Synthesis, antioxidant and cathepsin D inhibition activity of quaternary ammonium chitosan derivatives

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Abstract: Two (2-hydroxypropyl) trimethyl ammonium and/or imidazole-based quaternary ammonium chitosan derivatives (NHT-chitosan and Im-OHT-chitosan) were synthesized by using nucleophilic substitution reaction. These two synthesized chitosan derivatives were characterized by Fourier transform infrared spectroscopy, NMR spectra, and UV–visible spectra. The applications as antioxidant agents and cathepsin D inhibitors were further investigated. Both of quaternary ammonium chitosan derivatives exhibited good antioxidant activity upon scavenging against hydroxyl radical and hydrogen peroxide as well as the lipid peroxidation inhibition in the linoleic acid emulsion system. They also exhibited good inhibition activity of cathepsin D protease. NHT-chitosan and Im-OHT-chitosan are potential the natural, healthy and safe preservatives in food industry.

Keywords: Chitosan; Quaternary ammonium; Imidazole; Antioxidant activity; Cathepsin D inhibitor.

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1. Introduction

Postharvest animal and fish meat are highly perishable due to its biological composition. The spoilage of meat usually results from biological reactions, such as oxidation of lipids, the loss of protein functionality, and other reactions caused by the endogenous enzymes and exogenous microorganisms.

The most common strategy to extend shelf life of fresh meat is the use of antimicrobial and antioxidant additives of synthetic origin (butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butylhydroquinone (THBQ)), which are being questioned due to an increasing consumer demand for natural, healthy and safe preservatives. In meat industry, nitrates are commonly used in meat preservation because they not only help kill bacteria, but produce a characteristic flavor and give meat a pink or red color via breaking down in the meat into nitric oxide (NO), which then binds to the iron atom in the center of myoglobin's heme group, reducing oxidation (Harikumar et al., 1974). Nitrates also cause ir-reversible inhibition of chicken muscle cathepsin D via the formation of oxidative species such as nitrous acid, nitric oxide, and nitrogen dioxide (Harikumar et al., 1974). However, the use of nitrates in food preservation is controversial due to the potential harm resulted from the formation of nitrosamines when nitrates are present in high concentrations and the meat is cooked at high temperatures.

With a growing concern on the global environment and public health problems, numerous studies have focused on natural ingredients for food preservation. Chitosan is an important biocompatible, biodegradable, broadly antimicrobial, nutritional, and antioxidative polycationic polymer obtained from waste shell of the crab, shrimp, and

crawfish industries. It has been approved as a food additive in Japan since 1983, also applied as a food preservative and as a component of packaging material to improve the quality and shelf-life of food (Vasilatos & Savvaidis, 2013). Edible films based on chitosan with or without basil or thyme essential oils were studied on the storage and it was demonstrated that these films could maintain quality of pork meat products due to the antioxidant and antimicrobial properties of chitosan (Bonilla et al. 2014). Chitosan with high molecular weight was commonly dispersed in an acetic acid solution at 1 % (v/w) when it was used as bio-preservative agents in food and medicinal fields because of its insolubility in water. However, cathepsin D, which was considered to be one of most important endogenous proteases in postmortem degradation of muscle, is rather active at this acidic condition (pH 3.0 to 4.5) (Schwartz & Bird, 1977). That is to say, when high molecular weight chitosan/acetic acid solution was used as preservative, the activity of cathepsin D could, in vivo, potentially aid in the postmortem degradation of myofibrillar proteins to form the decomposition products, such as water-soluble peptides and amino acids, and further produce some harmful ingredients, such as amines, hydrogen sulfide, phenol, indole, fecal olfactory element, and curing alcohol, thus influencing meat tenderness and quality. Furthermore, increasing the pH of *post rigor* meat produced more tender cooked meat, similar to fresh meats (Alarcon-Rojo & Dransfield, 1995).

Therefore, numerous efforts have been devoted to preparing functional derivatives by chemical modification of chitosan to increase its solubility in water and to improve the antimicrobial and antioxidant activities, while remaining the biodegradability and biocompatibility (Rabea et al., 2003). Various functional groups, including alkyl (Zavaleta-Aveja et al., 2014), carboxymethyl (Liu et al., 2007), saccharides or

oligosaccharides, cyclodextrins, acids, as well as polyacrylic groups (Alves & Mano, 2008; Belalia et al., 2008; Calero et al., 2010; Chao et al., 2004; Curcio et al., 2009; Harte, 2010; Huang et al., 2011; Liu, 2014; Sashiwa et al., 2004; Siripatrawan & Kumar et al., 2004; Sobahi et al., 2014; Vachoud et al., 2001; Xie et al., 2002; Zavaleta-Aveja et al., 2014), have been introduced to ameliorate the properties of chitosan.

With the exception of bacterial spoilage, deterioration in quality during storage of meat products occurs by the action of hydrolytic enzymes in the products, so it should be considered to necessitate inactivation of the hydrolases during meat curing processes. Although the treatment with chitosan or chitosan derivatives could efficiently extend the shelf life of various fresh meats, rather few investigations on the inhibition activity of cathepsin D were involved.

It is known that *N*-methyl imidazole has been used as a caramel food coloring in the beverage industries, and imidazole groups presented in the structures of various natural or synthetic drug molecules have anticancer (Congio et al., 2008), antimicrobial (Aridoss et al., 2006; Nagarapu et al., 2008), antioxidant (Smith & Reeves, 1987), and enzyme inhibition (Siddiquee et al., 2007; Reddy et al., 2015) activities. Therefore, to combine both the advantages of imidazole groups and chitosan and to acquire new water-soluble chitosan derivatives, we designed two quaternary chitosan derivatives, (2-hydroxy-3-trimethylammonium)propyl-based and imidazole-based chitosans. Herein, we reported the synthesis, characterization and biological activities of these two water-soluble chitosan derivatives. Their biological activities were systematically investigated by the assessment of hydrogen peroxide (H₂O₂) scavenging activity, β -carotene-linoleate bleaching assay, hydroxyl radical scavenging activity, as well as the inhibition ability to

cathepsin D, as important indicators to achieve natural, healthy and safe food bio-preservative agents.

2. Materials and methods

2.1 Materials

Chitosan with the deacetylation of 96.1% and the centipoise viscosity of 20 mPa·s (1%, 20 °C) was purchased from Ningbo Zhenghai Haixin Biological Products Co., Ltd. (Zhejiang, China). Cathepsin D from bovine spleen (C 3138) was purchased from Sigma (America). Bovine haemoglobin was bought from TCI (Japan). Other reagents and solvents were purchased from Aladin Reagent Co., Ltd. (Shanghai, China), and used directly without further purification.

2.2 Preparation of *N*-(3-chloro-2-hydroxypropyl)trimethyl ammonium chitosan chloride

(**1**) (Scheme 1)

Scheme 1. Synthesis of *N*-(2-hydroxy-3-trimethylammonium)propyl chitosan chloride (**1**), *N*-chlorobutyryl chitosan (**2**) and *N*-(carboxybutyl-4-(3-methyl-imidazole))-6-*O*-(2-hydroxy-3-trimethylammonium)propyl chitosan chloride (**3**).

According to our previous report (Zhang et al., 2012), 20 mL of 2-propanol dispersion solution containing chitosan (2.00 g) was stirred for 0.5 h at room temperature,

followed by adding aqueous NaOH (5.00 g, 40 wt %). After stirring for 2 h, (3-chloro-2-hydroxypropyl) trimethyl ammonium chloride (CTA) (10.7 g, 57.1 mmol) was added to the mixture. The mixture was further stirred for 6 h at 40 °C. The resulting reaction mixture was then washed with 95% ethanol to afford a white ropy solid. After filtration, the solid was dissolved in water (100 mL). The mixture was filtered to remove insoluble gel-like particles. The filtrate was then purified by the addition of anhydrous ether with stirring. The white solid was collected and dried overnight under vacuum at 80 °C to afford N-(3-chloro-2-hydroxypropyl) trimethyl ammonium chitosan chloride (**1**) (1.26 g).

2.3 Preparation of *N*-(carboxybutyl-4-(3-methyl-imidazole))-6-*O*-(2-hydroxy-3-trimethylammonium)propyl chitosan chloride (**3**) (Scheme 1)

Chitosan (2.00 g) was dissolved in 60 mL of *N*-methylpyrrolidone (NMP) under nitrogen. 200 µL of 4-chlorobutyryl chloride dissolved in 5 mL of NMP was added dropwise to the above solution. After stirring on room temperature for 3 h, the resulting polymer was precipitated with diethyl ether, followed by washing with methanol and diethyl ether to afford *N*-chlorobutyryl chitosan (**2**). Compound **2** was then stirred in 200 mL of *N*-methyl-imidazole under nitrogen at 80 °C for 72 h. After diethyl ether was added to precipitate the chitosan derivative, the crude product was washed subsequently with methanol and diethyl ether, and dried in a freeze-dryer to give *N*-(1-carboxybutyl-4-(3-methyl-imidazole) chitosan chloride. Then this product (1.00 g) and 10 mL of isopropanol were mixed, the mixture was stirred for 0.5 h at room temperature. Aqueous sodium hydroxide (NaOH) (2.50 g, 40 wt %) was then added and the mixture was further stirred for 2 h. CTA (5.37 g, 28.5 mmol) was added into the resulting mixture and the mixture was reacted for 6 h at 40 °C. The resulting reaction mixture was cooled to room

temperature. Anhydrous ethanol was then added dropwise to precipitate the product. After filtration, the residue was dissolved in water (100 mL), and filtered to remove insoluble solid. The resulting chitosan derivative, *N*-(carboxybutyl-4-(3-methylimidazole))-6-*O*-(2-hydroxy-3-trimethylammonium)propyl chitosan chloride (**3**) was obtained (0.61 g, white solid) after lyophilization of the filtrate.

Characterization. ^1H and ^{13}C NMR Spectra were recorded at 343K on a Bruker AVANCE DRX 500. FT-IR spectra were recorded on a Nicolet Nexus 870 spectrometer with KBr pellets. UV-visible spectra were recorded on a UV-vis spectrophotometer (UV-2550, Shimadzu, Japan).

The degree of quaternization substitution (DS) of compound (**3**) was determined by ^1H NMR integration with Eq. 1 (Sajomsang, Tantayanon, Tangpasuthadol, & Daly, 2010).

$$\text{DQ}(\%) = (\text{Ar}/n) / [\text{H2} + 1/3\text{NHAc}] \times 100 \quad (1)$$

Where DQ (%) is the quaternization of *N*-substitution, Ar is the integral area of aromatic protons, n is the number of aromatic hydrogen atoms per-substituent, H2 is the integral areas of the protons at the C-2 carbon of GlcN, and NHAc is the integral area of GlcNAc protons.

Molecular weight distributions were measured on a conventional gel permeation chromatography (GPC) system equipped with a LC-20AD HPLC pump, a DGU-20A degassing system, a RID-20A RI detector, a 100F-LC injector, a Shodex SUGAR KS-804 linear chromatographic column in a CTO-20A thermostatic oven and LC-solution software. GPC measurements were carried out at 60 °C using an ultrapure water solution as the eluent with a flow rate of 1.0 mL/min. The system was calibrated with Pullulan standards.

2.4 Assay of H₂O₂ scavenging activity

The H₂O₂ scavenging activity was measured according to the literatural method (Zhang et al., 2012). An aliquot (1 mL) of the sample solution (2 mg/mL) and 0.6 mL of H₂O₂ (0.043 mol/L) solution were mixed with 2.4 mL of phosphate buffer (0.1 mol/L, pH 7.4). The mixture was shaken vigorously and the absorbance at 230 nm of the reaction mixture was immediately recorded from 0 to 240 min at room temperature. For each sample, phosphate buffer without addition of hydrogen peroxide was used for background subtraction. All samples were assayed in triplicate. The H₂O₂ scavenging activity was calculated as follows Eq. (2):

$$\text{Scavenging activity (\%)} = \left[1 - \frac{(A_0 - A_x)}{A'_0 - A'_x} \right] \times 100 \quad (2)$$

Where A'_0 is the initial absorbance of control; A_0 is the initial absorbance of sample; A'_x is the absorbance of control and A_x is the absorbance of sample at different times.

2.5 β -Carotene-linoleate bleaching assay

The ability of chitosan and quaternary chitosans to prevent bleaching of β -carotene was determined according to the method described by Amin et al. (Amin et al., 2004). 1 mL of β -carotene solution (1 mg/mL in chloroform) was mixed with 20 μ L of linoleic acid and 200 μ L of Tween 20. After stirring at room temperature for 5 minutes, chloroform was removed completely under vacuum in a rotary evaporator at 40 °C. Then 200 mL of distilled water was added slowly, and the resulting mixture was stirred vigorously to form an emulsion.

2 mL of sample solution with different concentrations ranging from 0 to 2.0 mg/mL dissolved in distilled water were transferred to different test tubes containing 2 mL of

emulsion. 2 mL of emulsion with 2 mL of distilled water was used as control. Tubes were then gently shaken and placed in a water bath at 50 °C for 2 h. The absorbance of samples and control was measured at 470 nm and recorded at 30 min intervals. All samples were assayed in triplicate. Antioxidant activity was measured in terms of successful bleaching of β -carotene using Eq. (3):

$$A_{oxA} (\%) = \left[1 - \frac{A_0 - A}{A'_0 - A'} \right] \times 100 \quad (3)$$

Where A'_0 is the initial absorbance of control, A_0 is the initial absorbance of sample, A' is the absorbance of control and A is the absorbance of sample after 2 hours.

2.6 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was measured according to the previous report (Liu et al., 2014). The reaction mixture containing 1 mL of ferrous sulfate (9 mmol/L), 1 mL of salicylic acid–ethanol (9 mmol/L), and 1 mL of sample with various concentrations (0–2.0 mg/mL) was initiated by 1 mL of hydrogen peroxide (0.3%, v/v). After incubating and shaken vigorously at 37 °C for 0.5 h, the absorbance of the reaction mixtures was measured at 510 nm. All samples were assayed in triplicate. The hydroxyl radical scavenging activity was calculated as the following formula:

$$\text{Scavenging activity (\%)} = \left[\frac{1 - (A_1 - A_2)}{A_0} \times 100\% \right] \quad (4)$$

Where A_0 is the absorbance of the control (water instead of sample), A_1 is the absorbance of the sample, and A_2 is the absorbance of the sample only (salicylic acid–ethanol solution instead of FeSO_4 and H_2O_2 solution).

2.7 Inhibition activity of cathepsin D

Effect of chitosan derivatives on the cathepsin D activity was investigated according to previous literatures (Esumi et al., 1980; Krause et al., 2011; McLay, 1980) with slight modifications. Acid-denatured haemoglobin substrate (1.5 g) was dissolved in 150 mL of sodium acetate buffer (0.05 mol/L, pH 3.5), following by filtration to remove insoluble particles. Cathepsin D (0.5 mL, 0.05 mg/mL) and chitosan sample (0.5 mL) with different concentration were mixed in the presence of 1.5 mL of sodium acetate buffer (0.05 mol/L, pH 3.5), subsequently incubated for 10 minutes at 45 °C. Then, 1.8 mL of haemoglobin solution (1% w/v) (equilibrated to the assay temperature for 10 minutes) was added to the resulting enzyme-chitosan mixture solution. After incubation for 30 minutes at 40 °C with vigorously stirring, the reaction was terminated by the addition of 2.15 mL trichloroacetic acid (TCA) solution (10% w/v). The mixture was centrifuged ($10000 \times g$, 10 min) (3–30 K, Sigma). The supernatant was transferred to a quartz cuvette and the absorbance was measured at 280 nm using a UV–vis spectrophotometer. The blank sample was prepared similarly but contained 0.5 mL sodium acetate buffer (0.05 mol/L, pH 3.5) instead of the chitosan solution. Effect of chitosan derivatives on the cathepsin D activity was calculated by the difference in absorbance of the chitosan derivatives sample and the blank. The cathepsin D inhibition constant (K_i) of chitosan derivatives was determined by using a Lineweaver-Burk and Double-Dixon plot. All analyses were carried out in triplicate.

2.8 Statistical analysis

The data were analyzed using analysis of variance (ANOVA) ($P < 0.05$). The mean differences were established via the Duncan's multiple range tests. The data were

analyzed by SPSS software (Version 18.0, SPSS Inc., Chicago, IL).

3. Results and discussion

3.1 Synthesis. As shown in Scheme 1, chitosan is a chemical structure including intrinsic oxygen- and nitrogen-based functional groups which can serve as the starting points for covalent modification, thereby giving chitosan derivatives with improved properties. In this study, 4-chlorobutryl chloride was successfully introduced to the primary amino groups of chitosan, followed by reacting with *N*-methyl imidazole to afford (1-carboxybutyl-4--(3-methyl-imidazole) chitosan chloride. The target quaternary ammonium compound *N*-(carboxybutyl-4-(3-methyl-imidazole))-6-*O*-(2-hydroxy-3-trimethylammonium)propyl chitosan chloride was then obtained by further coupling with *N*-(3-chloro-2-hydroxypropyl) trimethyl ammonium chloride. It is noteworthy that the purification procedure is quite simple, just by washing with anhydrous ether.

3.2 UV–Vis Spectra. The UV–vis absorptions of chitosan, NHT-chitosan and Im-OHT-chitosan are shown in Figure 1. An obvious band at 267 nm was observed, which is attributed to Im-OHT-chitosan due to $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ of imidazole ring, while pristine chitosan and NHT-chitosan without imidazole group were transparent in this region because there are no chromophores in the structures of both pristine chitosan and NHT-chitosan.

Figure 1. UV-vis spectra of (a) chitosan, (b) NHT-chitosan, (c) Im-OHT-chitosan.

3.3 FT-IR Spectroscopy. The resulting quaternary polymers were characterized using FT-IR. As shown in Figure 2, The FT-IR spectrum of (a) chitosan showed the characteristic C=O stretching (amide I) bands at 1654 cm^{-1} , N-H angular deformation band of amino groups at 1584 cm^{-1} , $-\text{CH}_2$ bending vibration at 1420 cm^{-1} , C-H (in plane) bending at 1364 cm^{-1} , and amide III band at 1428 cm^{-1} , as well as the band at 1080 cm^{-1} corresponding to the C-O stretching (primary alcoholic groups). The C-N stretching band is observed at 1155 cm^{-1} . The broad band at 3419 cm^{-1} corresponds to $-\text{OH}$ and $-\text{NH}$ stretching absorption, whereas the aliphatic C-H symmetric and asymmetric stretching band can be observed at 2875 cm^{-1} . The successful NHT-chitosan formation by the reaction of chitosan with (3-chloro-2-hydroxypropyl)trimethyl ammonium chloride can be proved by the disappeared characteristic amide (C=O) stretching band at 1654 cm^{-1} and 1584 cm^{-1} compared to pristine chitosan (a), implying that the epoxide groups of CTA have coupled with the NH_2 groups rather than the OH groups of chitosan (Nam, Kim, & Ko, 1999; Zhang et al., 2012). The secondary amide groups in the

backbone of NHT-chitosan displayed a characteristic absorption peak at 1628 cm^{-1} , while a peak at 1399 cm^{-1} is attributed to the characteristic symmetric absorption of $-\text{CH}_3$ bending, suggesting that CTA was introduced to the backbone of chitosan. The FTIR spectrum of Im-OHT-chitosan in Figure 2(c) shows an intermediate peak at 1686 cm^{-1} , which was attributed to the stretching vibration of the characteristic $\text{C}=\text{C}$ group of the imidazole, indicating the successful couple between imidazole and (1-carboxybutyl-4-(3-methyl-imidazole) chitosan chloride chain. a peak at 1616 cm^{-1} corresponded to the N-H bending. Additionally, the absorption peaks at 1462 cm^{-1} and 1393 cm^{-1} were the bending vibration of $\text{C}-\text{H}$, which were attributed to the CH_3 of N-methyl-imidazole and CH_3 of CTA, respectively.

Figure 2. FT-IR spectra of (a) chitosan, (b) NHT-chitosan, (c) Im-OHT-chitosan.

3.4 NMR Spectroscopy Analysis of Im-OHT-chitosan. The ^1H NMR and ^{13}C NMR spectra of Im-OHT-chitosan in D_2O are shown in Figure 3. Peaks at 3.77 ppm, 3.66 ppm, and multiplet from 3.14 to 3.03 ppm correspond to the methine and methylene protons of glucosamine which are characteristic of chitosan. New signal at 8.07 ppm is attributed to the $\text{N}=\text{CH}$ of imidazolium, while the signal at 5.98 ppm corresponds to $\text{CH}=\text{CH}$ of imidazolium, and the singlet peaks at 2.80 ppm and 2.90 ppm are attributed to methyl from CTA and *N*-methylimidazole, respectively. With the calculation based on the ^1H NMR integration data and according to Eq. 1, the degree of quaternization substitution (DS) of compound (**3**) was determined to be 36%. As shown in ^{13}C NMR spectra (Figure 3), the signals at δ 168.7 ppm, 168.4 ppm, and 130.8 ppm are ascribed to the carbons of imidazole, while the peak at 171.6 ppm is attributed to the carbon of carbonyl in glucosamine.

Figure 3. ^1H NMR and ^{13}C NMR spectra of Im-OHT-chitosan.

As a consequence, based on the data of UV, FT-IR, and NMR, it could be definitely concluded that *N*-methylimidazole and CTA groups were successfully introduced into the backbone of chitosan.

Molecular weight. Weight-average molecular weight (Mw) and number-average molecular weight (Mn) of chitosan and its derivatives were measured by GPC, and the results were summarized in Table S1. The polydispersity (PD) data are determined by the ratio of Mw to Mn to be 1.062, 1.069, and 1.092 for chitosan, NHT-chitosan and Im-OHT-chitosan, respectively, while Mw of them are 183735, 187662, and 170636, respectively. These data suggested that a series of samples fractions have narrow dispersity with similar molecular weight distributions.

3.5 Antioxidant activity

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen, including oxygen ions and peroxides. It is well known that excessive ROS may cause human diseases such as DNA damage, mutagenesis, and carcinogenesis, and even may be a major contributor to the functional decline that is characteristic of aging according to

the free radical theory (Van Raamsdonk & Hekimi, 2009; Cayuela 1995; Okezie 1998).

To evaluate antioxidant activity of the chitosan derivatives obtained herein, we conducted three different kinds of *in vitro* antioxidant assays: hydrogen peroxide scavenging, hydroxyl radical scavenging and β -carotene-linoleate bleaching.

Scavenging activity on hydrogen peroxide. Hydrogen peroxide is a non-radical oxidant which is inevitable produced by the dismutation reaction in a biological system (including humans and bacteria). According to the previous reports, hydrogen peroxide is the most potent and reactive ROS species because it generates the hydroxyl radical, especially at the presence of transition metal ions (Jonas et al., 1989; Halliwell & Gutteridge, 1984). Therefore, the measurement and search for the ideal antioxidant against hydrogen peroxide scavenging activity in food industry are important (Ozyurek et al., 2010).

As used in the most common UV-measurement method for the detection of H_2O_2 , the scavenging capacity depends on the change of the absorbance at 230 nm when H_2O_2 is consumed by scavenger compounds. Hydrogen peroxide scavenging activities of NHT-chitosan, Im-OHT-chitosan, and the pristine chitosan are shown in Figure 4. At the initial reaction, the efficient degradation of H_2O_2 by all tested samples was observed. Both NHT-chitosan and Im-OHT-chitosan exhibited significantly enhanced hydrogen peroxide scavenging activity by comparison with pristine chitosan ($P < 0.05$). Around 54% and 50% hydrogen scavenging activity were obtained for the Im-OHT-chitosan and NHT-chitosan, respectively, while that of pristine chitosan was only 10%.

Figure 4. The scavenging activities of (a) chitosan, (b) NHT-chitosan, (c) Im-OHT-chitosan against hydrogen peroxide.

β -Carotene-linoleate bleaching assay. It is well known that polyunsaturated fatty acids, such as linoleic acid, can be easily oxidized by oxygen in the air, thereby leading to the formation of aldehydes, ketones, acids, and alcohols, which are responsible for the changes in flavor, aroma, taste, and nutritional value (Lee et al., 2014). In the present study, the β -carotene-linoleic acid model system was used to evaluate the inhibition capacity of lipid peroxidation. As shown in Figure 5, the antioxidant activity of all samples was concentration-dependant. Im-OHT-chitosan and NHT-chitosan showed significant inhibition activity to β -carotene bleaching by comparison with pristine chitosan. Im-OHT-chitosan exhibited the highest inhibition capacity (74%, 2.0 mg/mL), followed by NHT-chitosan (72%, 2.0 mg/mL), and chitosan (27%, 2.0 mg/mL).

Figure 5. The inhibition activities of (a) chitosan, (b) NHT-chitosan, (c) Im-OHT-chitosan against linoleic acid oxidation.

Hydroxyl radical scavenging activity. Hydroxyl radical ($\bullet\text{OH}$) is the strongest reactive free radical, which can easily induce severe damage to a wide range of essential biomolecules (Cayuela 1995; Okezie 1998). The hydroxyl radical scavenging activity of chitosan, NHT-chitosan, and Im-OHT-chitosan are shown in Figure 6. Obviously, the hydroxyl radical-scavenging ability of NHT-chitosan and Im-OHT-chitosan is much higher than that of pristine chitosan at all concentrations. This result indicated that the scavenging activity of Im-OHT-chitosan significantly increased due to the synergic effect of the alien imidazole ring attached on the backbone of chitosan. At the concentration of 2 mg/mL, the hydroxyl radical scavenging activity of Im-OHT-chitosan reached 66% which was much higher than that of NHT-chitosan (35%) and pristine chitosan (11%). Notably, the measured value of the scavenging activity of NHT-chitosan against $\bullet\text{OH}$ (35%) was in accordance with the recent data reported by Mao group (30%) (Huang et al., 2014) and by Li group (43%) (Xing et al., 2008).

Figure 6. The scavenging activity of (a) chitosan, (b) NHT-chitosan, (c) Im-OHT-chitosan against hydroxyl radical.

The antioxidant activity of the chitosan and its derivatives mainly attributes to the active hydroxyl and amino groups, both of which have strong hydrogen-donating ability. Shon deduced that the clearance of hydroxyl radicals by polysaccharides are *via* two main mechanisms of (i) chelation with metal ions by alcohol hydroxyl groups against $\bullet\text{OH}$ formation, and (ii) cleaning hydroxyl radical by hydrogen-atoms of hydroxyl groups (Shon, 2003). NHT-chitosan has more hydroxyl groups than the pristine chitosan, thus exhibiting higher antioxidant ability. In the case of Im-OHT-chitosan, each imidazolium ring that was introduced onto the backbone of chitosan can easily leave 2 hydrogens of imidazolium out in the reaction system to form a carbene, so Im-OHT-chitosan showed the best antioxidant activity among all chitosans.

Chelation ability is important for the preservation of flavor and taste of food because the transition metal ions can initiate lipid peroxidation, then start a chain reaction and seriously deteriorate the quality of food. Previous studies demonstrated that chitosan, a long-chain polymer of *N*-acetyl glucosamine could be used as a potential antioxidant

agent because the active hydrogen atoms in hydroxyl or amino groups of chitosan could react with reactive oxygen species (ROS) to form a very stable macromolecular radical, and hydroxyl or amino groups could bind with transition metal ions, reducing the lipid peroxidation catalysis chance of metal ions.

Generally, antioxidant agents are able to prevent chain initiation, bind transition metal ions, decompose peroxides, and so on. Upon the results obtained above, the antioxidant activity of two (2-hydroxypropyl) trimethyl ammonium and/or imidazole-based quaternary chitosan derivatives (NHT-chitosan and Im-OHT-chitosan) was concentration-dependant, with the order of Im-OHT-chitosan > NHT-chitosan > pristine chitosan.

3.6 Cathepsin D Inhibition

As an aspartyl-type proteinase and a glycoprotein, Cathepsin D can be found in the cells and tissues of mammals ubiquitously (Zeece & Katoh, 1989). Cathepsin D plays an important role during meat processing, which may finally influence meat tenderness and quality traits (Cafe et al., 2010; Godiksen et al., 2009). A number of myofibrillar proteins, such as titin and connectin, were found to be susceptible to hydrolysis by cathepsin D. It is essential for proteolysis of proteins to help in regulating cell growth and tissue homeostasis, which is expressed in most cells and over-expressed in breast cancer cells (Masson et al., 2011; Srivastava et al., 2006; Tandon et al., 1990), even involved in tumor metastasis (Ollinger, 2000; Lah et al., 1996; Rochefort et al., 1996). Nowadays, protease inhibitors have been traditionally developed from natural product screening for lead molecules with subsequent optimization (Chaturvedi et al., 2013), thus, chitosan-based cathepsin D inhibitor is a potential.

Pepstatin ($K_i = 0.5$ nmol/L) (Knight et al., 1976) is a specific and powerful inhibitor of cathepsin D, which has been used to study the effect of endogenous proteases on meat aging (Alarcon-Rojas & Dransfield 1995). Subsequently, substrate analogues containing statine and analogues of pepstatin have been synthesized and documented the cathepsin D inhibition with the K_i value varying from 1.1 to 100 nmol/L (Agarwal & Rich, 1986). In this study, cathepsin D inhibition was assayed using a haemoglobin method. The results were presented in Figure 7. The cathepsin D inhibition activity of all samples exhibited dose-dependent. At the concentration of 2.4 mg/mL, the inhibition activity of Im-OHT-chitosan was 39%, followed by NHT-chitosan (36%), and chitosan (21%). Compared to unmodified chitosan, Im-OHT-chitosan exhibited good inhibition activity toward cathepsin D, implying that the introduction of imidazole group onto the backbone of chitosan played an important role in the behavior of cathepsin D inhibition activity. Upon the data of Lineweaver-Burk and Double-Dixon plot, the cathepsin D inhibition constant (K_i) of chitosan, NHT-chitosan, Im-OHT-chitosan was 6.48×10^{-3} mg/mL, 3.65×10^{-3} mg/mL and 2.82×10^{-3} mg/mL, respectively. Competitive inhibition was observed for these three inhibitors.

Figure 7. Cathepsin D inhibiting activity of (a) Chitosan, (b) NHT-chitosan, (c) Im-OHT-chitosan.

It is well known that the polymers with different molecular weight have different functions, and chitosan has shown scavenging activity against different radical species due to the capacity of metal ions chelation (Aranaz et al., 2009). Herein, two quaternary chitosan derivatives, NHT-chitosan and Im-OHT-chitosan were obtained in mild reaction conditions, thereby Mw were almost not changed compared with pristine chitosan. Nonetheless, Im-OHT-chitosan exhibited better antioxidant activity and cathepsin D inhibition activity than that NHT-chitosan and pristine chitosan did, indicating that the introduction of alien *N*-methylimidazole and (2-hydroxy-3-trimethylammonium)propyl groups played an important role in improving antioxidant activity and cathepsin D inhibition activity.

4. Conclusion

In this article, NHT-chitosan and Im-OHT-chitosan were synthesized and characterized. These two chitosan derivatives exhibited better antioxidant activities than the pristine chitosan, which indicated that the antioxidant ability of chitosan was strengthened by the introduction of alien *N*-methylimidazole and (2-hydroxy-3-trimethylammonium)propyl groups to natural chitosan. Based on the data of scavenging against hydroxyl radical and hydrogen peroxide, the lipid peroxidation inhibition in the linoleic acid emulsion system, as well as the inhibition of cathepsin D protease activity, these synthesized quaternary ammonium chitosan derivatives exhibited highly efficient antioxidant activity and cathepsin D protease inhibition activity. They also can bind the iron atom in the center of myoglobin's heme group, thus reducing oxidation. NHT-chitosan and Im-OHT-chitosan

may be potential and useful in applications as new healthy preservatives and antimicrobial reagents in the food and pharmaceutical industries.

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