Bioorganic & Medicinal Chemistry 20 (2012) 2905–2911

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Comparison in antioxidant action between α-chitosan and β-chitosan at a wide range of molecular weight and chitosan concentration

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ARTICLE INFO

Article history:
Received 4 January 2012
Revised 1 March 2012
Accepted 7 March 2012
Available online 15 March 2012

Keywords:
β-Chitosan
Jumbo squid pens
α-Chitosan
Antioxidant activity
Molecular weight

ABSTRACT

Antioxidant activity in α- and β-chitosan at a wide range of molecular weight (Mw) and chitosan concentration (CS) was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, reducing ability, chelating ability, and hydroxyl radical scavenging activity. The form of chitosan (FC) had significant (P <0.05) effect on all measurements except DPPH radical scavenging activity, and antioxidant activity was dependent on Mw and CS. High Mw (280–300 kDa) of β-chitosan had extremely lower half maximal effective concentrations (EC_{50}) than α-chitosan in DPPH radical scavenging activity and reducing ability. The 22–30 kDa of α- and β-chitosan showed significantly (P <0.05) higher activities in DPPH radical scavenging, reducing ability, and hydroxyl radical scavenging than samples at other Mw, while chelating ability was the highest in 4–5 kDa chitosan. CS had significant effect on all measurements and the effect was related to Mw. The antioxidant activity of 280–300 kDa chitosan was affected by coil-overlap concentrations (C^*) in the CS range of 4–10 mg/mL, forming entanglements. Reducing ability and hydroxyl radical scavenging activity were more predominant action in antioxidant activity of chitosan as shown by the lower EC_{50} values than those in other antioxidant measurements.

1. Introduction

Antioxidant activity is one of the well-known functionalities of chitosan. Many studies have shown that chitosan inhibit the reactive oxygen species (ROS) and prevent the lipid oxidation in food and biological systems. Several mechanisms about the antioxidant action of chitosan have been proposed.[1] Chitosan can scavenge free radicals or chelate metal ions from the donation of a hydrogen or the lone pairs of electrons.[2,3] The interaction of chitosan with metal ions could involve several complex actions including adsorption, ion-exchange, and chelation.[4] The hydroxyl groups (OH) and amino groups (NH_2) in chitosan are the key functional groups for its antioxidant activity, but can be difficult to be dissociated due to the semi-crystalline structure of chitosan with strong hydrogen bonds.[2] Chitin has two forms, named α- and β-chitin, in which α-chitin is very stable with intra-chain, intra-sheet, and inter-sheet hydrogen bonds from the antiparallel sheets along with c axis in orthorhombic cell, while β-chitin has no hydrogen bonds between two inter-sheets owing to their parallel directions.[5–7] Also, the initial crystallinity index (CI) of α- and β-chitin are different, 28.3% for α-chitin and 20.8% for β-chitin. Through deacetylation, CI of α-chitosan was slightly decreased, while CI of β-chitosan exhibited large reduction.[7–9] Similarly, Kurita et al. (1993) reported that α-chitin is rigid and can be less susceptible to deacetylation compared to β-chitin.[10] Therefore, the polymeric structures (e.g., CI) of chitosan deacetylated from different forms of chitin may not be identical and β-chitosan can have higher solubility with less crystallinity, thus providing better functionalities than α-chitosan in similar Mw and DDA. For this reason, we hypothesized that the form of chitosan (FC) may be a significant factor determining the antioxidant activity of chitosan. However, little study has compared the difference and/or similarity between α- and β-chitosan in their antioxidant activity.

Molecular weight (Mw) of chitosan is one of the most important factors affecting its antioxidant activity. Je et al. (2004) indicated that 1–5 kDa chitosan with 90% degree of deacetylation (DDA) has the highest radical scavenging activity.[11] Sun et al. (2007) reported that chitosan oligomers with low Mw (2.30, 3.27, and 6.12 kDa) have better antioxidant activity than that of higher Mw oligosaccharides (15.25 kDa).[12] Tomida et al. (2009) also showed that low Mw chitosan (2.8, 17.0, and 33.5 kDa) inhibits the oxidation of serum albumin, resulting in reduction of oxidative stress in uremia in comparison with higher Mw chitosan (62.6–931 kDa).[13] In the study of antioxidant effect of chitosan on salmon at Mw of 30, 90 and 120 kDa,[1] the lowest Mw of chitosan (30 kDa) showed

Abbreviations: FC, form of chitosan; Mw, molecular weight; CS, concentration of chitosan; C^*, coil-overlap concentration; DDA, degree of deacetylation; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EDTA, ethylenediaminetetraacetic acid; T (%), trans mittance; [η], intrinsic viscosity; EC_{50}, half maximal effective concentrations; Abs., absorbance; CI, crystallinity index.

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http://dx.doi.org/10.1016/j.bmc.2012.03.020

the strongest antioxidant activity, resulting in approximately 85% scavenging activity for free radicals. Chien et al. (2007) also found that lower Mw (12 kDa) chitosan increases antioxidant activity in apple juice, compared to higher Mw chitosan (95 and 318 kDa).[14] Also, some studies reported that Mw is dependent on its crystallinity. Kumar et al. (2004) reported decreased CI in lower Mw,[15] whereas Ogawa found increased CI in lower Mw.[16] Liu et al. (2006) reported increased crystallinity in high DDA and low Mw.[17] In respect to the effect of DDA, most studies reported that antioxidant property is enhanced with higher DDA.[11,18,19]

This study was aimed to investigate the antioxidant action of α- and β-chitosan obtained from shrimp shells and jumbo squid pens, respectively, at a wide range of Mw and chitosan concentration (CS). DDA effect was also considered. Different antioxidant measurements including DPPH radical scavenging activity, reducing ability, chelating ability, and hydroxyl radical scavenging were conducted to verify predominant antioxidant action in chitosan.

2. Experimental

2.1. Materials

Dried jumbo squid (*Dosidicus gigas*) pens were provided by Dosidicus LLC (USA). Commercial α-chitosan from shrimp shells was purchased from Primax (Iceland) with Mw of 300 kDa and DDA of 88%, determined in this study. NaOH, NaCl, ascorbic acid, and trichloroacetic acid were purchased from Mallinckrodt Chemicals Co. (USA). 2-Thiobarbituric acid and ferric chloride were from Sigma Chemical Co. (USA) and ammonium thiocyanate and deoxyribose from Alfa Aesar (USA). 1,1-Dephenyl-2-picrylhydrzyl (DPPH), free radical, and cellulase from *Aspergillus niger* were from TCI America (USA). Ferrous chloride and hydrogen peroxide were from J.T. Baker (USA) and VWR (USA), respectively. Potassium ferricyanide and ethylenediaminetetraacetic acid (EDTA) were from EM Science (USA). All the chemicals were of reagent grade.

2.2. Chitosan preparation

Three steps were applied for β-chitosan preparation, including deproteinization, deacetylation, and depolymerization. Dried squid pens were ground into about 18 mesh (ASTM) size by a grinder (Glenmilles Inc., USA), and then deproteinized in 5% NaOH for 3 d at room temperature. After washing with distilled water till neutral pH, chitin powder was dried at 40 °C oven (Thermo Fisher Scientific Inc., USA) for 24 h. β-Chitin was deacetylated by two treatment conditions: (1) 50% NaOH at 90 °C for 6 h to obtain 97% DDA; and (2) 40% NaOH at 90 °C for 4 h to obtain 86% DDA.[20] Samples were washed with distilled water till reaching neutral pH, and the filtrate was dried at 40 °C for 24 h. Both α- and β-chitosan were then depolymerized by enzymatic hydrolysis using cellulase. Briefly, chitosan was dissolved in 2% acetic acid solution at a ratio of 1:100 (chitosan: solvent) for α-chitosan and 1:200 for β-chitosan due to its higher viscosity. Solutions were adjusted to pH 5 by 10% NaOH. Cellulase was added in the same weight of chitosan in the solutions and reacted at 50 °C water bath for a given time determined from our preliminary studies to receive desired Mw. The hydrolyzates were boiled for 10 min to inactivate enzyme reaction, centrifuged at 8000*g* for 30 min, and filtered through a 0.45 μm membrane filter to remove denatured enzyme. A 10% NaOH was then added into the solution till about pH 7 for precipitation. Hydrolyzates of low and oligosaccharide chitosan were concentrated to about one-tenth of the original volume by a rotary evaporator with reduced pressure. After solution was adjusted to pH 7 by using 10% NaOH, ethanol was added for precipitation. Precipitated samples were washed again with distilled water and ethanol to remove other residues and dried at 40 °C oven for 24 h. Note that when preparing α- and β-chitosan samples with a wide range of Mw using enzyme hydrolysis, it was difficult to obtain exact same Mw in both forms of chitosan. Therefore, similar Mw of α- and β-chitosan samples was arranged into four groups: oligosaccharides (4–5 kDa), low Mw (22–30 kDa), med Mw (61–79 kDa), and high Mw (280–300 kDa) to compare their antioxidant activity. In addition, low DDA (86%) β-chitosan sample was prepared to compare its antioxidant activity with high DDA (97%) of β-chitosan and low DDA (88%) of α-chitosan at same sample Mw of 12–15 kDa.

2.3. Measurement of solubility

The pH-dependent solubility of chitosan solutions was measured by monitoring the changes of the solution turbidity (*T*, %) corresponding to different pH (3–11) adjusted by NaOH.[21] A 100 mg of chitosan sample was dissolved in 100 mL of 1% acetic acid, and 10% NaOH was added to increase pH gradually. The *T* (%) of the solutions was measured at 600 nm using UV160US Shimadzu spectrophotometer (Shimadzu, Japan).

2.4. Determination of viscosity-average molecular weight

The molecular weight of hydrolyzed chitosan was determined by using the Ubbelohde Dilution Viscometer (Cannon instrument Co., USA) with the capillary size of 0.58 nm. Approximate 100 mg of chitosan was dissolved in 10 mL of the mixture solution of 0.1 M CH_3COOH and 0.2 M NaCl. The intrinsic viscosity was measured by the intercept between the Huggins (reduced viscosity, η sp/C∼C) and Kraemer (relative viscosity, η rel/C∼C) plots when the concentration was 0.[22] The viscosity-average molecular weight of chitosan was calculated by Mark–Houwink–Sakurada (MHS) equation:

$$[\eta] = K(Mw)^a$$

where *K* and *a* were the constants, $K = 1.81 \times 10^{-3}$ and $a = 0.93$; and $[\eta]$ is the intrinsic viscosity obtained from two plots, Huggins and Kraemer. Coil-overlap concentrations (C^*) was calculated as $1/[\eta]$ in each group of Mw.

2.5. DPPH radical scavenging activity

Chitosan has been known to have different antioxidant mechanisms, such as free radical scavenging ability, chelating ability, and reducing ability,[2,23] thus four different antioxidant measurements including DPPH radical scavenging activity, reducing ability, chelating ability, and hydroxyl radical scavenging activity were conducted. DPPH free radical scavenging activity and hydroxyl radical scavenging activity were selected to evaluate free radical scavenging properties. Hydroxyl radicals are the most reactive among reactive oxygen species. Chitosan can donate electrons and hydrogen to prevent free radical chains from oxidation as it is oxidized, and its ability was determined by the measurement of reducing ability. Chelating ability also related to the lone pair of electron in amino groups as ligands forming chitosan–metal complex.

DPPH radical scavenging activity was measured by following the method of Shimada et al.[24] Each chitosan solution (2–10 mg/mL, 1.5 mL) in 0.2% acetic acid was added into 3 mL of DPPH in methanolic solution (0.09 mg/mL). The mixture was shaken by vortex mixer (Scientific Industries Inc., US) and stored for 30 min in the dark and measured at 517 nm, spectrophotometrically (Shimadzu, Japan). The scavenging ability was calculated as:[19]

Scavenging activity (%) = [(ΔA_{517} of control − ΔA_{517} of sample)/

ΔA_{517} of control] × 100. DPPH methanolic solution with 0.2% acetic acid solution was used as control. EC_{50} value (mg/mL) indicated the concentration showing 50% scavenging activity.

2.6. Reducing power

Chitosan solution (0.2–10 mg/mL, 2.5 mL) in 0.2% acetic acid was added into sodium phosphate buffer (2.5 mL, 200 mM in pH 6.5) with potassium ferricyanide (2.5 mL, 1%). The mixture was incubated at 50 °C for 20 min and then trichloroacetic acid (2.5 mL, 10%) was added. The mixture was centrifuged at 200*g* for 10 min. A 2 mL of supernatant was diluted with 2 mL of distilled water and 0.4 mL of ferric chloride (0.1%) was added. The absorbance was measured at 700 nm spectrophotometerically (Shimadzu, Japan). A higher absorbance indicated higher reducing power. EC_{50} value (mg/mL) was determined in the concentration at which the absorbance was 0.5.[19]

2.7. Chelating ability

Chelating ability on ferrous ions was determined by the method of Dinis et al. (1994).[25] Each chitosan solution (0.2–10 mg/mL, 1 mL) in 0.2% acetic acid was mixed with 3.7 mL of methanol and ferrous chloride (0.1 mL, 2 mM). The mixture was reacted with ferrozine (0.2 mL, 5 mM) for 10 min at room temperature. The absorbance was measured at 562 nm spectrophotometerically. The control was the mixture of reagent with 0.2% acetic acid solution. The chelating ability was calculated as:[19]

Chelating ability (%) = [(ΔA_{562} of control − ΔA_{562} of sample)/ΔA_{562} of control] × 100. EC_{50} value (mg/mL) was determined in the concentration at which chelating activity reached 50% effect.

2.8. Hydroxyl radical scavenging activity

Deoxyribose assay was used for determining hydroxyl radical scavenging activity.[26] Deoxyribose (16.8 mM), $FeCl_3$ (300 mM), EDTA (1.2 mM), H_2O_2 (16.8 mM), KH_2PO_4/KOH buffer (10 mM, pH 7.4), and ascorbic acid (0.6 mM) solutions were prepared, respectively. EDTA/$FeCl_3$ stock solution was made at a ratio of 1:1 (w/w) of EDTA and $FeCl_3$. Chitosan solutions (2–10 mg/mL, 200 μL) were reacted with all prepared solutions mentioned above, but ascorbic acid was added last among all solutions. The mixture was incubated at 37 °C for 60 min. TBA solution (1 mL, 1% in 50 mM NaOH) and TCA solution (1 mL, 2.8%) were added into the mixture and incubated at 80 °C for 20 min. Absorbance was measured at 532 nm in a spectrophotometer (Shimadzu, Japan). Hydroxyl radical scavenging activity was calculated as: [$1 - (A_a - A_b)/A_c$] × 100, where A_a was the absorbance of chitosan solution in deoxyribose assay; A_b was the absorbance of chitosan solution with 1 mL distilled water, 1 mL of TBA, and 1 mL of TCA; and A_c was the absorbance of control (0.2% acetic acid) in deoxyribose assay. EC_{50} value (mg/mL) was determined in the concentration at which hydroxyl radical scavenging activity reached 50%.

2.9. Experimental design and statistical analysis

Two experimental designs were applied in this study. First, a completely randomized factorial design was applied to investigate the effects of three independent factors of FC (α- and β-chitosan), Mw (oligosaccharides, low, med, and high), and CS (2, 4, 6, 8, and 10 mg/mL) on measured antioxidant activity. Three main effects and three two-way interactions between each individual factor were tested in each measurement. PROC GLM was applied to identify significant differences and interaction (P <0.05) among each factor using the SAS program (SAS 9.2, SAS Institute, Inc., USA) and Tukey's Studentized Range (HSD) test was used for the multiple comparisons among treatments within each factor. Secondly, a completely randomized design was employed to investigate the effect of DDA in a range of 86–97% at given Mw of 12–15 kDa. All experiments were repeated.

3. Results

3.1. Solubility

Changes of transmittance (T, %) in different Mw of α- and β-chitosan solutions at the pH range of 3–11 are reported in Figure 1, including a β-chitosan sample with Mw of 15 kDa and 86% DDA for evaluating the possible effect of different DDAs between α-chitosan (88%) and β-chitosan (97%) samples on the measured antioxidant activity. T (%) was not changed at pH 3–11 in both α- and β-chitosan samples with Mw of 4–5 kDa and 22–30 kDa except 22–30 kDa α-chitosan where T (%) decreased when pH was over 9. T (%) of both α- and β-chitosan at Mw of 61–79 kDa and 280–300 kDa was significantly decreased in the alkaline region. The solubility of chitosan decreased with increased Mw in the alkaline region. At 280–300 kDa, T (%) of α-chitosan showed faster decreasing trend than β-chitosan along with increased pH. In comparison of two β-chitosan samples with similar Mw, but different DDA (86% DDA and 15 kDa vs 97% DDA and 22–30 kDa), the one with low DDA exhibited reduced T (%) when pH reached about 9 despite of the lower Mw. This result demonstrated that the solubility of chitosan is decreased by reduced DDA.

3.2. Antioxidant activity

Mw and coli-overlap concentration of α- and β-chitosan samples are shown in Table 1. Mw of four groups of α- and β-chitosan samples were significantly different (P <0.05), while no difference (P >0.05) in Mw among high DDA (97%) of β-chitosan and low DDA of α-chitosan (88%) and β-chitosan (86%) samples. At Mw of 4–5, 22–30, 61–79, and 280–300 kDa, C^* value was 179.86–271.00, 38.40–55.77, 14.72–20.55, and 4.27–6.11 mg/mL, respectively. The long chain of 280–300 kDa chitosan samples could be entangled in CS range of 4–10 mg/mL since C^* of 4.27–6.11 mg/mL was overlapped within the tested CS in this study.

ANOVA results of all antioxidant activity measurements are reported in Table 2. FC, Mw, and CS had significant (P <0.05) effect on the change of reducing power, chelating ability, and hydroxyl radical scavenging activity, but DPPH radical scavenging activity was significantly (P <0.05) affected by only Mw and CS. There was significant two-way interaction between FC and Mw on all measured antioxidant activity except chelating ability, and the interactions between Mw and CS on DPPH radical scavenging activity and chelating ability (P <0.05).

The multiple comparison results among different treatments within each main factor are shown in Table 3. In comparison of FC, reducing ability and hydroxyl radical scavenging activity were significantly higher in β-chitosan samples than that in α-chitosan, whereas α-chitosan showed higher chelating ability. In respect to the effect of Mw, the 22–30 kDa samples were significantly higher in DPPH radical scavenging activity, reducing ability, and hydroxyl radical scavenging activity, whereas 4–5 kDa sample showed the highest chelating ability. For CS, all measured antioxidant activities were increased with increased CS. DPPH radical scavenging activity and chelating ability were significantly increased when CS was >8 mg/mL, while reducing ability and hydroxyl radical scavenging activity was significantly increased when CS was >6 mg/mL (P <0.05).

Half maximal effective concentrations (EC_{50}) are shown in Table 4 along with R^2 values, the coordination between EC_{50} and CS. In

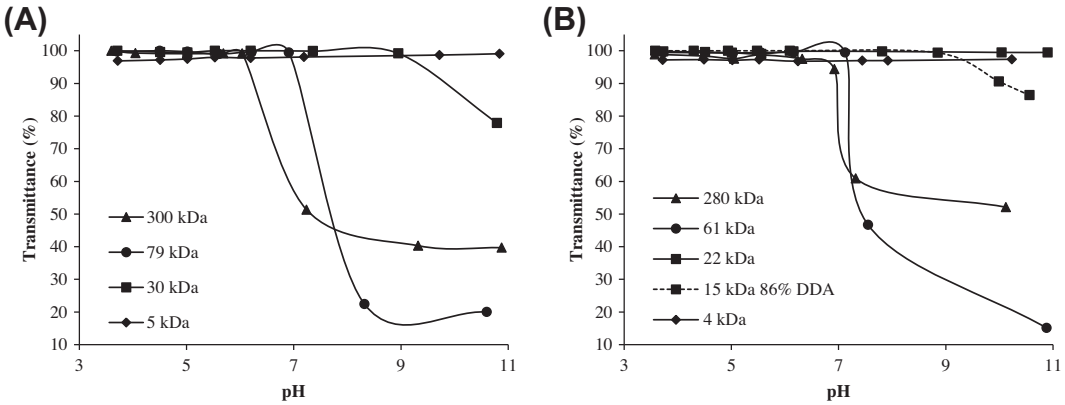

Figure 1. Transmittance (*T*, %) of different molecular weights of α-chitosan (88% DDA) and β-chitosan (97% DDA, plus additional sample with 86% DDA) solutions at pH range of 3–11.

Table 1
Differences in molecular weight (Mw) and coil-overlap concentration (C^*) between α- and β-chitosan samples in two experiments

Experiment	Treatment factors	Mw (kDa)		$^{++}C^*$ (mg/mL)	
		α	β	α	β
A	High Mw	300[a+]	280[a]	4.3[e]	6.1[e]
	Med Mw	79[b]	61[b]	14.7[d]	20.6[d]
	Low Mw	30[c]	22[c]	38.4[c]	55.8[c]
	Oligosaccharides	5[e]	4[e]	226.7[a]	309.1[a]
B	Higher DDA (97%)	—	14[d]	—	81.6[b]
	Lower DDA (86–88%)	12[d]	15[d]	89.3[b]	72.3[b]

[+] Means preceded by the same small letter in the same column within each form of chitosan in same experimental design were not significantly different (P >0.05).
[++] C^* stands for coil-overlap concentrations, and is the lowest concentration of chitosan getting entangled in solutions. It was calculated as $1/[\eta]$.

Table 2
Analysis of variance (ANOVA) table (P = 0.05) for analyzing main treatment effect and interactions among individual factors including the form of chitosan (FC), molecular weight (Mw), and chitosan concentration (CS)

Source of variation	DPPH radical scavenging activity (%)			Reducing power (Abs.)			Chelating ability (%)			Hydroxyl radical scavenging activity (%)		
	df	*F* value	*P* value	df	*F* value	*P* value	df	*F* value	*P* value	df	*F* value	*P* value
Main effects												
The form of chitosan (FC)	1	1.14	0.2915	1	19.23	<.0001	1	6.07	0.0171	1	24.67	<.0001
Molecular weight (Mw)	3	30.75	<.0001	3	30.38	<.0001	3	29.20	<.0001	3	13.47	<.0001
Chitosan concentration (CS)	4	55.43	<.0001	4	22.79	<.0001	4	16.06	<.0001	4	21.08	<.0001
First-order interaction												
FC × Mw	3	14.00	<.0001	3	23.17	<.0001	3	5.83	0.0016	3	9.42	<.0001
Mw × CS	12	4.38	<.0001	12	2.00	0.0434	12	7.06	<.0001	12	1.48	0.1635
FC × CS	4	0.83	0.5103	4	0.62	0.6509	4	1.64	0.1788	4	3.76	0.0093
Model	27	15.30	<.0001	27	11.02	<.0001	27	9.87	<.0001	27	7.79	<.0001
Error	52			52			52			52		
Corrected total	79			79			79			79		

DPPH measurement, EC_{50} was consistently lower in 22–30 kDa samples in both α- and β-chitosan, but extremely higher (46.09 mg/mL) in 280–300 kDa of α-chitosan. R^2 of α-chitosan was relatively lower than that of β-chitosan, demonstrated less dependence on CS. EC_{50} of reducing ability was less than 5 mg/mL at tested Mw range in both α- and β-chitosan except 280–300 kDa of α-chitosan, and R^2 was relatively lower in 22–30 kDa samples in which EC_{50} was less than 2 mg/mL, exhibited higher reducing ability at Mw of 22–30 kDa regardless of CS. In chelating ability, EC_{50} showed negative values in 280–300 kDa samples and 4–5 kDa chitosan had lower EC_{50} which was consistent with ANOVA result. EC_{50} of hydroxyl radical scavenging activity was relatively lower at tested Mw range in both α- and β-chitosan except 4–5 kDa of α-chitosan. Hydroxyl radical scavenging activity and reducing ability was more predominant than chelating ability in comparison of EC_{50} values, but DPPH radical scavenging activity was more dependent on FC and Mw.

The potential DDA effect between the two forms of chitosan was studied at 12–15 kDa chitosan samples (Fig. 2). DPPH radical scavenging activity and reducing ability were not significantly affected by DDA in low Mw chitosan. Hydroxyl radical scavenging activity was increased significantly at higher DDA, whereas chelating ability was decreased along with increased DDA. The 9% difference in DDA between α- and β-chitosan samples showed no effect on DPPH radical scavenging activity and reducing ability.

4. Discussion

The key compounds contributing to the antioxidant activity in chitosan is oxygen and hydrogen from hydroxyl groups, and

Table 3
Multiple comparison using Tukey's Studentized Range (HSD) test among main treatment factors including the form of chitosan (FC), molecular weight (Mw), and chitosan concentrations (CS)

Main factors	Treatments	DPPH free radical scavenging activity (%)	Reducing ability (abs.)	Chelating ability (%)	Hydroxyl radical scavenging activity (%)
The form of chitosan	α-Form	29.21[a+]	0.58[b]	14.16[a]	55.70[b]
	β-Form	27.73[a]	0.75[a]	10.79[b]	68.88[a]
Molecular weight (kDa)	4–5	28.94[b]	0.73[b]	22.04[a]	51.08[c]
	22–30	36.54[a]	0.95[a]	13.72[b]	73.34[a]
	61–79	30.40[b]	0.51[c]	9.49[bc]	66.34[ab]
	280–300	17.98[c]	0.47[c]	4.65[c]	58.09[bc]
Chitosan concentrations (mg/ml)	2	12.87[d]	0.34[c]	3.94[c]	41.25[c]
	4	21.44[c]	0.57[b]	9.07[bc]	56.25[b]
	6	29.67[b]	0.72[ab]	13.83[ab]	68.56[a]
	8	36.52[a]	0.80[a]	15.84[a]	70.78[a]
	10	41.85[a]	0.89[a]	19.70[a]	74.61[a]

[+] Means preceded by the same letter in the same column within each factor were not significantly different (P >0.05).

Table 4
Half maximal effective concentrations (EC_{50}, mg/mL) of all antioxidant activity measurements in α- and β-chitosan at different Mw

Mw (kDa)	DPPH radical scavenging activity (mg/mL)[+]				Reducing ability (mg/mL)[++]				Chelating ability (mg/mL)[+]				Hydroxyl radical scavenging activity (mg/mL)[+]			
	α	R^{2+++}	β	R^2	α	R^2	β	R^2	α	R^2	β	R^2	α	R^2	β	R^2
4–5	30.54	0.56	10.66	0.93	1.73	0.99	2.14	0.99	9.75	0.97	7.11	0.86	12.23	0.9	<2	0.36
22–30	7.78	0.73	4.93	0.99	<2	0.17	<2	0.57	44.69	0.31	28.29	0.19	3.52	0.98	<2	0.76
61–79	8.23	0.9	5.85	0.99	2.52	0.91	4.55	0.92	21.71	0.87	17.38	0.95	4.91	0.94	<2	0.18
280–300	46.09	0.79	7.32	0.99	28.65	0.53	<2	0.96	ND[*]	—	ND	—	3.38	0.53	2.61	0.69

[+] EC_{50} value (mg/mL) indicated the concentration showing 50% scavenging activity, compared to the control.
[++] EC_{50} value (mg/mL) was determined in the concentration at which the absorbance was 0.5.
[+++] R^2 values were obtained by interpolation from linear regression analysis and can be interpreted with the dependence of CS in each form of chitosan at a wide range of Mw.
[*] ND indicated that EC_{50} was negative value.

nitrogen and hydrogen from positively charged amino groups. Hydrogen or the lone pair of electrons can scavenge free radicals, and the lone pair of electron in oxygen and nitrogen chelates metal ions, forming chitosan–metal ion complex since those functional groups act as ligands.[14,27–29] FC, Mw, and CS were considered as main factors affecting antioxidant activity and the effect of DDA was studied in low Mw (12–15 kDa). Also, each antioxidant property had shown different results depending on the measurements related to the antioxidant mechanisms.

4.1. Solubility

Mw of chitosan was a significant factor affecting solubility. Kubota et al. (2000) reported that intermolecular attraction force was lowered with decrease of Mw.[30] Similarly, lower Mw chitosan remained high T (%) over a wide pH range.[31] The altered solubility between two different forms of chitosan can be related to their different crystal structures, crystallinity, and crystal imperfection, as Cho et al. (2000) reported that the solubility has a close relationship to polymeric structure.[32] However, the exact reasons why different form of chitosan showed different solubility can be more complicated since the solubility is highly related to Mw and DDA as well.

4.2. The effect of different forms of chitosan

Different polymeric structures between α and β-chitin might alter the antioxidant activity of the two different forms (α and β) of chitosan. Lima et al. (2004) reported that crystallinity index (CI) of α-chitin is higher than β-chitin. In the process of deacetylation, CI of chitosan derived from α-chitin was slightly changed, whereas CI of chitosan from β-chitin was significantly decreased.[33] Therefore, altered crystallinity of chitosan after deacetylation between the two forms of chitosan might be one of the reasons why β-chitosan had significantly higher reducing ability and hydroxyl radical scavenging activity at high Mw. At Mw of 280–300 kDa, α-chitosan could have more rigid and stiff structure due to the covalent bonds or interactive force (Van der waals)[1] with higher crystallinity than β-chitosan, thus resulting in highest EC_{50} in DPPH radical scavenging activity and reducing ability. The dissociation energy of O–H and N–H can be increased along with higher crystallinity and Mw, thus α-chitosan can be more difficult to be dissociated than β-chitosan. Also, β-chitosan could have more available functional groups free from crystalline polymeric structure with hydrogen bonds at high Mw. Chelating ability was not exhibited at 280–300 kDa chitosan, showing no EC_{50} detected.

4.3. The effect of Mw

Increased DPPH radical scavenging activity and reducing ability in 22–30 kDa samples may be due to the collapse of crystalline region by decrease in Mw, resulting in increase of solubility and reactivity in solution. It was reported that depolymerization is associated to decrease in CI.[15] With decrease of intermolecular interaction (Van der Waals) and hydrogen bonds in low Mw, the lone pair of electrons or hydrogen compounds from C–O and \N–H may be more available. Feng et al. (2007) found that low Mw chitosan has higher solubility due to the low van der Waals forces.[31] Similarly, Kim & Thomas (2007) reported the highest DPPH radical scavenging activity in 30 kDa chitosan due to the increase in polymer mobility with lower Mw. According to Chen et al. (2003), the number of amino groups in chitooligosaccharides should be more than two for providing antioxidant activity in hydrolyzed chitosan.[31] Low Mw chitosan showed higher antioxidant activity than that of oligosaccharides in this study, which might be related to the number of available amino groups. Oligosaccharide chitosan might not provide enough amino groups to exert similar antioxidant activity as lower Mw chitosan. Also,

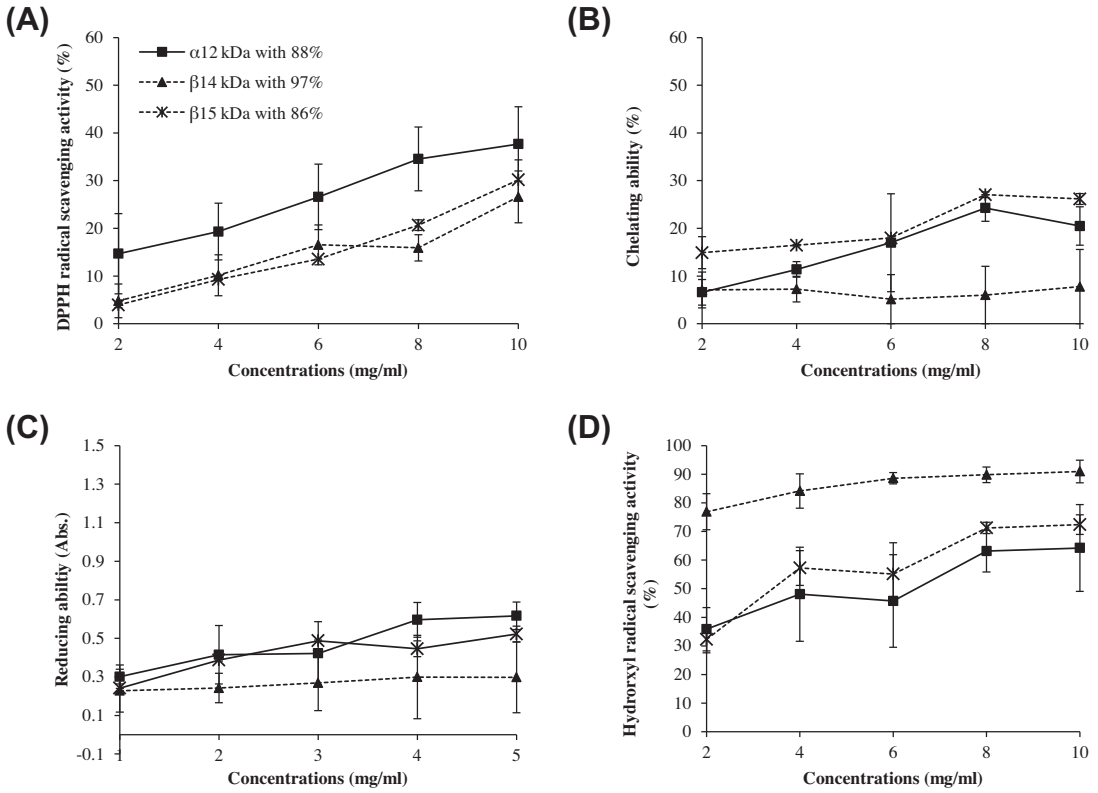

Figure 2. Effect of DDA on measured antioxidant activity of α- and β-chitosan at low Mw of 20–30 kDa.

hydroxyl or amino groups could be destroyed from further depolymerization from lower Mw samples. Therefore, antioxidant activity was overall increased as Mw decreased, but should be higher than about 20 kDa to provide enough amino groups existed in depolymerized chitosan. Chelating ability was shown higher activity in lower Mw than other measurements. Similarly, low Mw chitosan could easily form chitosan–Fe^2 complexes and low Mw polysaccharide radicals from more lone pairs of electrons.[23,34] Significant interaction effect between Mw and CS can be explained by different C^* depending on Mw. C^* was increased with reduced Mw. As CS is higher than C^* in 280–300 kDa samples, chitosan polymer can be entangled in solutions, thus decreasing antioxidant activity due to the less susceptibility from intermolecular interactions. CS had no relationship with Mw in hydroxyl radical scavenging activity since R^2 showing CS dependence was relatively lower than other measurements.

4.4. Other effects

CS significantly interacted with FC in only hydroxyl radical scavenging activity (ANOVA), as shown by significantly lower R^2 in EC_{50} of β-chitosan than that of α-chitosan (Table 4). Unlike other measurements, EC_{50} of hydroxyl radical scavenging activity in β-chitosan was poorly coordinated with CS and less dependent on Mw (Table 4). As shown by the multiple comparison results in Table 3, chitosan samples at CS of 10 mg/mL exhibited significantly higher antioxidant activity in all measurements. However, it may be inappropriate to conclude in this way since there was possible interaction between CS and Mw at a certain range of CS, in which long chain of chitosan at high Mw could be entangled, thus inhibiting antioxidant activity in CS overlapped with C^*. R^2 in EC_{50} of hydroxyl radical scavenging activity was relatively lower in high Mw of α-chitosan (Table 4), demonstrating the interactions among FC, Mw, and CS. Therefore, to maximize the antioxidant ability of high Mw chitosan, CS has to be lower than C^*.

DDA has been considered as a critical factor impacting the antioxidant activity of chitosan.[11,18,19] Antioxidant property could be increased by increasing DDA since the amino group can be more available at high DDA. Due to the initial DDA difference between α- and β-chitosan samples in this study, potential impact of DDA on the antioxidant activity was studied in low Mw (12–15 kDa) chitosan samples. Results showed that hydroxyl radical scavenging activity was dependent on DDA. Je et al. (2004) also reported increased radical scavenging activity with increased DDA among nine hetero-oligosaccharides.[11] However, DPPH radical scavenging was not affected by DDA, and it can be assumed that hydroxyl radical scavenging activity of chitosan is more closely related to the amount of amino groups than DPPH radical scavenging. On the other hand, chelating ability was decreased with increased DDA. Chitosan metal interacting characteristics could be related to the distribution of acetyl groups as well as DDA and Mw.[4] At reduced DDA, the distribution characteristics of acetyl groups could become a significant factor impacting the metal chelating of chitosan. In the future studies, the effect of DDA in high Mw of chitosan samples should be studied since DDA may have different effect on antioxidant activity at high Mw with high crystallinity in terms of polymeric structure. Related to this hypothesis, Trung et al. (2006) found that the crystallinity of chitosan at high Mw (810 kDa) can be increased with increased DDA.

EC_{50} was relatively lower in reducing ability and hydroxyl radical scavenging activity, meaning more predominant than DPPH radical scavenging activity and chelating ability in the action of antioxidant activity of chitosan. Though overall EC_{50} of chelating ability was relatively higher than other measurements, EC_{50} of 4–5 kDa chitosan was lower than DPPH radical scavenging activity, similar to the result by Feng et al. (2007) that the highest chelating ability was observed at the lowest Mw of 1.7 kDa among samples tested at Mw of 1.7–281 kDa.[31] This result indicated antioxidant mechanisms can be exerted differently in chitosan depending on Mw.

5. Conclusion

This study demonstrated the higher reducing ability and hydroxyl radical scavenging activity in β-chitosan than those in α-chitosan at high Mw. DPPH radical scavenging activity, reducing ability, and hydroxyl radical scavenging activity were higher in 22–30 kDa α- and β-chitosan samples, but chelating ability was the highest in 4–5 kDa chitosan. There was no significant difference between α- and β-chitosan in DPPH radical scavenging, but EC_{50} of β-chitosan was extremely lower than that of α-chitosan at Mw of 280–300 kDa. Increasing CS generally enhanced the antioxidant activity, but has to be interpreted by the interaction effect with Mw and FC. Mw of 280–300 kDa chitosan samples at CS of 4–10 mg/mL can be entangled in solutions, thus lowering antioxidant activities from intermolecular interactions. Increase of DDA enhanced hydroxyl radical scavenging activity, but decreased chelating ability in low Mw (12–15 kDa) chitosan. Hydroxyl radical scavenging activity might have a close relationship with the amount of amino groups (DDA) than DPPH radical scavenging in low Mw based on DDA dependence in hydroxyl radical scavenging activity. Reducing ability and hydroxyl radical scavenging activity were more predominant as shown by the lower EC_{50} than others. In the future study, the difference of the polymeric structures in the two forms of chitosan prepared with a wide range of Mw and DDA should be investigated by using FTIR or X-ray diffraction. Also, DDA effect on the antioxidant activity will be studied in high Mw chitosan since DDA can affect crystallinity at high Mw.

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