absorbance of the resulting solution was read at 517 nm against a blank. The inhibitory percentage of DPPH was calculated according to the following equation:

$$\text{Scavenging effect (\%)} = (1 - \text{absorbance}_{\text{sample}} / \text{absorbance}_{\text{control}}) \times 100$$

2.5. Hydrogen peroxide scavenging assay

Hydrogen peroxide scavenging activity was measured by the modified method of Yen and Chung (1999). Briefly, 1 ml of sample was first mixed with 400 μ l of 4 mM H_2O_2

solution and incubated for 20 min at room temperature. It was then supplemented with 600 μ l of HRPase – phenol red solution (HRPase 300 μ g/ml and phenol red 4.5 mM in 100 mM phosphate buffer). After another 10 min of incubation and then 10 min on ice to stop the reaction, the sample absorbance at 610 nm was monitored by an automated microplate reader. The scavenging effect was then calculated according to the following equation:

Scavenging effect (%)

$$= (1 - \text{absorbance}_{\text{sample}} / \text{absorbance}_{\text{control}}) \times 100$$

2.6. Superoxide anion radical scavenging assay

The superoxide scavenging ability of chitosans was assayed by the method of Robak and Gryglewski (1988). The reaction mixture, contained chitosans (0.2–20 mg/ml). First the reagents were prepared in 100 mM phosphate buffer (pH 7.4). The reaction mixture containing 50 μ l of test sample, 50 μ l of 300 μ M nitro blue tetrazolium, 50 μ l of 936 μ M NADH and 50 μ l of 120 μ M phenazine methosulfate was incubated at room temperature for 5 min and then the absorbance was read at 560 nm against a blank. The capability of scavenging of superoxide radical was calculated using the following equation:

Scavenging effect (%)

$$= (1 - \text{absorbance}_{\text{sample}} / \text{absorbance}_{\text{control}}) \times 100$$

2.7. Metal ion chelating assay

The ferrous ion-chelating activity of chitosans was measured according to the method of Yen and Chung (1999), wherein the Fe^{2+} -chelating ability of chitosan was monitored by the absorbance of the ferrous iron–ferrozine complex at 562 nm. Briefly, the reaction mixture, containing chitosans of different concentrations, FeCl_2 (2 mM), and ferrozine (5 mM) was adjusted to 5 ml with water, shaken well and incubated for 10 min at room temperature. The absorbance of the mixture was measured at 562 nm against a blank. EDTA was used as positive control.

The ability of sulfated chitosans to chelate ferrous ion was calculated using the following equation:

Chelating effect (%)

$$= [1 - \text{absorbance}_{\text{sample}} / \text{absorbance}_{\text{control}}] \times 100$$

2.8. ABTS assay

Total antioxidant capacity was evaluated according to the ABTS modified assay (Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans, 1999; Arts, Dallinga, Voss, Haenen, & Bast, 2004; Wu et al., 2006). In the most recent version of the Trolox equivalent antioxidant capacity (TEAC) assay, an antioxidant is added to a pre-formed ABTS radical solution and, after a fixed time period, the remaining

ABTS $^{\bullet+}$ is quantified spectrophotometrically (Van den Berg, Haenen, van den Berg, & Bast, 1999). The reference compound in the TEAC assay is Trolox. The reduction in ABTS $^{\bullet+}$ concentration, induced by a certain concentration of antioxidant, is related to that of Trolox and gives the TEAC value of that antioxidant (Arts et al., 2004).

The ABTS $^{\bullet+}$ was generated by ABTS salt, 2.45 mmol of potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$) was reacted with 7 mmol ABTS salt in 0.01 M phosphate-buffered saline, pH 7.4, for 15 h at room temperature in the dark. The resultant ABTS $^{\bullet+}$ radical cation was diluted with 0.01 M phosphate-buffered saline, pH 7.4, to give an absorbance of around 0.70 at 734 nm. The standard or sample was diluted 100 times with the ABTS $^{\bullet+}$ solution to a total volume of 1 ml and allowed to react for 6 min. Absorbance was measured at different time intervals. A control (without a standard or sample) was used as a blank and 990 μ l of PBS were added to these control samples instead. The absorption peak of ABTS $^{\bullet+}$ is at 734 nm. Addition of antioxidant reduced ABTS $^{\bullet+}$ to its colourless form. The extent of decolorisation, as percentage of inhibition of ABTS $^{\bullet+}$, is determined as a function of concentration and calculated relative to the reactivity of Trolox.

2.9. Statistical analysis

In this study, three analyses of each sample were made and each experiment was carried out in triplicate ($n = 3$). The mean value and standard deviation were calculated from the data obtained. These data were then compared by the Duncan's multiple range method SAS (2001).

3. Results and discussion

3.1. Scavenging of DPPH radical by DMWC

DPPH, decreases significantly upon exposure to proton radical scavengers (Yamaguchi, Takamura, Matoba, & Terao, 1998). The total DPPH scavenging potential of the DMWC and ascorbic acid, used as the positive scavenger, was measured at various concentrations. The DMWC was found to influence the DPPH radical-scavenging activities (Fig. 1). The LMWC exhibited the highest radical-scavenging activity, followed by ascorbic acid, MMWC and HMWC. Notably, the DPPH radical-scavenging effect generally increased with the concentration of DMWC. For instance, as a concentration of LMWC rose from 0.2 to 1.0 mg/ml, scavenging activity toward DPPH radicals increased from 25% to 53%, indicating that LMWC exhibited stronger scavenging activity toward DPPH than ascorbic acid.

Fig. 1b plots the DPPH scavenging potential of DMWC in apple juice. In the DPPH test, the apple juice reduced the DPPH radicals, exhibiting 20% scavenging activity. Here, LMWC in apple juice exhibited excellent scavenging activity toward DPPH radical, which fact is attributable to its stronger hydrogen-donating capacity than that of apple

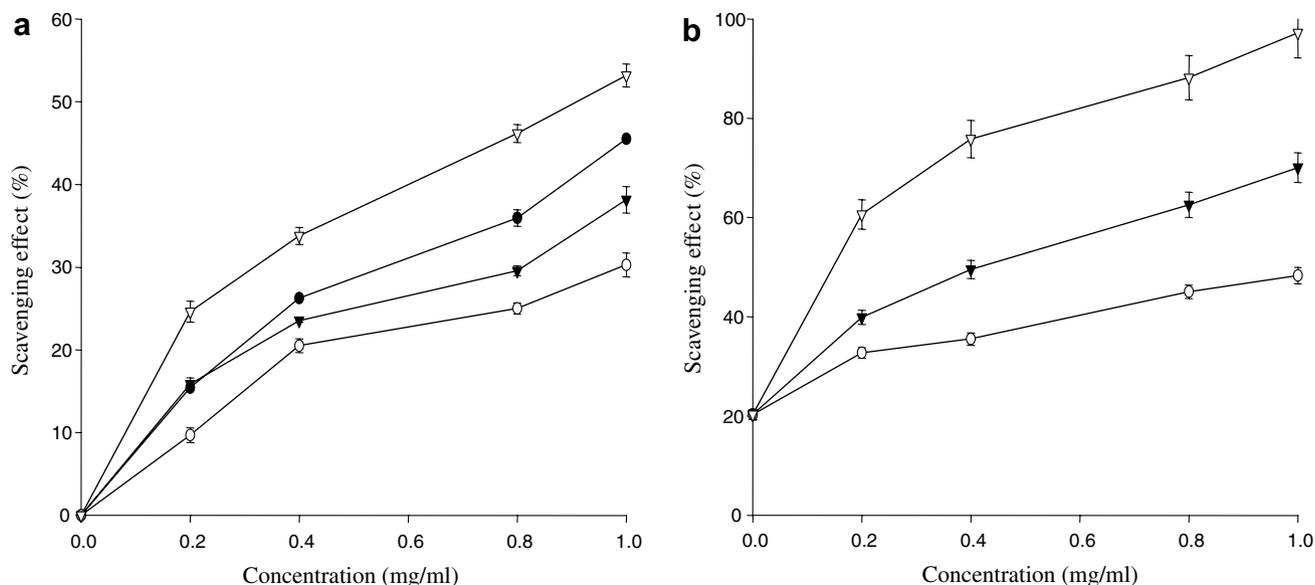


Fig. 1. (a) Scavenging effects of chitosans with different molecular weights and ascorbic acid on DPPH radicals. Each value is presented as mean \pm SD ($n = 3$). Ascorbic acid, ●; HMWC, ○; MMWC, ▼; LMWC, ∇. (b) Scavenging effects of chitosans with different molecular weights in apple juice on DPPH radicals. Each value is presented as mean \pm SD ($n = 3$). Apple juice, ●; Apple juice + HMWC, ○; Apple juice + MMWC, ▼; Apple juice + LMWC, ∇.

juice (control), MMWC or HMWC. At a concentration of 1.0 mg/ml, the LMWC in apple juice exhibited 97% scavenging activity toward DPPH radicals. The data herein on the DPPH scavenging potential of LMWC in apple juice reveal that LMWC probably contributed significantly toward the observed antioxidant effect.

3.2. Scavenging activity of DMWC toward superoxide anion radical

Superoxides are radicals whose unpaired electrons are located on oxygen. Although relatively weak oxidants, superoxides exhibit limited chemical reactivity, but can generate more dangerous species, including singlet oxygen and hydroxyl radicals, which cause the peroxidation of lipids (Halliwell & Chirico, 1993).

Fig. 2a summarizes the effect of DMWC and ascorbic acid on the superoxide anion radical-scavenging activities. The figure shows that the tested LMWC has the highest scavenging activity toward superoxide anion radicals ($P < 0.05$). The order of scavenging activities toward superoxide anion radicals was LMWC > ascorbic acid > MMWC > HMWC. At a concentration of 0.8–1.0 mg/ml, HMWC did not exhibit any significant scavenging activity toward superoxide anion radicals ($P > 0.05$). These findings resembled that of Yin et al. (2002) and may be caused by intramolecular hydrogen bonds. Chitosan has many hydrogen bonds on O₃–O₅ and N₂–O₆. HMWC has compact structures, whose intramolecular hydrogen bonds are stronger than LMWC. The strong effect of the intramolecular hydrogen bonds weakens the activities of the hydroxyl and amino groups. In contrast, LMWC has a less-compact structure, so the effect of the intramolecular hydrogen bonds is weak. However, the

superoxide anion radical is a zwitterionic radical. It reacts with free hydroxyl and amino groups in chitosan, and is thus eliminated. LMWC has more free hydroxyl and amino groups than HMWC, explaining why its scavenging activities toward superoxide anion radicals was stronger (Xing et al., 2005).

Fig. 2b presents the effect of DMWC on superoxide anion radical scavenging activities in apple juice. The data indicate that HMWC exhibited greater scavenging activity toward superoxide anion radicals at concentrations of 0.8–1.0 mg/ml than at 0.2 mg/ml ($P < 0.05$). However, LMWC exhibited an excellent capacity to scavenge superoxide anion radicals in apple juice. It exhibited the highest scavenging activity in the elimination of superoxide anion radicals ($P < 0.05$). At a concentration of 0.8 mg/ml, LMWC in apple juice exhibited 93.0% scavenging activity toward superoxide anion radicals. It may have better solubility than HMWC. These findings clearly indicate that the antioxidant activity of LMWC increased its capacity to scavenge superoxide anion radicals when in apple juice.

3.3. Hydrogen peroxide scavenging activity of DMWC

Biological systems can produce hydrogen peroxide. Hydrogen peroxide can attack many cellular energy-producing systems; for instance, it deactivates the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Hyslop et al., 1988).

Fig. 3a summarizes the effect of DMWC and ascorbic acid on hydrogen peroxide scavenging activities. At a concentration of 1.0 mg/ml, ascorbic acid, HMWC, MMWC and LMWC exhibited 87.4%, 68.2%, 75.0% and 90.5% scavenging activity toward hydrogen peroxide, respectively, indicating that all of the tested DMWC exhibited

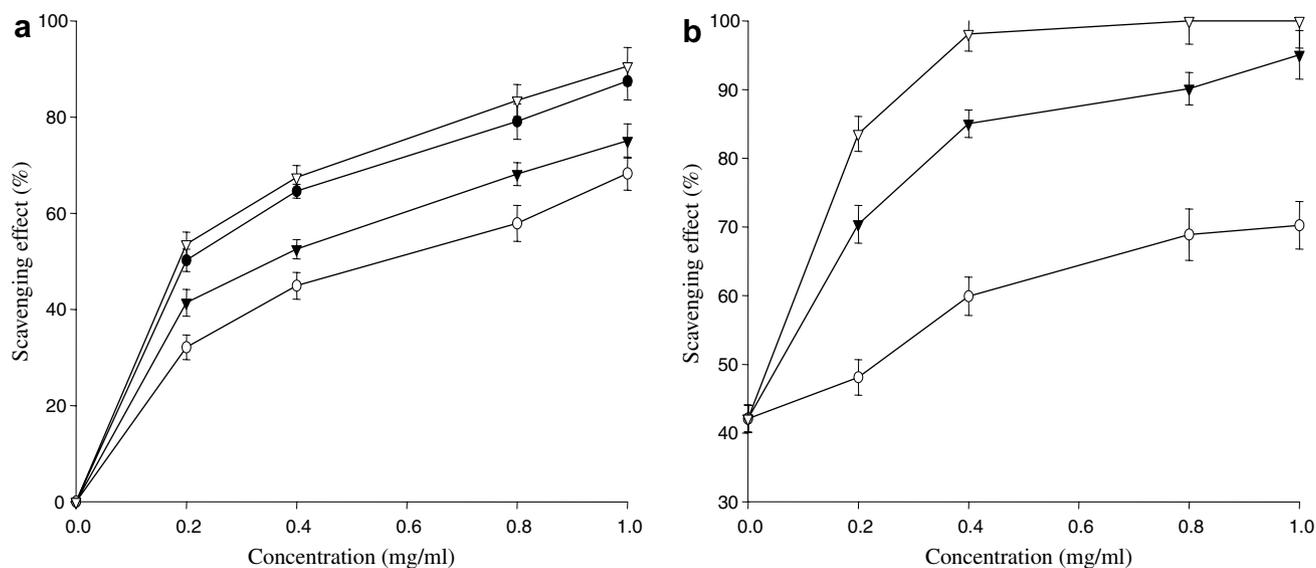


Fig. 2. (a) Scavenging effects of chitosans with different molecular weights and ascorbic acid on superoxide anion radicals. Each value represents mean \pm SD ($n = 3$). Ascorbic acid, ●; HMWC, ○; MMWC, ▼; LMWC, ▽. (b) Scavenging effects of chitosans with different molecular weights in apple juice on superoxide anion radicals. Each value is presented mean \pm SD ($n = 3$). Apple juice, ●; Apple juice + HMWC, ○; Apple juice + MMWC, ▼; Apple juice + LMWC, ▽.

H_2O_2 -scavenging activity. Oxidative destruction of chitosan has been previously reported to occur in the presence of hydroxy radicals, formed from H_2O_2 (Kabali'Nova et al., 2001; Qin, Du, & Xiao, 2002). Accordingly, the decrease in the amount of hydrogen following this reaction may account for the H_2O_2 -scavenging effect of DMWC observed in this work.

Fig. 3b presents hydrogen peroxide scavenging activities of DMWC in apple juice. Apple juice exhibited 42% scavenging activity; DMWC exhibited increased H_2O_2 -scavenging activity in apple juice. Additionally, the scavenging rate

of the chitosans other than HMWC increased with concentration. At a concentration of 0.4 mg/ml, LMWC in apple juice exhibited 98.1% scavenging activity toward hydrogen peroxide. LMWC was more effective in scavenging H_2O_2 in apple juice than apple juice alone (control), HMWC or MMWC ($P < 0.05$).

4. Chelating of metal ions by DMW chitosans

EDTA exhibited an excellent ferrous ion-chelating capacity of approximately 78.8% at a concentration of

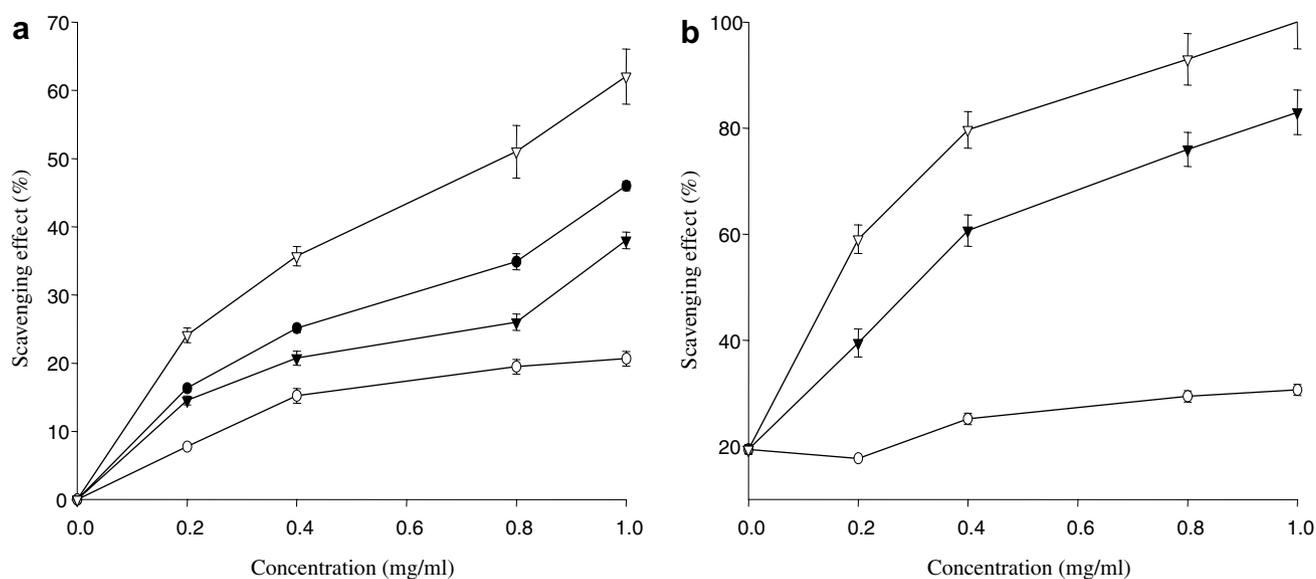


Fig. 3. (a) Scavenging effects of chitosans with different molecular weights and ascorbic acid on hydrogen peroxide. Each value is presented as mean \pm SD ($n = 3$). Ascorbic acid, ●; HMWC, ○; MMWC, ▼; LMWC, ▽. (b) Scavenging effects of chitosans with different molecular weights in apple juice on hydrogen peroxide. Each value is presented as mean \pm SD ($n = 3$). Apple juice, ●; Apple juice + HMWC, ○; Apple juice + MMWC, ▼; Apple juice + LMWC, ▽.

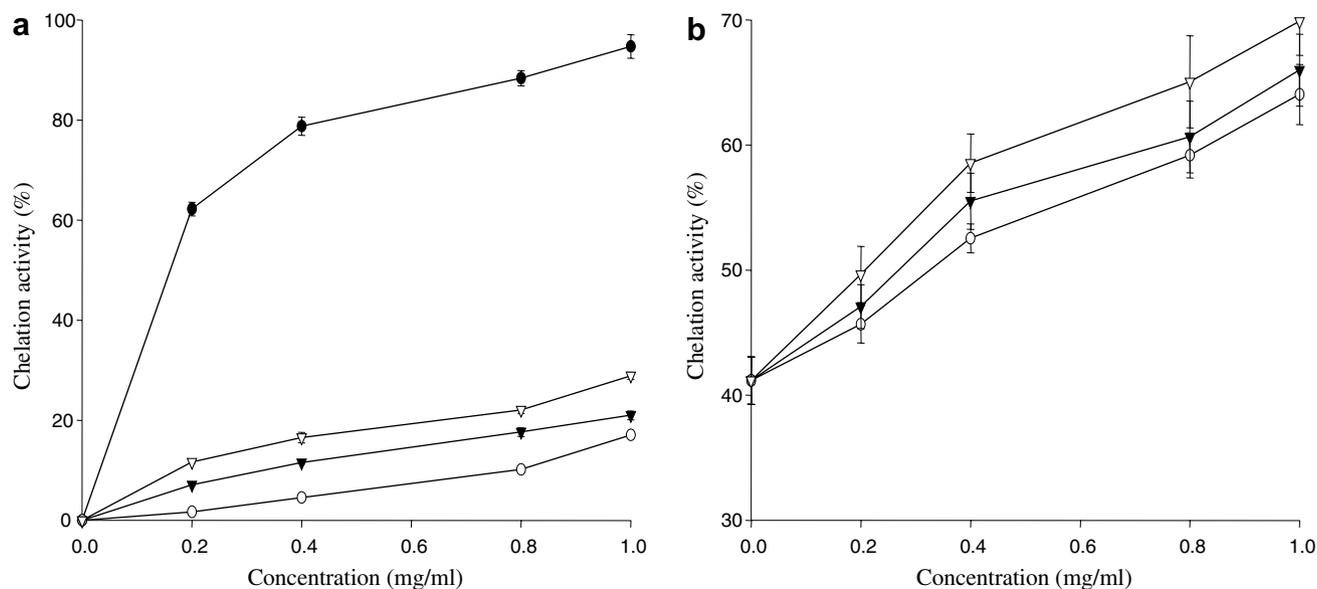


Fig. 4. (a) Chelating effects of chitosans with different molecular weights and EDTA on ferrous ions. Each value is presented as mean \pm SD ($n = 3$). EDTA, ●; HMWC, ○; MMWC, ▼; LMWC, ▽. (b) Chelating effects of chitosans with different molecular weights in apple juice on ferrous ions. Each value is presented as mean \pm SD ($n = 3$). Apple juice, ●; Apple juice + HMWC, ○; Apple juice + MMWC, ▼; Apple juice + LMWC, ▽.

0.4 mg/ml. The chelating effect of, HMWC, MMWC and LMWC slowly increased with concentration (Fig. 4a). Factors affecting the ion-chelating ability of chitosan are rather complex. It has been reported that although the metal ion absorption capability of chitin is closely related to its amino acid content, other factors such as affinity for water and crystallinity also affected ion-chelating activity (Kurita, Sannan, & Iwakura, 1979). Qin (1993) indicated that the ion-chelating activity of chitosan is strongly affected by the degree of acetylation, with the fully acetylated chitosan showing very little chelating activity.

Fig. 4b reveals that the ferrous ion-chelating effect of DMWC in apple juice was higher than that of apple juice alone (41.2%). At a concentration of 1.0 mg/ml, LMWC in apple juice exhibited a 70.0% chelating activity. The most effective pro-oxidants present in food systems are ferrous ions and the strong chelating effect of LMWC would be beneficial in food.

4.1. Effect of Trolox equivalent antioxidant capacity (TEAC) on DMWC

Table 1 summarizes the effect of DMWC on antioxidant capacity, expressed as TEAC. The behaviours of DMWC alone and DMWC in apple juice differ. The TEAC values of HMWC (0.89 ± 0.03) declined slightly, whereas, the TEAC values of LMWC (3.24 ± 0.21) increased in apple juice. The antioxidant activities (TEAC) were in the order: LMWC > MMWC > HMWC. A possible explanation of the considerable rise in TEAC in apple juice could be the higher antioxidant potential polyphenols in the intermediate stages of oxidation. However, further studies must be conducted to explain the antioxidant mechanisms of LMWC in apple juice.

Table 1

The Trolox equivalent antioxidant capacity (TEAC) value of HMWC, MMWC, LMWC in apple juices

Compounds ^b	TEAC persulfate decolorization assay (μ M Trolox equivalent) ^a
Trolox	1.00 ± 0.01
HMWC	0.89 ± 0.03
MMWC	1.46 ± 0.05
LMWC	2.15 ± 0.08
AJ	1.03 ± 0.07
AJ + HMWC	1.75 ± 0.12
AJ + MMWC	2.08 ± 0.16
AJ + LMWC	3.24 ± 0.21

The concentration range was 0.2–1.0 mg/ml for each DMWCs. Various concentrations of each DMWCs were tested and the TEAC is expressed as mean \pm SD. Experiments were performed three times ($n = 3$).

^a Applying the ABTS \bullet^+ decolorization assay (based on potassium persulfate), the value derived from the area under the time-dependency curve.

^b AJ : apple juice ; AJ + HMWC : apple juice with high molecular weight chitosan ; AJ + MMWC : apple juice with middle molecular weight chitosan ; AJ + LMWC: apple juice with low molecular weight chitosan.

5. Conclusions

The results of this work demonstrate that DMWC exhibit antioxidant activity and free radical scavenging activity, including activity toward DPPH radicals, hydrogen peroxide and superoxide anion radicals. They also exhibit ferrous ion-chelating activity toward ABTS radicals. The assays were useful in establishing the antioxidant capacities of DMWC, which have important applications in food industries. This work shows that LMWC can increase antioxidant activity in apple juice. However, *in vivo* antioxidant activity and the various antioxidant mechanisms must be investigated further.

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