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Antioxidant properties of chitosan from crab shells

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ABSTRACT

Crab chitosan was prepared by alkaline *N*-deacetylation of crab chitin for 60, 90 and 120 min and its antioxidant properties studied. Chitosan exhibited showed antioxidant activities of 58.3–70.2% at 1 mg/mL and showed reducing powers of 0.32–0.44 at 10 mg/mL. At 10 mg/mL, the scavenging ability of chitosan C60 on 1,1-diphenyl-2-picrylhydrazyl radicals was 28.4% whereas those of other chitosans were 46.4–52.3%. At 0.1 mg/mL, scavenging abilities on hydroxyl radicals were 62.3–77.6% whereas at 1 mg/mL, chelating abilities on ferrous ions were 82.9–96.5%. All EC_{50} values of antioxidant activity were below 1.5 mg/mL. With regard to antioxidant properties assayed, the effectiveness of chitosans C60, C90 and C120 correlated with their *N*-deacetylation times. Overall, crab chitosan was good in antioxidant activity, scavenging ability on hydroxyl radicals and chelating abilities on ferrous ions and may be used as a source of antioxidants, as a possible food supplement or ingredient in the pharmaceutical industry.
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1. Introduction

Chitin, found in the shell of crustaceans, the cuticles of insects, and the cell walls of fungi, is the second abundant biopolymer in the nature (Knorr, 1984). Structurally, chitin is a straight-chain polymer composed of β-1,4-*N*-acetylglucosamine and classified into α-, β- and γ-chitin (Cabib, 1981; Cabib, Bowers, Sburlati, & Silverman, 1988). Chitosan derived by partial *N*-deacetylation of chitin is also a straight-chain polymer of glucosamine and *N*-acetylglucosamine (Muzzarelli, Rochetti, Stanic, & Weckx, 1997). α-Chitin, the most abundant in nature, has a structure of antiparallel chains and is found in the crab, shrimp and lobster whereas β-chitin found in squid has intrasheet hydrogen bonding by parallel chains (Jang, Kong, Jeong, Lee, & Nah, 2004; Minke & Blackwell, 1978). However, γ-chitin found in the cell walls of fungi has a mixture of parallel and antiparallel chains, which is a combination of α-chitin and β-chitin (Jang et al., 2004).

Because chitin and chitosan possesses many beneficially biological properties such as antimicrobial activity (Kobayashi, Watanabe, Suzuki, & Suzuki, 1990; Tokoro et al., 1989), biocompatibility, biodegradability, hemostatic activity and woundhealing property, much attention has been paid to its biomedical applications (Farkas, 1990; Fleet & Phaff, 1981). Due to these unique properties, chitosan and its derivatives have been proposed for applications in biomedical, food, agriculture, biotechnology and pharmaceutical fields (Felse & Panda, 1999; Kumar, 2000; Shahidi, Arachchi, & Jeon, 1999).

Antioxidant properties of chitosan derivatives have been studied (Lin & Chou, 2004; Xie, Xu, & Liu, 2001; Xing et al., 2005). Furthermore, antioxidant properties of fungal chitosan from shiitake stipes have also been studied (Yen, Tseng, Li, & Mau, 2007). However, antioxidant properties of chitosan derived from crab shells are not available. Accordingly, the objective of this study was to assess the antioxidant properties of chitosan prepared from crab chitin by *N*-deacetylation using a concentrated sodium hydroxide solution. Antioxidant properties assayed were antioxidant activity by the conjugated diene method, reducing power, scavenging abilities on radicals and chelating ability on ferrous ions.

2. Materials and methods

2.1. Chemicals

Methanol was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Ascorbic acid, butylated hydroxyanisole (BHA), citric acid, crude crab chitin, crab chitosan, 5,5-dimethyl pyrroline-*N*-oxide (DMPO), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ethylenediaminetetraacetic acid (EDTA), ferrozine, linoleic acid, potassium ferricyanide, potassium permanganate and α-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO). Ferrous chloride and hydrogen peroxide were obtained from Merck Co. (Darmstadt, Germany). Ferric chloride, oxalic acid, sodium hydroxide, sodium phosphate and trichloroacetic acid were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Acetic acid was ob-

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tained from Union Chemical Works (Hsinchu, Taiwan). Commercial crab α-chitosan form the snow crab (*Chionoecetes opilio*) was obtained from Dalian City, Liaonin, China. Ethanol (95% pure) was supplied by Taiwan Tobacco & Wine Monopoly Bureau (Taipei). Other reagents were of analytical grade.

2.2. Preparation of crab chitosan

Crude chitin from crab shells (~200 g each) was ground using a mill (Retsch ultracentrifugal mill and sieving machine, Haan, Germany) to obtain coarse powder (65 mesh) and treated with the method of Kurita, Tomita, Tada, Nishimura, and Ishii (1993) with some modification. The powder of crab chitin was treated with 1 N HCl solution at room temperature for 6 h to remove minerals and then treated with aqueous sodium hydroxide solution at the ratio of 1:10 (w/v) at 100 °C for 3 h to remove protein. The mixture was filtered and washed with deionized water to neutral. For the purpose of decolorization, the precipitate thus obtained was treated further with 1% potassium permanganate solution for 1 h, and then reacted with 1% oxalic acid solution for 1 h (Chang, 1982). Following decolorization, the precipitate was washed with deionized water to neutral and freeze dried to obtain purified crab chitin.

For the purpose of *N*-deacetylation, 1 g of purified crab chitin was treated with 30 mL of 40% sodium hydroxide solution at 105 °C for 60, 90 and 120 min, respectively. After filtration, washing to neutral with deionized water and freeze drying, the corresponding chitosans obtained were designated as chitosans C60, C90 and C120. Chitosan samples used for assays of antioxidant properties were chitosans C60, C90 and C120 and crab chitosan from Sigma (CS) and crab chitosan from China (CC). The degrees of *N*-deacetylation (DD) of crab chitosans C60, C90 and C120 were determined to be 83.3%, 88.4% and 93.3%, respectively (Yen, 2006).

2.3. Antioxidant activity

The antioxidant activity was determined by the conjugated diene method (Lingnert, Vallentin, & Eriksson, 1979). Each chitosan sample (0.1–10 mg/mL, 100 μl) in 0.2% acetic acid solution was mixed with 2 mL of 10 mM linoleic acid emulsion in 200 mM sodium phosphate buffer (pH 6.5) in test tubes and placed in darkness at 37 °C to accelerate oxidation. After incubation for 15 h, 6 mL of 60% methanol in deionized water was added, and the absorbance of the mixture was measured at 234 nm against a blank in a Hitachi U-2001 UV/vis spectrophotometer (Hitachi, Tokyo, Japan). The antioxidant activity was calculated as follows: Antioxidant activity (%) = $[(\Delta A_{234}$ of control $- \Delta A_{234}$ of sample)/ΔA_{234} of control$] \times 100$. A control consisted of methanol and the reagent solution without chitosan. An antioxidant activity value of 100% indicates the strongest antioxidant activity. EC_{50} value (mg/mL) is the effective concentration at which the antioxidant activity was 50% and was obtained by interpolation from linear regression analysis. Ascorbic acid, BHA and α-tocopherol were used for comparison.

2.4. Reducing power

The reducing power was determined according to the method of Oyaizu (1986). Each chitosan sample (0.1–10 mg/mL, 2.5 mL) in 0.2% acetic acid solution was mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide, and the mixture was incubated at 50 °C for 20 min. Thereafter, 2.5 mL of 10% trichloroacetic acid was added and the mixture was centrifuged at 200*g* for 10 min. The upper layer (5 mL) was mixed with 5 mL of deionized water and 1 mL of 0.1% ferric chloride, and the absorbance was measured at 700 nm against a blank. A higher absorbance indicates a higher reducing power. EC_{50} value (mg/mL) is the effective concentration at which the absorbance was 0.5 for reducing power. Ascorbic acid, BHA and α-tocopherol were used for comparison.

2.5. Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radicals

Each chitosan sample (0.1–10 mg/mL, 4 mL) in 0.2% acetic acid solution was mixed with 1 mL of methanolic solution containing DPPH radicals, resulting in a final concentration of 10 mM DPPH. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank (Shimada, Fujikawa, Yahara, & Nakamura, 1992). The scavenging ability was calculated as follows: Scavenging ability (%) = $[(\Delta A_{517}$ of control $- \Delta A_{517}$ of sample)/ΔA_{517} of control$] \times 100$. EC_{50} value (mg/mL) is the effective concentration at which DPPH radicals were scavenged by 50%. Ascorbic acid, BHA and α-tocopherol were used for comparison.

2.6. Scavenging ability on hydroxyl radicals

The hydroxyl radicals reacted with the nitrone spin trap DMPO and the resultant DMPO-OH adducts were detected with an electron paramagnetic resonance (EPR) spectrometer. The EPR spectrum was recorded 2.5 min after mixing each chitosan sample (0.1–20 mg/mL, 200 μl) in 0.2% acetic acid solution with 200 μl of 10 mM hydrogen peroxide, 200 μl of 10 mM ferrous chloride and 200 μl of 10 mM DMPO using a Bruker EMX-10 EPR spectrometer (Bruker-Biospin, Rheinstetten, Germany) at the following settings: 0.3480-T magnetic field, 1.0×10^{-4} T modulation amplitude, 0.5 s time constant and 200 s scan period (Shi, Dalal, & Jain, 1991). The scavenging ability was calculated by subtracting the relative EPR signal intensity from 100. The relative EPR signal intensity was calculated as follows: Relative EPR signal intensity (%) = $[h\,\Delta H^2$ (sample)/$h\,\Delta H^2$ (control)$] \times 100$; h is the width of the peak and ΔH is the length of the peak. EC_{50} value (mg/mL) is the effective concentration at which hydroxyl radicals were scavenged by 50%. BHA was used for comparison.

2.7. Chelating ability on ferrous ions

Chelating ability was determined according to the method of Dinis, Madeira, and Almeida (1994). Each chitosan sample (0.1–10 mg/mL, 1 mL) in 0.2% acetic acid solution was mixed with 3.7 mL of methanol and 0.1 mL of 2 mM ferrous chloride. The reaction was initiated by the addition of 0.2 mL of 5 mM ferrozine. After 10 min at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. A lower absorbance indicates a higher chelating ability. EC_{50} value (mg/mL) is the effective concentration at which ferrous ions were chelated by 50%. Citric acid and EDTA were used for comparison.

2.8. Statistical analysis

For each chitosan preparation, three samples were prepared for assays of every antioxidant attribute and component. The experimental data were subjected to an analysis of variance for a completely random design to determine the least significant difference at the level of 0.05.

3. Results and discussion

3.1. Antioxidant activity

Using the conjugated diene method, all crab chitosans exhibited showed moderate to high antioxidant activities of 58.3–70.2% at

1 mg/mL and high antioxidant activities of 79.9–85.2% at 10 mg/mL (Fig. 1). All chitosans showed consistent antioxidant activity with increased concentration. In addition, the antioxidant activities of chitosans C60, C90 and C120 correlated with their *N*-deacetylation times. However, at 0.1 mg/mL, the antioxidant activities were 89.2%, 88.9% and 32.0% for BHA, α-tocopherol and ascorbic acid, respectively.

Yen et al. (2007) used the same alkaline *N*-deacetylation method to prepare fungal chitosans from shiitake stipes and found that their antioxidant activities were 61.6–82.4% at 1 mg/mL and 82.3–89.9% at 10 mg/mL. Similarly, the antioxidant activities of fungal chitosans C60, C90 and C120 correlated with their *N*-deacetylation times. In other words, the longer the *N*-deacetylation time, the higher the antioxidant activities were observed. At longer *N*-deacetylation time, the chitosan was obtained with higher DD and had more amino groups on C2 to inhibit the oxidation of linoleic acid, and thereby, increasing their antioxidant activities.

3.2. Reducing power

Reducing power of all crab chitosans were 0.32–0.44 at 10 mg/mL (Fig. 2). However, BHA, α-tocopherol and ascorbic acid showed reducing powers of 0.96, 0.45 and 0.68 at 0.1 mg/mL, respectively. Yen et al. (2007) mentioned that fungal chitosans showed slight (0.13–0.29) at 1 mg/mL and moderate reducing power (0.42–0.57) at 10 mg/mL. It seems that chitosan from crab shells and shiitake stipes was not effective in reducing power. Xing et al. (2005) found that at 0.1 mg/mL, the regioselective sulfated chitosan TSCTS (12.1% S, 11.7×10^4 Da, white color and easy soluble in water) exhibited a reducing power of 1.07 whereas other sulfated chitosan was not effective. Therefore, sulfated chitosan B and C may have this improved antioxidant attribute.

3.3. Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radicals

At 10 mg/mL, the scavenging ability of crab chitosan C60 on DPPH radicals was 28.4% whereas those of other crab chitosans were in the range of 46.4–52.3% (Fig. 3). However, at 0.1 mg/mL, BHA and α-tocopherol showed moderate scavenging abilities of 65.1% and 69.4%, respectively. At 0.1–10 mg/mL, ascorbic acid showed a plateau of scavenging abilities of 35.4–44.3%. Yen et al. (2007) reported that fungal chitosans scavenged DPPH radicals by 28.4–53.5% at 10 mg/mL. Obviously, chitosan from crab shells and shiitake stipes was also not an effective scavenger for DPPH radicals.

Xing et al. (2005) found that at 0.05 mg/mL, the sulfated chitosan TSCTS exhibited a high scavenging ability of 83.4% whereas other sulfated chitosans were not the scavenger for DPPH radicals. Lin and Chou (2004) found that the *N*-alkylated disaccharide chitosan derivatives with different degrees of substitution (DS) of 20–30% exhibited the highest DPPH radical scavenging abilities of 80–95% at 0.1 mg/mL, followed by the derivatives with DS of 40–50% and 60–70%. Apparently, the scavenging ability of chitosan might be reduced after sulfation or might be enhanced after *N*-alkylation of disaccharide.

3.4. Scavenging ability on hydroxyl radicals

At 0.1 mg/mL, scavenging abilities of crab chitosans C60, C90, C120, CS and CC on hydroxyl radicals were 62.3%, 66.3%, 75.8%, 76.3% and 77.6% whereas at 10 mg/mL, all crab chitosans showed high scavenging abilities of 88.7–94.1% (Fig. 4). It seems that scavenging abilities of crab chitosans C60, C90 and C120 correlated with their *N*-deacetylation times. These values were much higher than those for BHA when used at 20 mg/mL (22.8%). Yen et al. (2007) reported that at 0.1 mg/mL, scavenging abilities of fungal chitosans C60, C90 and C120 on hydroxyl radicals were 61.9–63.6%, 68.3–69.9% and 77.2–77.7%, respectively, whereas at 1 mg/mL, the scavenging abilities were in the range of 80.2–86.5%. Obvi-

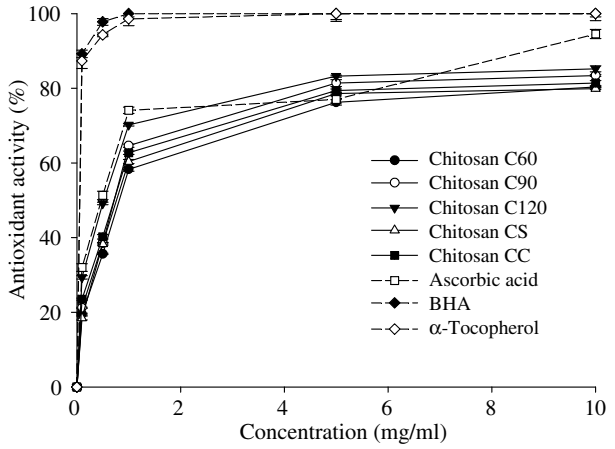

Fig. 1. Antioxidant activity of chitosans from crab shells. Each value is expressed as mean ± *SD* (*n* = 3).

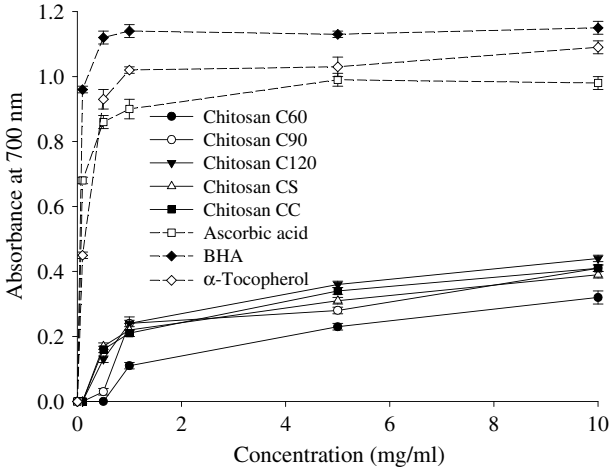

Fig. 2. Reducing power of chitosans from crab shells. Each value is expressed as mean ± *SD* (*n* = 3).

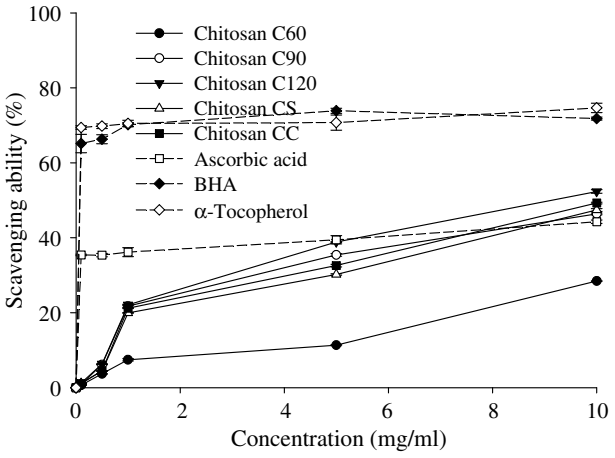

Fig. 3. Scavenging ability of chitosans from crab shells on 1,1-diphenyl-2-picrylhydrazyl radicals. Each value is expressed as mean ± *SD* (*n* = 3).

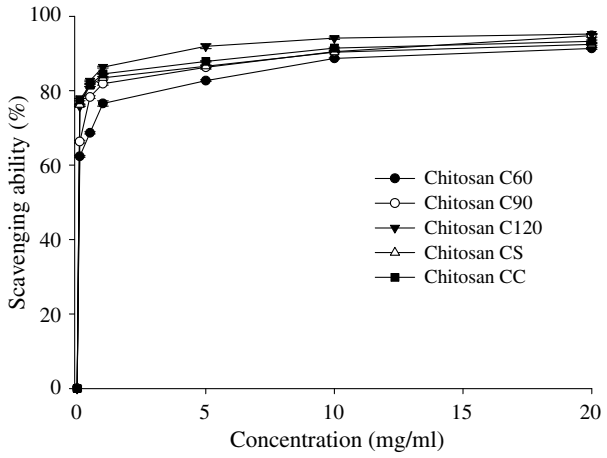

Fig. 4. Scavenging ability of chitosans from crab shells on hydroxyl radicals. Each value is expressed as mean ± *SD* (*n* = 3).

ously, chitosan from crab shells and shiitake stipes was an effective scavenger for hydroxyl radicals.

Actually, previous studies have shown that chitosan is a good scavenger for hydroxyl radicals regardless of chemical modification. For example, Esumi, Takei, and Yoshimura (2003) showed that the gold-chitosan nanocomposites have a catalytic activity 80-fold higher than that of ascorbic acid. Xing et al. (2005) studied the scavenging abilities of sulfated chitosan on hydroxyl radicals, generated by reaction of iron–EDTA complex with hydrogen peroxide finding that sulfated chitosan effectively scavenged hydroxyl radicals at 3.2 mg/mL and protected deoxyribose from oxidative damage. Lin and Chou (2004) reported that at 0.5 mg/mL, *N*-alkylated disaccharide chitosan derivatives scavenged more than 80% of hydrogen peroxide.

3.5. Chelating effects on ferrous ions

At 1 mg/mL, chelating abilities of all crab chitosans on ferrous ions were in the range of 82.9–96.5% (Fig. 5). At 10 mg/mL, ferrous ions were all chelated. It seems that chelating abilities of crab chitosans C60, C90 and C120 also correlated with their *N*-deacetylation times. However, EDTA showed a high chelating ability of 97.9% at a concentration as low as 0.1 mg/mL while the value for citric acid was only 6.8% when used at 20 mg/mL.

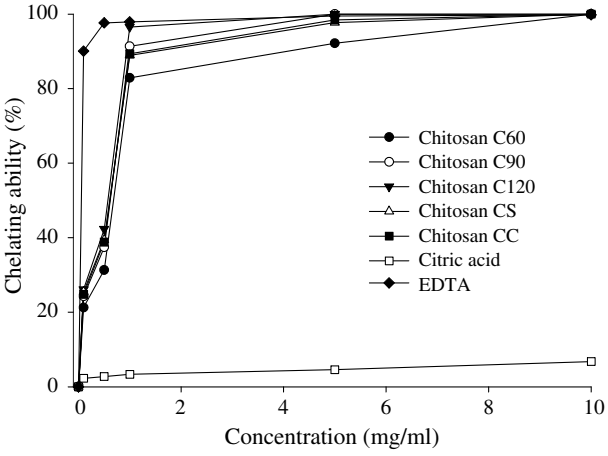

Fig. 5. Chelating ability of chitosans from crab shells on ferrous ions. Each value is expressed as mean ± *SD* (*n* = 3).

Similarly, Yen et al. (2007) reported that at 1 mg/mL, chelating abilities of fungal chitosans were 88.7–100% whereas at 10 mg/mL, ferrous ions were all chelated. Xing et al. (2005) found that the ferrous ion-chelating effect of all kinds of sulfated chitosan was concentration related with sulfated chitosan TSCTS being the most effective (~72% at 0.25 mg/mL). Lin and Chou (2004) reported that the chelating abilities of *N*-alkylated disaccharide chitosan derivatives were less than that observed with EDTA. However, at 1.0 mg/mL, EDTA chelated ~60% of cupric ions (Lin & Chou, 2004).

Factors affecting the ion-chelating ability of chitosan are rather complex. Inoue, Baba, Yoshizuka, Noguchi, and Yoshizaki (1988) suggested that chelating of Cu^{2+} involves the binding of Cu^{2+} with the hydroxyl group on C6 and amino group on C2 of the chitosan molecule. Similarly, ferrous ion chelation might also involve the binding of Fe^{2+} with the hydroxyl group on C6 and amino group on C2 of the chitosan molecule. Qin (1993) indicated that the ion-chelating ability of chitosan is strongly affected by the degree of acetylation, with the fully acetylated chitosan showing very little chelating activity.

Transition metal ions can initiate lipid peroxidation and start a chain reaction, which lead to the deterioration of flavor and taste in food (Gordon, 1990). It has also been proposed that the catalysis of metal ions might correlate to cancer and arthritis (Halliwell, Murcia, Chirico, & Aruoma, 1995). Since ferrous ions are the most effective pro-oxidants in the food system (Yamaguchi, Tatsumi, Kato, & Yoshimitsu, 1988), the high ferrous ion-chelating abilities of chitosan would be beneficial if they were formulated into foods.

3.6. EC_{50} in antioxidant properties

The antioxidant properties assayed herein were summarized in Table 1, and the results were normalized by computing the effective concentration of chitosan samples at which the effect was 50% or the absorbance was 0.500 and expressed as EC_{50} values (mg chitosan per mL) for comparison. Effectiveness in antioxidant properties inversely correlated with EC_{50} value. With regard to antioxidant activity, all EC_{50} values were below 1.5 mg/mL with crab chitosan C120 being the most effective. Both EC_{50} values of reducing powers and scavenging abilities on DPPH radicals were in the range of 9.13–20.0 mg/mL, indicating that crab chitosans were less effective in these two antioxidant attributes. All EC_{50} values of scavenging abilities on hydroxyl radicals were below 0.1 mg/mL, indicating that crab chitosans were the most effective in this antioxidant attribute. All EC_{50} values of chelating abilities on ferrous ions were in the range of 0.57–0.68 mg/mL. Overall, crab chitosans were good in antioxidant activity, scavenging ability on hydroxyl radicals and chelating abilities on ferrous ions.

With regard to antioxidant activity, the effectiveness was in the descending order of chitosan C120 > C90 = CC > CS > C60. With regard to scavenging ability on hydroxyl radicals and chelating ability on ferrous ions, all crab chitosans were comparable. With regard to antioxidant properties assayed, the effectiveness of crab chitosans C60, C90 and C120 correlated with their *N*-deacetylation times with chitosan C120 being the most effective. Chitosan prepared from different *N*-deacetylation times exhibited different DD. Yen (2006) found that crab chitosans C60, C90 and C120 showed DD of 83.3%, 88.4% and 93.3%, respectively. It is obvious that crab chitosan with higher DD had more amino groups on C2 to enhance their antioxidant properties.

Although BHA and α-tocopherol were good in antioxidant activity, reducing power and scavenging ability on DPPH radicals and EDTA was excellent for chelating ferrous ions, they are additives and used or present in mg levels in foods. However, chitosans C60, C90 and C120 prepared from crab shells could be used in g levels as food or a food ingredient. Therefore, these crab chitosans

Table 1
EC_{50} values of chitosans from crab shells in antioxidant properties

	EC_{50} [a] (mg extract/mL)				
	C60	C90	C120	CS	CC
Antioxidant activity	1.32 ± 0.01 A[b]	0.72 ± 0.02 C	0.52 ± 0.01 D	0.77 ± 0.01 B	0.72 ± 0.01 C
Reducing power	20.00 ± 0.03 A[c]	13.46 ± 0.02 E[c]	13.75 ± 0.01 D[c]	16.25 ± 0.02 C[c]	16.43 ± 0.01 B[c]
Scavenging ability on DPPH radicals	16.30 ± 0.08 A[c]	11.67 ± 0.02 B[c]	9.13 ± 0.02 E	10.75 ± 0.02 C[c]	10.22 ± 0.01 D[c]
Scavenging ability on OH radicals	0.08 ± <0.01 A	0.08 ± <0.01 A	0.07 ± <0.01 B	0.07 ± <0.01 B	0.06 ± <0.01 C
Chelating ability on ferrous ions	0.68 ± 0.02 A	0.62 ± 0.01 B	0.57 ± 0.01 D	0.61 ± 0.01 C	0.61 ± <0.01 C

[a] EC_{50} value: the effective concentration at which the antioxidant activity was 50%; the absorbance was 0.5 for reducing power; 1,1-diphenyl-2-picrylhydrazyl (DPPH) or hydroxyl (OH) radicals were scavenged by 50%; and ferrous ions were chelated by 50%, respectively. EC_{50} value was obtained by interpolation from linear regression analysis.
[b] Each value is expressed as mean ± standard deviation ($n = 3$). Means with different letters within a row are significantly different ($P < .05$).
[c] Obtained by extrapolation from linear regression analysis.

might serve as possible protective agents to help human reduce oxidative damage.

4. Conclusion

The results showed all crab chitosans were good in antioxidant properties, especially antioxidant activity, scavenging ability on hydroxyl radicals and chelating ability on ferrous ions. In addition, the prolonged *N*-deacetylation resulted in chitosan with more effective antioxidant properties. All crab chitosans exhibited comparable antioxidant properties. On the basis of the results obtained, crab chitosan with presumed antioxidant properties may be used as a source of antioxidants, as a possible food supplement or ingredient or in the pharmaceutical industry.

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