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Biochemical activities of low molecular weight chitosans derived from squid pens

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ABSTRACT

Chitosans were prepared by H_2O_2 oxidative depolymerization from squid pens with low molecular weights (LMW) of 13,025, 7011, 4169, 2242 and 963 Da. The bile acid binding capacities and antioxidant properties of LMW chitosans were studied *in vitro*. LMW chitosans exhibited stronger bile acid binding capacities than that of chitosan. The scavenging ability of LMW chitosans against DPPH radicals improved with increasing concentration, and EC_{50} values were below 1.3 mg/mL. The EC_{50} values of LMW chitosans against hydroxyl radicals ranged from 0.93 to 3.66 mg/mL. All LMW chitosans exhibited a strong ferrous ion chelating effect and reducing power. At 1 mg/mL, the scavenging ability of chitosan-963 towards superoxide radicals was 67.76%. These results indicated that LMW chitosans which have stronger bile acid binding capacity and antioxidant activities may act as potential antioxidants *in vitro*.

1. Introduction

Chitosan, a nontoxic copolymer consisting of β-(1,4)-2-amino-2-deoxy-D-glucose units (Muzzarelli, 1988), is a natural cationic polysaccharide made from alkaline *N*-deacetylation of chitin. α-Chitin, has a structure of antiparallel chains and is extracted from shrimp or crab shells, whereas β-chitin, found in squid or loligo pens, has parallel chains joined through intrasheet hydrogen bonding (Minke & Blackwell, 1978). β-Chitin is characterized by weak intermolecular forces (Rudall, 1963) and has been confirmed to exhibit higher reactivity under various modification conditions as well as higher affinity for solvents than α-chitin (Kurita et al., 1993). These results indicate that chitosan derived from β-chitin may have potential as a novel functional biopolymer. Because chitosan possesses many beneficially biological properties such as antioxidant activity, biocompatibility, biodegradability (Muzzarelli et al., 2007; Zhao, Huang, Hu, Mao, & Mei, 2011), hemostatic activity and wound healing properties (Muzzarelli, 2009), much attention has been paid to its biomedical (Muzzarelli et al., 2000) and pharmaceutical applications (Muzzarelli, 2010; Muzzarelli & Muzzarelli, 2002). However, because of its high molecular weight and water-insolubility, the applications of chitosan are severely limited. Therefore, its potential for chemical modification and depolymerization to obtain water-soluble derivatives over a wide pH range has been investigated (Peng, Han, Liu, & Xu, 2005). Compared with ordinary chitosan, LMW chitosans have improved water-solubility and some special biological functions such as immuno-enhancing effects, antitumor and antifungal activities (Hong, Na, Shin, & Samuel, 2002). Chitosan with an average molecular weight in the range of 5000–10,000 Da possesses strong bactericidal and superior biological activities (Kittur, Vishu Kumar, & Tharanathan, 2003). Chitosan with a molecular weight of 20 kDa prevents progression of diabetes mellitus and exhibits higher affinity for lipopolysaccharides than 140 kDa chitosan (Kondo, Nakatani, Hayashi, & Ito, 2000). So it is of increasing interest to depolymerize chitosan into low molecular weight fragments under appropriate conditions to study the properties of the smaller types.

Antioxidants can reduce oxidative damage that is caused by ROS (reactive oxygen species). ROS in the forms of superoxide anion, hydroxyl radical and hydrogen peroxide are produced by sunlight, ultraviolet light, ionizing radiation, chemical reactions and metabolic processes which have a wide variety of pathological effects, such as cancer, cardiovascular diseases, diabetes and atherosclerosis (Willett, 1994). Antioxidants exert their effects by scavenging ROS or preventing the generation of ROS. Recently, the antioxidant activity of chitosan and its derivatives has been investigated due to their natural abundance and biological properties (Castagnino et al., 2008; Zhao et al., 2011). However, few studies on the biochemical activities of LMW chitosans prepared from squid

Abbreviations: LMW, low molecular weight; ROS, reactive oxygen species; EDTA, ethylenediaminetetraacetic acid; NBT, nitro blue tetrazolium; PMS, phenazine methosulfate; NADH, nicotinamide adenine dinucleotide-reduced; DPPH, 1,1-diphenyl-2-picrylhydrazyl.

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Table 1
Reaction conditions, molecular weight (M_w), yield and bile acid binding capacity of chitosan and LMW chitosans.

Sample	H_2O_2	Time (h)	Temperature (°C)	M_w (Da)	Yield (%)	Bile acid binding (mg/g)
Chitosan	–	–	–	6.5×10^5	–	14.02
COS-13025	3%	2	45	13,025	77.78	20.47
COS-7011	6%	3	45	7011	59.7	29.25
COS-4169	9%	4	45	4169	38.65	38.67
COS-2242	3%	4	65	2242	49.85	54.51
COS-963	6%	5	65	963	57.1	63.53

cartilage are available. In order to investigate the effect of molecular size on antioxidant activity and bile acid binding capacity of chitosan, five LMW chitosans were prepared by H_2O_2 oxidation from squid pens. Antioxidant activity was investigated by scavenging DPPH, hydroxyl and superoxide anion radicals. As well, their reducing power, chelating ability against ferrous ions and *in vitro* bile acid binding capacity were evaluated.

2. Materials and methods

2.1. Chemicals

Squid pens were provided by Hangzhou Baokai Biochemical Co., Ltd. Bile acid (derived from taurocholate), furfural, 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide (H_2O_2), potassium ferricyanide ($K_3Fe(CN)_6$), trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), ferrozine, ethylenediaminetetraacetic acid (EDTA), ascorbic acid, nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), nicotinamide adenine dinucleotide-reduced (NADH), and ferrous sulfate ($FeSO_4$) were purchased from Aladdin-reagent Co., Ltd. (Shanghai, China). All other chemicals were analytical grade and used without further purification. All water used in extraction and analysis had been distilled and deionized.

2.2. Preparation of β-chitin from squid pens

Squid pens were pulverized and ground to a powder with a cutting mill. The squid pens were submitted to the usual sequence of treatments used for β-chitin extraction: demineralization and deproteinization. β-Chitin was extracted from squid pen as follows: HCl concentration was set to 6%, solid to liquid ratio (w/v) 1:14, and demineralization treatment time was 2 h; NaOH concentration was 10%, solid to liquid ratio (w/v) 1:10, treatment temperature was 100 °C, and deproteinization time was 1 h. The product obtained was filtered under reduced pressure, washed with deionized water, dried under vacuum at 60 °C for 12 h. The β-chitin recovery from squid pens was about 24%.

2.3. Preparation of chitosan

With the assistance of ultrasonics (25 kHz), a total of 20.0 g of β-chitin was suspended in 200 mL of 50% sodium hydroxide, and the system was subjected to a continuous reaction for 2 h at 80 °C. The reaction product was filtered immediately, and then washed thoroughly with water to neutral. Chitosan was collected by filtration followed by drying under vacuum at 60 °C for 12 h prior to analysis. Chitosan with a degree of deacetylation of more than 90% was prepared from squid pens with a viscosity average-molecular weight of 6.5×10^5 Da. The chitosan yield from β-chitin was approximately 70%.

2.4. Preparation of LMW chitosans

The preparation of LMW chitosans was carried out using a modification of the method of Sun, Zhou, Xie, and Mao (2007). According to Table 1, chitosan (4.0 g) was suspended in different volumes of distilled water. After equilibrating in a water bath with different temperatures, 30% H_2O_2 solution was added into the mixture and was ultrasonicated for 2–5 h. Then the mixture was adjusted to pH 10.0, filtered, and the water insoluble parts were decanted. The filtrate was subjected to precipitation by adding five amounts of ethanol, then filtered and the obtained filtrate was dried to obtain the LMW chitosans.

2.5. Determination of the molecular weight of LMW chitosans

The average molecular weights (M_w) of the LMW chitosans were determined using the viscosity method as previously described (Chandumpai, Singhpibulporn, Faroongsarng, & Sornprasit, 2004; Sun et al., 2007). The samples were dissolved in 0.1 M sodium acetate/0.2 M acetic acid, filtered through a sinter glass, and the viscosity of the solution was measured using an Ubbelohde capillary viscometer at 25 ± 1 °C. Then, M_w was calculated from the Mark–Houwink–Sakurada equation:

$$[\eta] = K_m M_w^{\alpha}$$

where $[\eta]$ is intrinsic viscosity, K_m and α are viscometric constants and their values are 6.589×10^{-3} and 0.88, respectively (Wang, Bo, & Qin, 1990).

2.6. FT/IR spectroscopy

Fourier transform infrared (FTIR) spectra of LMW chitosans were obtained using a Nicolet FTIR spectrometer (Magna-IR 760 ESP, Nicolet Instrument Corp., Madison, WI).

2.7. Bile acid binding assay

Using the method of Muzzarelli et al. (2006) with minor modifications, the bile acid binding capacity of LMW chitosans was investigated *in vitro*. Sample (0.05 g) was mixed with 2 mL of 5 mg/mL bile acid, and the mixtures were adjusted to a total volume of 25 mL with distilled water. The mixtures were incubated for 2 h at 37 °C, and then filtered. The resulting samples (1.0 mL) were mixed with 1 mL 1% (w/v) furfural and 45% (v/v) sulfuric acid, and then the mixtures were incubated for 20 min at 70 °C, and the absorbance was measured at 605 nm.

2.8. DPPH radical scavenging ability

The scavenging effect of LMW chitosans on DPPH radicals was measured using a modification of the method of Yamaguchi, Takamura, Matoba, and Terao (1998). A total of 2.5 mL of the ethanol solution of DPPH (50 mg/L) was incubated with 2.5 mL LMW chitosan samples at different concentrations (0.4–2.0 mg/mL). The reaction mixture was shaken thoroughly and incubated for 30 min at 33 °C, and the absorbance was measured at 517 nm against a blank. Ascorbic acid was used as the reference standard. The EC_{50} value (mg/mL) is the effective concentration at which DPPH

radicals were scavenged by 50%. The radical scavenging activity was calculated using the following equation:

$$\text{Scavenging effect}\ (\%) = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100$$

where A_{control} is the absorbance of the control (distilled water instead of LMW chitosan).

2.9. Hydroxyl radical scavenging ability

The scavenging ability of LMW chitosans towards hydroxyl radicals was determined according to the method of Lee et al. (2005) with minor modifications. The reaction mixture contained $FeSO_4$ (5 mM), salicylic acid (5 mM), LMW chitosan samples at different concentrations (1–5 mg/mL) and H_2O_2 (20 mM). The reaction mixture was shaken thoroughly and incubated for 1 h at 37 °C. The absorbance of the mixtures was measured at 510 nm against a blank. Ascorbic acid was used for comparison. The EC_{50} value (mg/mL) is the effective concentration at which hydroxyl radicals were scavenged by 50%.

2.10. Measurement of reducing power

The reducing power of LMW chitosans was measured by the method of Oyaizu (1986). The reaction mixture contained different concentrations of LMW chitosan samples (2.5 mL), 0.2 mol/L 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, and then centrifuged at 4000 rpm for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and 0.1% (w/v) ferric chloride solution (0.5 mL). The absorbance values of the reaction mixtures were determined at 700 nm. The absorption indicated the intensity of the reducing ability, and increased absorbance of the reaction mixture indicated increased reducing power.

2.11. Metal ion chelating assay

Ferrous ions are the most effective pro-oxidants (Yamaguchi, Tatsumi, Kato, & Yoshimitsu, 1988) and they are commonly found in food systems where they can initiate lipid peroxidation and start a chain reaction that leads to the deterioration of food (Lin, Wei, & Chou, 2006). The ferrous chelating ability of LMW chitosans was determined according to the method of Carter (1971) with minor modifications. Each sample (2-10 mg/mL) was mixed with 9.25 mL of methanol, 0.25 mL of 1.51 mM $FeSO_4$ and 0.5 mL of 2.4 mM ferrozine. After 20 min at 37 °C, the absorbance of the mixtures was determined at 562 nm against a blank. A lower absorbance indicates a higher chelating ability. EDTA was used for comparison. The ability of chitosan oligosaccharides to chelate ferrous ion was calculated using the following equation:

$$\text{Chelating ability}\ (\%) = \left(1 - \frac{A_1}{A_0}\right) \times 100$$

where A_0 and A_1 were the optical density at 562 nm without and with samples, respectively.

2.12. Superoxide radical scavenging assay

The superoxide scavenging ability of LMW chitosans was assessed by the method of Li, Zheng, Liu, and Jia (1992) with some modifications. Each LMW chitosan sample (1–5 mg/mL) was mixed separately with PMS (163 μM), NADH (471.6 μM), and NBT (342.5 μM) in 0.05 M Tris–HCl buffer (pH 8.0). After 5 min at room temperature, the absorbance was measured at 560 nm against a blank and the scavenging ability against superoxide radical was calculated. The EC_{50} value (mg/mL) is the effective concentration at which superoxide radicals were scavenged by 50%.

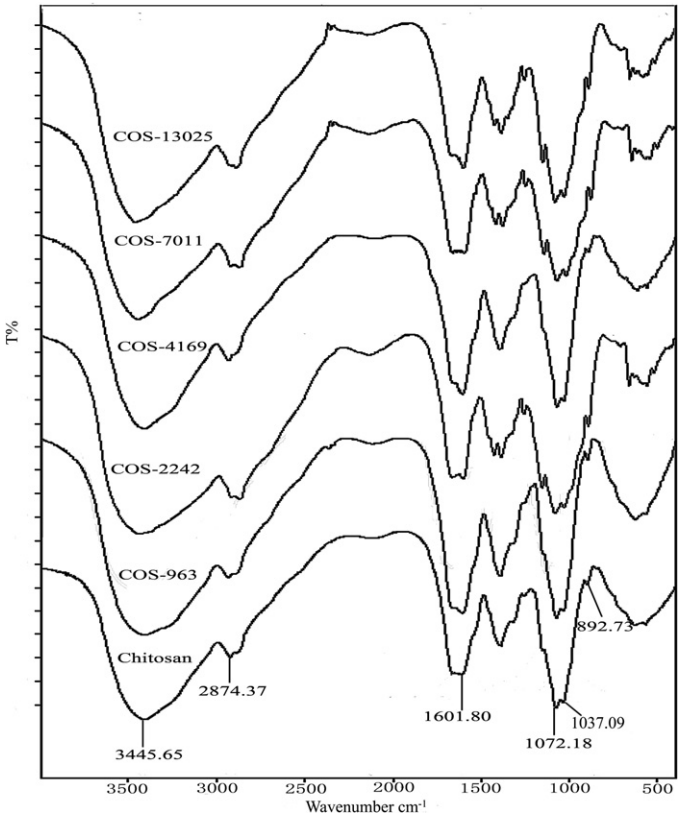

Fig. 1. IR spectra of LMW chitosans and native chitosan. Molecular weights of COS-13025, COS-7011, COS-4169, COS-2242 and COS-963 were 13025, 7011, 4169, 2242, and 963 Da, respectively.

2.13. Statistical analysis

All of the analyses were performed in triplicate. Each experimental data point represents the mean from three independent experiments. The derivation from the mean at the 95% significance level was used to determine the differences in biological activity.

3. Results and discussion

3.1. Infrared spectra analyses

Infrared spectroscopy has been used to determine the structure of chitosan and chitin (Muzzarelli, 1988). Fig. 1 displays the FT-IR spectrum of LMW chitosans and initial chitosan. As shown in Fig. 1, the main absorption bands of initial chitosan were 3445.65 cm^{-1} (O–H stretch), 2874.37 cm^{-1} (C–H stretch), 1601.80 cm^{-1} (N–H bend), 1156.09 cm^{-1} (bridge O stretch) and 1072.18 cm^{-1} (C–O stretch). The peak of chitosan at 892.73 cm^{-1} belonging to the pyranose ring group was identified. Compared with initial chitosan, the basic structural unit of LMW chitosans after depolymerization was not changed, and had the characteristic absorption peaks of the saccharide unit at 1072.18 and 892.73 cm^{-1}.

3.2. In vitro bile acid binding capacity

Bile acids are steroid carboxylic acids synthesized in liver from cholesterol, and the primary bile acids are cholic and chenodeoxycholic acids. Binding of bile acids and subsequent excretion in feces has been recognized as a significant mechanism to

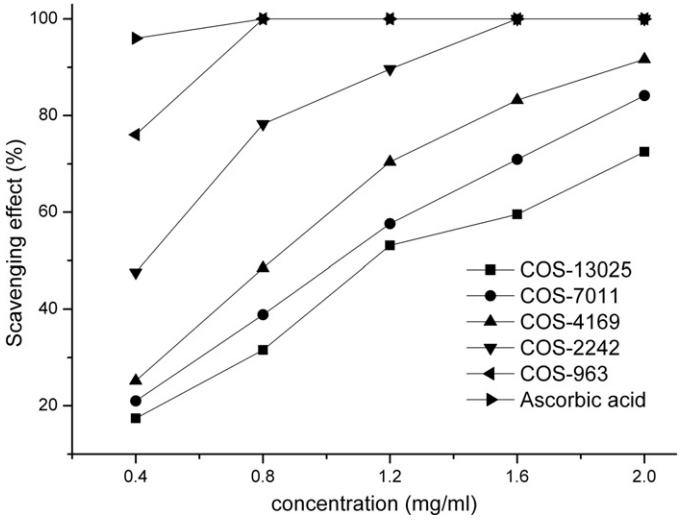

Fig. 2. Scavenging effects of LMW chitosans towards DPPH radicals. Molecular weights of COS-13025, COS-7011, COS-4169, COS-2242, and COS-963 were 13025, 7011, 4169, 2242, and 963 Da, respectively.

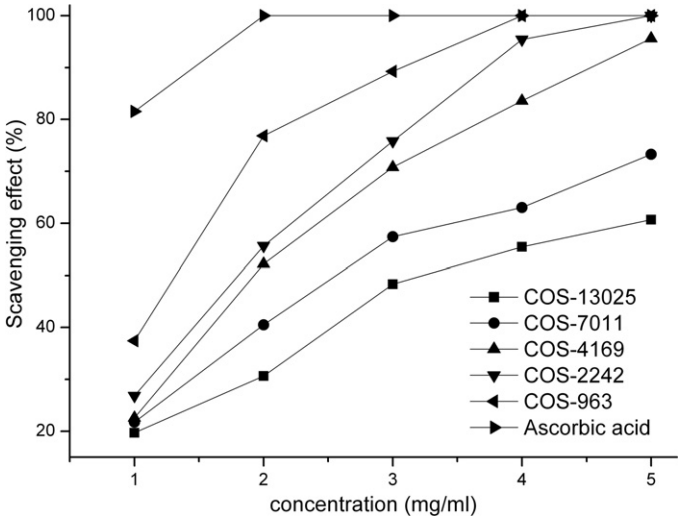

Fig. 3. Scavenging effects of LMW chitosans towards hydroxyl radicals. Molecular weights of COS-13025, COS-7011, COS-4169, COS-2242, and COS-963 were 13025, 7011, 4169, 2242, and 963 Da, respectively.

eliminate excess cholesterol (Muzzarelli, 1996; Parvathy, Susheelamma, Tharanathan, & Gaonkar, 2005). Therefore, the high binding capacities of bile acids suggest a possible ability to lower cholesterol in the body.

The *in vitro* bile acid binding capacities of chitosan and LMW chitosans are shown in Table 1. LMW chitosans had significantly higher bile acid binding capacities than that of initial chitosan, implying they had more cholesterol-lowering effects. The bile acid binding capacities of COS-13025, COS-7011, COS-4169, COS-2242, and COS-963 were 20.47 mg/g, 29.25 mg/g, 38.67 mg/g, 54.51 mg/g, and 63.53 mg/g, which were 1.46-fold, 2.09-fold, 2.76-fold, 3.89-fold, and 4.53-fold higher than that of chitosan, respectively.

It is reported that water-soluble dietary fibers are more effective in lowering cholesterol levels than water-insoluble dietary fibers (Brown, Rosner, Willett, & Sacks, 1999). Thus, water solubility appears to be involved in biological activities such as bile acid binding capacity. Furthermore, a few previous studies indicated that amination of chitosan could increase its bile acid binding capacity possibly due to introduction of cationic groups into the polysaccharide molecules (Shin, Lee, Lee, & Lee, 2005). The strong intramolecular and intermolecular hydrogen bonds in chitosan inhibit its reactivity with hydroxyl and amino groups. After depolymerization by H_2O_2, the inner structure of chitosan becomes severely disrupted and its ability to form hydrogen bonds declines sharply, hence activating the amino and hydroxyl groups. Compared with LMW chitosans, high-molecular weight chitosans have compact structures and the effect of their intramolecular hydrogen bonds is stronger. As the LMW chitosan molecular weight decreases, the more amino and hydroxyl groups are activated. The amino group activation of chitosan contributes to improving its bile acid binding capacity.

3.3. Scavenging effect on DPPH radicals

DPPH scavenging effects of various LMW chitosans at different concentrations are shown in Fig. 2. The scavenging ability of LMW chitosans against DPPH radicals increased with their increasing concentrations, and EC_{50} values of COS-13025, COS-7011, COS-4169, and COS-2242 were 1.29, 1.09, 0.87, and 0.31 mg/mL, respectively. At 0.4 mg/mL, the scavenging abilities of COS-963 and ascorbic acid towards DPPH radicals were 76.04% and 96.01%. The scavenging ability of LMW chitosans on DPPH was weaker than ascorbic acid. Compared with ascorbic acid, LMW chitosans were not effective scavengers for DPPH radicals.

DPPH possesses a proton free radical with a characteristic absorption which decreases significantly on exposure to proton radical scavengers (Yamaguchi et al., 1998). Furthermore, it is well accepted that DPPH free radical scavenging by antioxidants is due to their hydrogen-donating ability (Chen & Ho, 1995). However, the mechanism of how LMW chitosans scavenge DPPH radicals needs to be studied further.

3.4. Hydroxyl radical scavenging activity

The hydroxyl radicals generated by the Fenton reaction were scavenged by chitosan and LMW chitosans *in vitro*. Fig. 3 shows the hydroxyl radical scavenging activity of LMW chitosans at various concentrations. All LMW chitosans exhibited effective scavenging activities against hydroxyl radicals. EC_{50} values of COS-13025, COS-7011, COS-4169, COS-2242, and COS-963 towards hydroxyl radical were 3.66, 2.91, 2.16, 1.89, and 0.93 mg/mL, respectively. At 1 mg/mL, the scavenging ability towards ascorbic acid reached 81.53%. As shown in Fig. 3, LMW chitosans exhibit stronger hydroxyl radical scavenging activity when its molecular weight decreased.

Hydroxyl radicals are one of the most reactive free-radicals and can be formed from superoxide anion and hydrogen peroxide in the presence of metal ions such as copper or iron. Hydroxyl radical scavenging activity of LMW chitosans 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). Furthermore, LMW chitosans have short chains and thus their ability to form intramolecular hydroxyl bonds declines sharply, that is, the hydroxyl and amino groups remain activated and this would be helpful to the radical scavenging process (Sun et al., 2007).

3.5. Reducing power

Fig. 4 depicts the reducing power of different molecular weight chitosans using the potassium ferricyanide reduction method. The reducing power of all sizes of chitosans correlated well with increasing concentrations. As shown in Fig. 4, the reducing power of LMW chitosans increased with increasing concentrations.

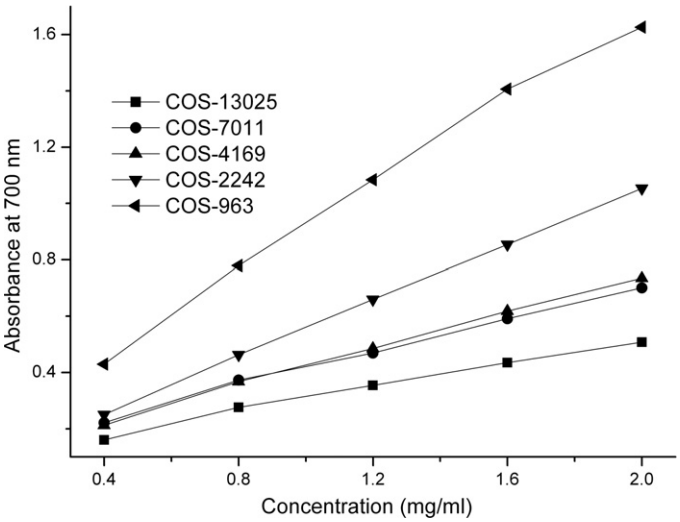

Fig. 4. Reducing power of LMW chitosans with different molecular weights. Molecular weights of COS-13025, COS-7011, COS-4169, COS-2242, and COS-963 were 13025, 7011, 4169, 2242, and 963 Da, respectively.

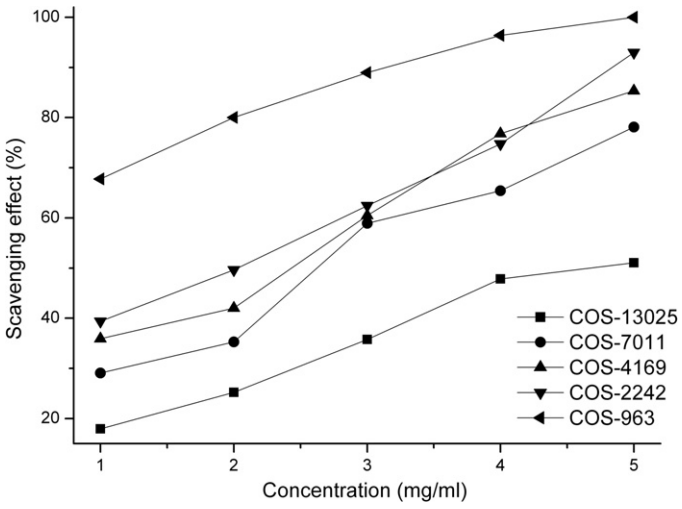

Fig. 6. Scavenging effects of LMW chitosans towards superoxide radicals. Molecular weights of COS-13025, COS-7011, COS-4169, COS-2242, and COS-963 were 13025, 7011, 4169, 2242, and 963 Da, respectively.

Moreover, at 2 mg/mL, the reducing power of COS-13025, COS-7011, COS-4169, COS-2242, and COS-963 were 0.508, 0.701, 0.735, 1.053, and 1.626, respectively. The reducing properties are generally associated with the presence of reductones (Duh, 1998), which have been shown to exert antioxidant action by breaking the free radical chain through donation of a hydrogen atom (Shimada, Fujikawa, Yahara, & Nakamura, 1992). The reducing power of LMW chitosans suggested that it was likely to contribute significantly towards the observed antioxidant effect.

3.6. Chelating effects on ferrous ions

Ferrous ion chelating effects of the LMW chitosans were determined by measuring the decrease in the absorbance at 562 nm of the iron (α)-ferrozine complex. The chelating effects on ferrous ions of LMW chitosans are shown in Fig. 5. The chelating effects increased with increasing concentrations and LMW chitosans exhibited high chelating ability. When the concentration of LMW chitosan was 6 mg/mL, the chelating effects of COS-13025, COS-7011, COS-4169, COS-2242, and COS-963 were 43.73%, 80.42%, 88.9%, 90.72%, and 100%, respectively. At 2 mg/mL, the chelating effect of EDTA on ferrous ions was 100% and was not concentration dependent in the range of tested concentrations. The most effective pro-oxidants present in food systems are ferrous ions (Yamaguchi et al., 1988). The high ferrous ion-chelating ability of LMW chitosans may be beneficial if they were formulated into foods.

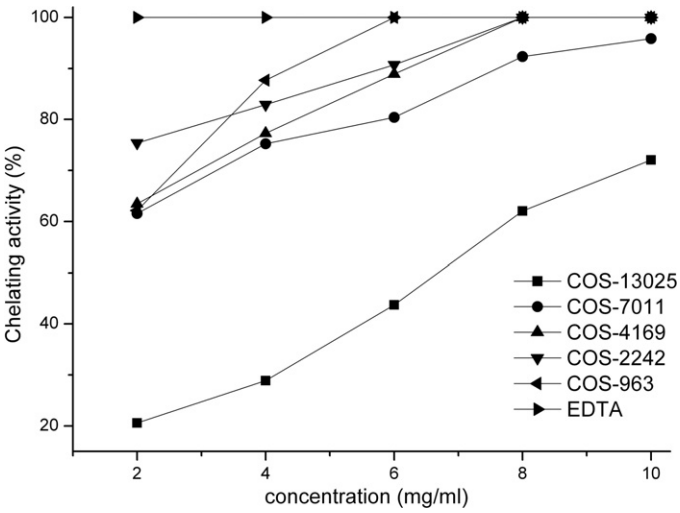

Fig. 5. Chelating effects of LMW chitosans on ferrous ions. Molecular weights of COS-13025, COS-7011, COS-4169, COS-2242, and COS-963 were 13025, 7011, 4169, 2242, and 963 Da, respectively.

3.7. Superoxide radical scavenging activity

Superoxide anion is a reduced form of molecular oxygen created by the addition of one electron. Superoxide anion is known to be very harmful to cellular components as a precursor of more reactive oxidative species, such as single oxygen and hydroxyl radicals. Compared with other oxygen radicals, superoxide anion has a longer lifetime, and thus has the potential to do more harm. Therefore, it is very important to study the scavenging of superoxide anion (Sun, Yao, Zhou, & Mao, 2008). Fig. 6 shows the superoxide anion scavenging activity of LMW chitosans at different concentrations. The EC_{50} values of COS-13025, COS-7011, COS-4169, and COS-2242 were 4.62, 2.74, 2.24, and 1.95 mg/mL, respectively. At 1 mg/mL, the scavenging activity against superoxide radicals by COS-963 was 67.76%. As shown in Fig. 6, COS-963 has the highest scavenging ability towards superoxide radicals compared with the other LMW chitosans.

Chitosan has two hydroxyl groups and one amino group in its basic unit. Polysaccharides with scavenging effects on superoxide anion all have one or more alcohol or phenolic hydroxyl groups. High-molecular weight chitosan has a compact structure and thus, the effect of intramolecular hydrogen bond is stronger. On the contrary, LMW chitosans have a less compact structure and the effect of intramolecular hydrogen bond is relatively weak (Xing et al., 2005). With a decrease in LMW chitosan molecular weight, more hydroxyl groups are activated, and this is beneficial to the superoxide radical scavenging.

4. Conclusion

Chitosans with different molecular weights were prepared from β-chitin of squid pens, and their bile acid binding capacities and antioxidant activities were assessed *in vitro*. Our results revealed that the bile acid binding capacities of LMW chitosans were better than that of the native chitosan sample and the highest bile acid binding capacity of LMW chitosans reached 63.53 mg/g

compared with the previously reported value for chitosan of 14.02 mg/g (Zhao et al., 2011). Antioxidant activities of high-molecular weight chitosans were poorer than that of LMW chitosans, especially in terms of their reducing power, scavenging ability towards DPPH and hydroxyl radicals, and their antioxidant properties, which increased with increasing concentrations. On the basis of the results obtained, LMW chitosans with presumed antioxidant properties and enhanced bile acid binding capacity may be used as a source of antioxidants, and be useful as a means to possibly reduce cholesterol and blood pressure.

Acknowledgments

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