AN ABSTRACT OF THE DISSERTATION OF

Jooyeoun Jung for the degree of Doctor of Philosophy in Food Science and Technology presented on May 29, 2013.

Title: New Development of β-Chitosan from Jumbo Squid Pens (*Dosidicus gigas*) and its Structural, Physicochemical, and Biological Properties

Abstract approved: _____
Yanyun Zhao

β-chitin/chitosan extracted from newly utilized jumbo squid (***Dosidicus gigas***) pens were investigated in respect to their physicochemical properties, polymeric structures, deacetylation and depolymerization characteristics, and antioxidant and antibacterial activities. These functional properties were further compared with α-chitin/chitosan prepared from shrimp shells. Due to the low mineral content (< 1%) and negligible pigment in squid pens, demineralization and depigmentation steps could be omitted when extracting β-chitin. Molecular weight (Mw) and moisture content of deproteinized β-chitin were significantly higher than those of α-chitin. Crystallographic structure of β-chitin was distinguished from that of α-chitin, in which β-chitin had less inter-molecular hydrogen bond than α-chitin along with lower crystallinity (CI). β-chitin could convert into α-form as the result of alkali treatment, and the resulted α-chitin exerted significantly higher moisture absorption ability than the native α-chitin, thus retaining higher susceptibility of native β-chitin by means of the polymorphic destruction. The Kurita method using NaOH was an effective deacetylation treatment to obtain β-chitosan with high Mw and a wide range of DDA, and cellulase was more susceptible than lysozyme to

degrade β-chitosan for obtaining low Mw of product. To obtain similar DDA of α- and β-chitosan, lower concentrations of NaOH and shorter reaction times were required for β-chitin than that for α-chitin. In addition, β-chitosan was more susceptible to cellulase hydrolysis than α-chitosan. High Mw (280-300 kDa) of β-chitosan exerted extremely lower half maximal effective concentrations (EC_{50}) than α-chitosan, i.e., higher antioxidant activity based on DPPH radical scavenging activity and reducing ability. The 75% DDA/31 kDa β-chitosan exerted higher inhibition against *E. coli* (lower MIC) than that of 75% DDA/31 kDa α-chitosan, whereas opposite result was observed in 90% DDA/74-76 kDa α- and β-chitosan. This difference could be due to the impact of the different structural properties between α- and β-chitosan on chitosan conformations in the solution, altering the surface phenomenon of protonated chitosan with negatively charged bacterial cells in the suspension. Therefore, jumbo squid pens can be commercially employed to extract functional β-chitin/chitosan with desirable structural, physicochemical, and biological activities.

New Development of β-Chitosan from Jumbo Squid Pens (*Dosidicus gigas*) and its Structural, Physicochemical, and Biological Properties

by

Jooyeoun Jung

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APPROVED:

_____

Major Professor, representing Food Science and Technology

_____

Chair of the Department of Food Science and Technology

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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CONTRIBUTION OF AUTHORS

Dr. Yanyun Zhao assisted with the experimental design, data analysis, and writing of each chapter. Dr. John Simonsen assisted with data collection of Fourier-Transform infrared (FT-IR) spectroscopy. Dr. George Cavender was involved with the data analysis and writing of Appendix I.

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CHAPTER 1

INTRODUCTION TO CURRENT WORK

Chitin is a polymer linked *N*-acetyl-D-glucosamine and D-glucosamine by β-(1-4) glycosidic bonds. Chitosan polymer, deacetylated form of chitin, is predominantly occupied with D-glucosamine (Fig. 1.1). It is difficult to obtain fully deacetylated or acetylated forms, and two terms including degree of acetylation (DA, %) or degree of deacetylation (DDA, %) are interchangeable depending on the determined monomers.

Although chitin and chitosan can be extracted from various aquatic organisms, terrestrial organisms, and some microorganisms [1], commercial α-chitin/chitosan are mostly extracted from crustacean wastes (e.g., crab, shrimp, or krill shells) [2-4]. Hence, most of chitin/chitosan studies are based upon α-chitin/chitosan extracted from readily accessible crustacean shells. In contrast, study of β-chitin/chitosan has been sparse, probably due to relatively low source of the materials for extracting β-chitin/chitosan.

Squid pens, feather-shaped internal structure in squid species including *Loligo lessonicana, Loligo formosana, Loligo vulgaris, Ommasterphes bartrami,* and *Illex argentines*, are the only marine sources for isolating β-chitin/chitosan that have distinguished structures and physicochemical properties from α-chitin/chitosan[5-11]. In particular, jumbo squid (*Dosidicus gigas*) pens have received our attention because of increased catch of this squid speice (from 14 tons in 1974 to over 250,000 tons in 2005) [12] unique functionality, and limited previous research.

To date, β-chitin/chitosan extracted from other species of squids have been studied for their optimal deproteinization or deacetylation conditions to produce chitin or chitosan [5-9, 13-20], structures and physicochemical changes during deprotenization or

deacetylation processes including the conversion phenomenon from β-chitin to α-chitin [5-7, 10, 11, 13, 21-23], chitosan derivatives [24, 25] and nanofibers [26, 27], and applications as wound dressing and scaffolds in tissue engineering or biodegradable films [8, 26, 28-32]. However, only a few studies reported antioxidant and antimicrobial activities of β-chitosan [33, 34]. So far, no reporting study compared the biological activity between α- and β-chitosan and its coordination with their polymeric structures and physicochemical properties. Hence, it is necessary to investigate the feasibility of using jumbo squid pens as a source of material to produce functional β-chitin/chitosan, its structural, physicochemical, and biological properties in comparison with α-chitin/chitosan isolated from crustacean shells[7, 13, 23].This dissertation research sought to quantify the differences in the structural, physicochemical, and biological properties between α- and β-chitin/chitosan extracted from shrimp shells and jumbo squid pens, respectively. Initiating the study by investigating the deacetylation and depolymerization characteristics of newly employed raw material (jumbo squid pens), further studies were carried for comparing alkali or acid-induced structural modifications and biological activities of β-chitin/chitosan with those of commercial α-chitin/chitosan in association with their different structural properties.

The work is divided into seven chapters, including this introduction. Chapter 2 provides an in-depth review to discuss the different characteristics of β-chitin/chitosan in terms of their resources, extraction methods and procedures, structural, physicochemical, and biological properties. Chapter 3 examins the deacetylation and depolymerization characteristics of β-chitin from jumbo squid (*Dosidicus gigas*) pens to obtain samples with different DDA and Mw, and the optimal deacetylation and depolymerization conditions for desirable β-chitin/chitosan is further investigated. This article was

published in Carbohydrate Research in 2011 [35]. Chapter 4 investigates alkali or acid-induced structural modifications, moisture absorption ability, and deacetylating reactions of β-chitin and compared with those of α-chitin. Although the polymorphic destruction of α-chitin and the conversion of the β-form to the α-form as the result of alkali and acid treatments have been investigated in previous studies [36-39], no study was attempted to compare alkali or acid induced polymorphic changes between the two forms of chitin. This manuscript has been submitted to Food Chemistry in 2013. Chapter 5 examines the antioxidant action of α- and β-chitosan prepared with different Mw and concentrations through determining 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, reducing ability, chelating ability, and hydroxyl radical scavenging activity. This chapter was published in Bioorganic & Medicinal Chemistry in 2012 [40]. Chapter 6 investigates the depolymerizing reactions of β-chitosan in comparison with α-chitosan and their antibacterial action against *Listeria innocua* and *Escherichia coli* by studying the minimum inhibitory concentration (MIC) and inhibition ratios (IR, %). Strucural properties of derived α- and β-chitosan are also investigated by using Fourier-Transform infrared (FT-IR) spectroscopy and X-ray diffraction (XRD). This chapter was submitted to Journal of Agricultural and Food Chemistry in 2013. Chapter 7 summarizes the important findings of this dissertation study and offered insights of future research opportunities and speculates as to how such areas may best be explored. Appendix I includes further examination about the contribution of acidulant type to the antibacterial activity of acid-soluble α- and β-chitosan solution and film as a series of the studies to investigate the antibacterial action of β-chitosan. This manuscript was submitted to the International Journal of Food Microbiology in 2013.

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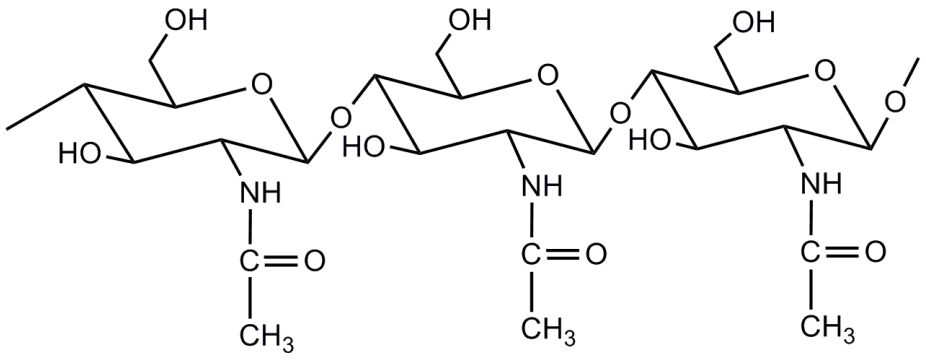
[A]



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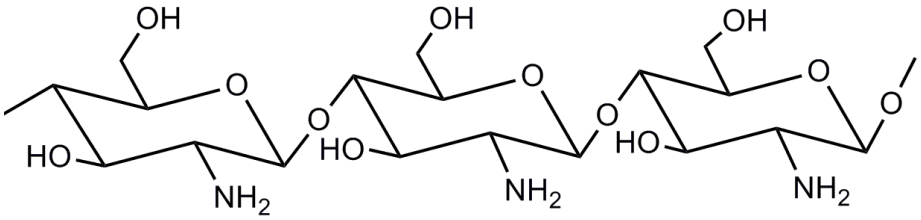

Fig. 1.1. Structures of chitin and chitosan

CHAPTER 2

FEASIBILITY OF USING SQUID PENS AS THE RAW MATERIAL OF PREPARING β-CHITIN/CHITOSAN AND THEIR DIFFERENCES FROM α-CHITIN/CHITOSAN: A LITERATURE REVIEW

Jung, J., Zhao, Y. To be submitted to *Review in Carbohydrate Polymers*

ABSTRACT

This review focused on discussing the unique characteristics of β-chitin/chitosan by comparing with α-chitin/chitosan in respect to their structure, preparing procedures, and physicochemical and biological properties. There are several commercial advantages for obtaining β-chitin/chitosan from squid pens. The amount of mineral and carotenoid in squid pens is negligible and derived β-chitin is more susceptible to alkali deproteinizing and deacetylating reactions due to its loose crystallographic structure and lower crystallinity (CI), compared with α-chitin. The antioxidant and the antimicrobial activities of β-chitosan could be compatible with or even better than those of α-chitosan based on a few previous studies. Therefore, further utilization to the large-scale production of β-chitin/chitosan from squid pens and development of in-depth understanding of its functionalities are necessary and important.

INTRODUCTION

Chitin was first identified in 1884 [1]. The subsequent research has found three different forms of chitin including α-, β-, and γ-forms. These three forms of chitin occur in the exoskeleton of arthropods or in the cell walls of fungi and yeast, but the commercial α-chitin/chitosan have been extracted from crustacean shells only [2]. Hence, scientific studies have been based upon α-chitin/chitosan extracted from readily accessible crab or shrimp shells.

The squid pen has feather-shaped internal structure in squid species and contains β-chitin [3]. Previously, β-chitin was extracted from the following squid species: *Loligo sp.*, *Ommasterphes bartrami*, and *Illex argentines* [4-7]. However, the commercial harvest of jumbo squids (*Dosidicus gigas*) has been increased from 14 tons in 1974 to over 250,000 tons in 2005 [8], and its utilization was less than 11% of the total catch [9]. In addition, the valuable components including collagen, gelatin, chitin, and chitosan are disposed despite its affordability and nutritional values [10, 11]. Hence, the development of new β-chitin/chitosan is able to enhance the utilization of jumbo squid pens, which need the fundamental investigations to understand their structures and functional properties in comparison with chitin and chitosan extracted from crustacean shells and other squid species. It has been also demonstrated that the different nature sources and species are able to alter the functional properties of resulting chitin and chitosan [1, 12-15]. Therefore, these studies can not only increase the utilization of squid pens, but also differentiate the characteristics of chitin and chitosan extracted from various nature sources and species.

Studies about β-chitin/chitosan have been rare in comparison with α-chitin/chitosan in association with the lower commercial demand to squid. However, it has been

demonstrated that α- and β-chitin are distinguished based on their polymorphic structures [16-18]. Due to the structural differences, α- and β-chitin responded differently to alkali deprotenization and deactylation, resulting in α- and β-chitosan with different functional properties, specifically in the degree of solubility and chitosan conformations in the solution (Fig. 2.1) [4, 6, 7, 19-21], which can indirectly impact their biological activities.

This review discussed the originated sources, structures, physicochemical properties, extraction procedures, and biological activities of β-chitin/chitosan and compared with those of α-chitin/chitosan. The important definitions and analytical methods widely applied in chitin and chitosan studies were also reviewed.

COMMON DEFINITION

When chitosan is extracted from chitin by using strong alkali treatment in the deacetylation process, the unique polymorphic structures of α- and β-chitin may no longer exist due to structural modifications. However, the term of α- and β-chitosan was used to distinguish chitosan extracted from different nature sources, following its original form of chitin. Previously, the term of α- and β-chitosan has been applied in a few studies [22-26].

Three parameters, such as degree of deacetylation (DDA), molecular weight (Mw), and crystallinity (CI), have been investigated in chitin and chitosan studies in respect to the structures, physicochemical properties, and biological activities. However, terms indicated in previous studies have been variously described, thus must be defined before any discussions. The following terms are of note:

Chitin and Chitosan: Chitin consists of about 60-100% of *N*-acetyl-D-glucosaminemonomer (NADG) in the polymeric chains. Chitosan is extracted from chitin by hydrolyzing acetyl groups ($COCH_3$) from NADG and forming D-glucosamine (DG). The amount of NADG in chitosan usually becomes less than 40-50%. Chitin is classified into α- and β-form based on the crystallographic properties of polymorphic structures. However, chitosan extracted from α- and β-chitin may no longer retain the unique crystal structure of α- and β-form due to the destructions of aligned polymeric sheets by means of alkali treatments. Despite the concern that α- or β-chitosan was unable to represent the polymorphic characteristic of α- and β-chitin, derived chitosan from each form of chitin was described with α- or β-chitosan, following their original forms of chitin, to distinguish chitosan obtained from different marine sources and the form of chitin.

Molecular Weight (Mw): In polymers such as chitin and chitosan, four different types of Mw including weight-average molecular weight (Mw), number-average molecular weight (Mn), viscosity-average molecular weight (Mv), and z-average molecular weight (Mz) can be determined and represented differently in the distribution of Mw. Mz is relatively higher than Mw, Mw is lower than Mn, and Mv is close to Mw but slightly lower than Mn [27]. As a polymer is generally a mixture of various molecular weights and sizes, it is difficult to measure apparent Mw of polymer. The relative Mw or Mv has been determined to achieve the comparative investigation among prepared chitosan samples.

Degree of Deacetylation (DDA): DDA and degree of acetylation (DA) represent the amount of either DG or NADG monomer in polymeric chains of chitin and chitosan, respectively. They can be measured by colloidal titration methods, UV spectroscopy, Fourier-transform infrared (FT-IR) spectroscopy, and X-ray diffraction (XRD). DDA and DA are interchangeable by the following equation: DDA (%) = 100-DA (%).

Crystallinity (CI): Semi-crystalline chitin and chitosan have amorphous and crystalline regions. Parallel and antiparallel alignments of polymeric chains or sheets form crystallographic structures with various crystal planes, and the portion of crystal regions in the polymorphic structure in chitin and chitosan is described as CI.

CI of chitin and chitosan are important as the reactivity and solubility increase with decrease of CI [28]. Hence, CI can be indirectly related to biological properties of chitosan solutions due to its influence on the degree of solubility and chitosan conformations in the solution. CI can be determined through the FT-IR and XRD, typically ranging 10-80%. However, it is difficult to fully understand the polymorphic characteristics of chitin and chitosan based on CI alone. Hence, structural properties of chitin and chitosan have been interpreted using CI and crystallographic structures including the orientation of the molecular chains, crystal distance, and the crystal imperfection along with chitosan conformations and interactions between amorphous chains.

METHODS

It is important in the studies of chitin and chitosan that the viscosity-average Mw, DDA, and polymorphic structures are appropriately analyzed and interpreted due to their

effects on the functional properties of chitin and chitosan. Following is a brief introduction on the instrumental methods frequently used.

Ubbelohde Capillary Viscometer: Intrinsic viscosity [η] was measured by using the Ubbelohde capillary viscometer and the viscosity-average Mw was calculated by using Mark-Houwink-Sakurada (MHS) equation: $[\eta] = K (Mw)^{a}$, where K and a are constants and depend on the solution used to solubilize chitin and chitosan [29-32]. The constant values (k and a) for MHS equation were selected based on the preliminarily study, in which relativeMw was the closest to the Mw of commercial chitosan. Hence, relative Mw can be compared among prepared chitin and chitosan samples.

Fourier Transforms Infrared (FT-IR) Spectroscopy: Using FT-IR, the crystal structure of α-chitin was able to be distinguished with that of β-chitin by means of the presence of C=O band around at ~1620-1660 cm^{-1}. The peaks of C=O band in α-chitin were split into 1627 and 1660 cm^{-1} due to the presence of strong inter-sheet hydrogen bonds, whereas those in β-chitin were shifted to a single peak at 1656 cm^{-1} as no inter-sheet hydrogen bond was formed with C=O (Fig. 2.2) [27]. Hence, the crystallographic α-chitin was rigid and highly stable by means of strong inter-molecular hydrogen bonds, whereas β-chitin was more reactive due to much weaker inter-molecular hydrogen bonds [20, 33, 34]. Moreover, the assigned bands between 2200 and 3700 cm^{-1} attributed to the vibration of OH or NH and CH stretching, indicating intra- or inter-molecular hydrogen bonds and available OH groups dissociated from hydrogen bonds of hydroxymethyl (CH_2OH) groups in chitosan. FT-IR was also able to determine DDA using adequate

equations for different samples [35, 36]. CI of chitin and chitosan could be also estimated by comparing peak intensities between the two functional groups, such as CH bending and C-CH_3 deformation (A_{1382}) and CH stretching (A_{2920}) [37].

X-ray Diffraction (XRD): Similar to FT-IR, XRD was able to determine CI of chitin and chitosan [37, 38]. The form of chitin was able to be identified based on the image plate of XRD, representing different diffraction patterns [39]. The investigation of XRD patterns at a wide angle (5-40 º, 2θ) could interpret the polymorphic structures and modifications based on the shape, the shift, and the intensity of appeared peaks [40, 41]. In addition, the *d*-spacing representing the space distance within the crystal plane, the apparent crystal size (D_{ap}), and the relative intensity (%) of various crystal planes (e.g. (020), (110), (120), (101), and (103)) were further examined to analyze the crystal properties of chitin and chitosan [42].

DIFFERENT CHARACTERISTICS BETWEEN β-CHITIN/CHITOSAN AND α-CHITIN/CHITOSAN

Since α-chitin/chitosan are commercially produced [43], most of studies have fairly focused on α-chitin/chitosan. In contrast, β-chitin/chitosan is not commercially available so far, thus their studies have received much less attention despite the industrial advantage and unique characteristics in structures and functional properties. To explore the investigation of β-chitin/chitosan, this review discussed structures, physicochemical properties, the extraction methods and procedures, and biological activities of β-chitin/chitosan and compared with those of α-chitin/chitosan.

Originated Nature Sources of α- and β-Chitin and their Polymorphic Structures: Table 2.1 summarizes the originated sources of α- and β-chitin. The α-chitin has more extensive nature sources than β-chitin. So far, β-chitin is mainly extracted from a few species of squid, including *Loligo sp.*, *Todarodes sp.*, and *Oligobrachia sp.*

The polymorphic structures of α- and β-chitin have been investigated by Dwelts [17], Minke [16], Gardener [44], and Blackwell [18] since 1960s. Subsequent researches were varied depending on the originated source and species, the extraction method, and the chemical derivative. Regarding to the properties of crystal structures, α-chitin has antiparallel polymeric sheets, making rigid formation by means of inter-sheet hydrogen bonds, whereas β-chitin consists of loose-packed crystal structure due to much weaker hydrogen bonds between the parallel polymeric sheets [16-19]. It was also demonstrated that crystallinity (CI) of α-chitin (28.3%) was higher than that of β-chitin (20.8%) [45]. Moreover, α- and β-chitin has different crystallographic characteristics. The α-chitin has the orthorhombic unit cell with dimensions of a=0.476 nm, b=1.885 nm, and c=1.028 nm and a space group of $P2_12_12$, whereas β-chitin shows the monoclinic unit with dimensions of a=0.485 nm, b=0.926 nm, and c=1.038 and a space group of $P2_1$. Hence, β-chitin has distinguished polymorphic structures from α-chitin, and these structural differences are strongly associated with other characteristics of chitin/chitosan, such as physicochemical properties, extraction procedures, and biological activities.

Reactivity of α- and β-Chitin toward Solvents and the Relationship with its Polymorphic Structure: To extract chitosan from natural sources, acid and alkali treatments are commonly applied to prepared raw materials. The extracting conditions

depend on the form of chitin due to the different polymorphic properties and its impact on the reactivity of chitin toward applied acidic or alkali solvents. β-Chitin has been found more reactive to acid and alkali treatments than α-chitin since applied solutions are easily able to penetrate into loose crystal structure and lower CI of β-chitin. Kurita et al. (1993 and 1994) found that unlike α-chitin, β-chitin could be swelled in methanol and pyridine solutions, was more susceptible to alkali solutions, and the derived β-chitosan had lower CI and loose crystallites than α-chitosan after the deacetylation process [6, 7]. The similar result was observed by Kumirska et al. (2010) [46]. Therefore, the utilization of squid pens can be cost-effective for producing β-chitin/chitosan since it needs smaller amount of reagents and shorter reaction times than crustacean shells to produce α-chitin/chitosan.

Extraction Procedures: Fig. 2.3 illustrates the extraction procedures of α- and β-chitin/chitosan from crustacean shells and squid pens, respectively. Typically, five consecutive processes are required to extract α-chitosan from crab and shrimp shells, including demineralization, deproteinization, decoloration, deacetylation, and depolymerization [2, 47, 48 49-51]. In contrast, only three stages are necessary to isolate β-chitosan from squid pens, including deprotenization, deacetylation, and depolymerization due to the ignorable mineral content and pigment in squid pens [4, 32, 52]. The extraction of β-chitin/chitosan from squid pens can be beneficial to the industrial-scale production since it reduces the chemical usage, production cost and time [43]. The following sections compared different extraction procedures between α- and β-chitin and discussed the structural and physicochemical properties of resulted products.

Deproteinization: To produce α- and β-chitin, deprotenization process is required to eliminate proteins greatly presented in both crustacean shells and squid pens. According to Abdou et al. (2008), the amount of protein was ~35% in shrimp shells, ~17% in crab shells, and ~45% in squid pens, respectively [52]. Proteins are soluble in alkali pH, hence lower concentration of alkali treatment under milder temperature can be applied to solubilize protein, whereas the insolubilized chitin is obtained after filtration and drying of the samples. However, for isolating α-chitin from crustacean shells, further processes including the demineralization and decoloration are required.

The optimal deprotenization conditions to obtain α- and β-chitin from different nature sources and species from previous studies are summarized in Table 2.2 [4, 13, 20, 53-58]. Lower NaOH concentrations and temperatures are needed to prepare β-chitin in comparison with that for preparing α-chitin, which might be related to the different polymorphic properties between α- and β-chitin as described above. The alkali deproteinization could be more susceptible to β-chitin than α-chitin since β-chitin had loose-packed crystallographic structures and lower CI.

It has been found that the deproteinizing reaction is divided into two stages (possibly three stages for crustacean shells) along with different reaction rate constants in each stage. According to Chaussard et al. (2004), 90% of proteins was eliminated from squid pens within 15 min in the first stage, whereas shrimp shells needed more than 2 h to reach the equivalent level of proteins similar to squid pens [55]. Hence, crustacean shells required three stages with longer reaction times than squid pens. In addition, β-chitin was successfully extracted from squid pens during the first stage of the process, thus the second stage had slow slop of the reaction constant rate. Likewise, the reaction rate

constant of the first stage was 10 times higher in squid pens than in shrimp shells [55, 59]. Therefore, the deproteinization of squid pens can be cost-effective as it requires smaller amounts of reagent and shorter reaction times than that of crustacean shells.

It is generally believed that the alkali treatment during the deproteinization process could induce the degradation of Mw and increase of DDA in resulting chitin [60-62]. However, α- and β-chitin were successfully obtained from spider crab shells, shrimp shells, and squid pens under the treatment of 0.3 N NaOH, preserving the original DA (94-100%) with no polymorphic modifications [21]. Moreover, biological methods including enzymatic treatments [63, 64] and microbial processes using *Lactobacillus* [65, 66] or *Pseudomonas aeruginos*a K-187 [67] have been applied to alternate chemical treatments.

According to No et al. (2003), the deproteinization of chitin enhanced the quality of derived chitosan, showing that fat or dye-binding capacities and antibacterial activity improved in chitosan extracted after the deproteinization process, compared with chitosan without the process [32]. Therefore, both crustacean shells and squid pens require the deproteinization process to prepare functional α- and β-chitin, but the optimal treatment conditions can depend on the originated nature sources and species.

Demineralization: This process removes greater amount of minerals existing in the crustacean shells. According to Kurita (2006), krill, crab, and shrimp shells consist of 20-50% mineral contents [68]. In contrast, mineral content in squid specie of *Loligo vulgaris* was only 1.70% [13] and slightly different depending on squid species [4]. By reason of this unique characteristic in squid pens, Chandumpai et al. (2004) [4], Chaussard et al. (2004) [59], and Lamarque et al. (2004) [19, 69] successfully produced acceptable β-chitin from squid

pens without the demineralization process. Therefore, the utilization of squid pens as the raw material of β-chitin/chitosan can assist not only to reduce the extracting costs as the demineralization step is not required, but also to produce higher Mw of β-chitin in comparison with α-chitin causing the acid hydrolysis during the demineralization process. Similarly, Tolaimate et al. (2003) demonstrated that Mw of β-chitin is 2-3 times higher than that of α-chitin.

Decoloration: α-chitin/chitosan can be tinted due to the presence of carotenoid (e.g. astaxanthin) compounds in the crustacean shells [53, 70], therefore, the decoloration step is required to eliminate carotenoids to produce commercially acceptable chitin and chitosan. In contrast, β-chitin/chitosan extracted from squid pens is neither pigmented nor contaminated by heavy metals since the amount of carotenoid is negligible in squid pens [32, 52]. According to Tolaimate et al. (2003) and Abdou et al. (2008), colorless β-chitin was successfully extracted from squid pens without the decoloration process, whereas the crustacean shells had to process pinkish α-chitin under the bleaching or the oxidizing treatment ($KMnO_4$ + oxalic acid + H_2SO_4) [13, 52].

Deacetylation: To obtain chitosan, chitin is deacetylated by using strong alkali treatments. The *N*-aceetyl-D-glucosamine (NADG) monomer in polymeric chains of chitin are hydrolyzed and transformed into D-glucosamine (DG) in polymeric chains of chitosan. The deacetylation has been commonly done by two methods, homogeneous and heterogeneous processes based on deacetylating mechanisms. For the homogenous deacetylation, swelling and dissolving of chitin suspended in the intermediate

concentrations of alkali solutions led to homogenous amorphous polymorphic chitin through freeze-thaw cycle, and resulted in chitosan with the regular distribution of the residual *N*-acetyl-D-glucosamine along the polymeric chains [71]. In contrast, the heterogeneous deacetylation at high concentrations of alkalis (40-50% NaOH or KOH) with high temperature (~90-100 ℃) was more rapidly induced in amorphous regions than crystallographic parts and the residual $COCH_3$ groups were randomly distributed along the polymeric chains. The heterogeneous deactylation has been widely investigated and used in the industrial extraction.

The optimal deacetylating treatments have been studied in α- and β-chitin to produce high DDA of chitosan. Focher et al. (1990) successfully extracted almost free *N*-acetyl groups of α-chitosan by the treatment of 40% NaOH for 30-270 sec at the saturated high temperature stream (140-190 ℃) [37]. Similarly, high DDA of α-chitosan was isolated from black tiger shrimp shells under the treatment of 50% NaOH for 45 min at 100 ℃ by using the intermediate washing [72]. In addition, preconditioning with 0.016 M benzoic acid to α-chitin helped to increase DDA of α-chitosan [73]. The deacetylation process altered not only DDA, but also structures, physicochemical properties, and biological activities of α-chitosan. According to Burkhanova et al. (2000), the crystal structure of α-chitin was largely degraded into amorphous α-chitosan as the result of strong alkali treatments. It was also reported that the inter-molecular hydrogen bonds weakened as DDA increased [74]. The polymorphic structure and DDA of chitosan extracted from lobster chitin were similar to those of commercial chitosan extracted from crab and shrimp shells [75]. Tajik et al. (2008) applied different deacetylating treatments to α-chitin, demonstrating that physicochemical (e.g., minerals, nitrogen, and Mw) and biological properties (e.g., water-

binding capacity and antibacterial activity) of α-chitosan depended on the combination of treatment conditions [43]. Similarly, Hongpattarakere et al. (2008) reported that the antibacterial activity of chitosan was enhanced in higher DDA of chitosan processed in higher concentration of alkali and temperatures with longer reaction times [76]. However, since the Mw of chitosan could be decreased with prolonging reaction times, the deacetylating time should be adjusted to extract high Mw with acceptable DDA[57]. The enzymatic deacetylation was also utilized to alternate chemical methods for lessening the polymer degradation and environmental concern [77].

With regards to β-chitin, high DDA and Mw of β-chitosan could be extracted by using two different types of reagents, so-called Kurita method with NaOH and Broussignac method with KOH [78]. Similar to α-chitin, Methacanon et al. (2003) demonstrated that DDA of β-chitosan increases with higher alkali concentrations and temperatures along with longer reaction times. In addition, various optimum conditions for obtaining 90% DD chitosan were predicted from the best fit regression equation[79]. Recently, Delezuk et al. (2011) extracted high DDA (~93%) of β-chitosan by using the ultrasound-assisted deacetylation method for 30 min at 50–80 ℃ [80]. In addition to the changes of DDA and Mw, structural and physicochemical properties of the resulted β-chitosan were altered after the deacetylation of β-chitin. Chandumpai et al. (2003) reported that chitosan obtained from squid pens was classified as 'Class III: moderately hygroscopic', where moisture contents of β-chitosan essentially increased under the condition of 80% relative humidity [4]. Chen et al. (2004) investigated the structural changes of chitosan at different DDAs and found that a specific absorption peak of NH_2 in FT-IR spectrum significantly increases as DDA increased. XRD patterns of

deacetylated chitosan with DD values of 17.5 and 44.7% have three significant diffraction peaks, whereas there are only two diffraction peaks in chitosan with higher DDA of 76.5 and 94.7% since the crystallographic structure of chitosan collapsed in high DDA of β-chitosan [4, 81].

The deacetylating characteristics and resulting chitosan can be different between α- and β-chitin due to the impact of different polymorphic structures on the alkali reactions. DDA of derived chitosan was compared between α- and β-chitin processed under same treatment conditions and is summarized in Table 2.3. Overall, it was observed that DDA of β-chitosan was higher than that of α-chitosan. As previously mentioned, the crystallographic structure of β-chitin is less rigid with much weaker inter-molecular hydrogen bonds than that of α-chitin, allowing alkali solutions to penetrate easier into crystal lattice of β-chitin [4, 13, 19]. Hence, the same deacetylating treatment was able to produce higher DDA of chitosan in β-chitin than chitosan from α-chitin. Similarly, Rhazi et al. (2000) demonstrated that β-chitin was more reactive to alkali treatments than α-chitin, obtaining higher DDA of β-chitosan within 2 h. No further increase of DDA appeared after 2 h, and Mw of β-chitosan degraded with prolonging reaction times [21]. Tolaimate et al. (2003) reported that Mw and DDA of β-chitosan was higher than those of α-chitosan under the same deacetylating treatment [13]. By deacetylating at 121 ℃ for 15 min, β-chitosan with about 90% DDA was extracted from squid pens, and DDA of β-chitosan was higher than that of α-chitosan [52]. To extract similar DDA of α- and β-chitosan, β-chitin required lower NaOH concentrations and shorter reaction times [82]. The structural modifications were also compared between α- and β-chitin during the deacetylation process. The crystallographic β-chitin became amorphous faster and easier

by means of milder treatments along with a statistical distribution of DG and NADG in the polymeric chains of β-chitosan. In contrast, the deacetylating reaction of α-chitin was mainly induced in amorphous regions and each monomer was randomly distributed along the polymeric chains of α-chitosan, causing less polymorphic modification in comparison with β-chitin [19, 69]. The influence of NaOH concentration (30-55% (w/v)) and sodium hydroxide hydrates depended on the form of chitin, reporting that mono- and di-hydrates were more reactive in β-chitin than in α-chitin[83].

In summary, DDA of chitosan extracted from β-chitin was higher than that of chitosan from α-chitin under the same deacetylating conditions. Likewise, the deacetylating treatment of β-chitin could be relatively milder than that for α-chitin to prepare similar DDA of chitosan. Therefore, structural and physicochemical properties of α- and β-chitosan can be different since α- and β-chitin are able to respond differently to the deacetylation process due to the different structures between the two forms of chitin.

Depolymerization: The depolymerization process is selectively carried out to produce a certain range of Mw, depending on purposes of applications. It has been reported that the influence of Mw on the biological activities of chitosan varies with several factors [84-89]. As it was reported, Mw of native chitin/chitosan is larger than 1000 kDa, the depolymerizing process is required to produce the commercial chitosan with Mw of 100-200 kDa [88, 90]. Hence, α- and β-chitosan need to process the depolymerization step to enhance their functionality.

Enzymatic, chemical, and physical hydrolysis are the three common methods for deploymerization of chitosan. Although the chemical method is faster and cheaper than

other methods, it has limited control over the extent of hydrolysis due to its harsh conditions as well as environmental concern of using high concentrations of chemical reagents [91]. The physical method can result in irregular Mw, but it is safe and easy to handle [92]. Finally, the enzymatic method is able to compensate the disadvantages referred in other two methods and is more applicable as it controls the extent of reaction with less environmental and safety concerns.

The depolymerization of β-chitosan has been investigated in a few studies. The chemical hydrolysis by using hydrogen peroxide induced much faster degradation of β-chitosan than the ultrasonic hydrolysis, and resulted in high efficiency similar to the enzymatic hydrolysis [93]. Kurita et al. (2000) investigated the impact of DDA on the depolymerizating efficiency of β-chitosan processed in lysozyme hydrolysis. The degradation of β-chitin was greater with decreasing DDA due to the higher affinity of lysozyme to lower DDA of β-chitosan [94]. The ultrasound-assisted depolymerizing method could produce lower Mw β-chitosan, lasting primary structures of chitosan [95]. In addition, *Trichoderma harzianum* chitinase hydrolysis was able to produce oligosaccharides (< 10 kDa) from β-chitosan, which exerted high antibacterial effects against various bacterial strains [24].

The depolymerization reaction between α- and β-chitosan can be different due to their different structural properties, which in turn may impact the degree of solubility and chitosan conformations in the solution. However, no previous study has demonstrated the different characteristics of depolymerizing reactions between α- and β-chitosan.

Biological Properties: Since the different structural and physicochemical properties between α- and β-chitin can alter the degree of deacetylation and/or chitosan conformations in the solution (Table 2.4), the biological activity of the resulting chitosan can vary depending on the form of chitosan. The antioxidant and antimicrobial activities were the two biological functionalities discussed in this review as they are the most interesting functional properties of chitosan. To exert antioxidant properties, the hydroxyl (OH) groups in C_3 and C_5 and amino (NH_2) groups in C_2 need to donate available hydrogen and unpaired electrons to scavenge free radicals and to chelate metal ions for preventing oxidative stress [24, 96, 97]. Antimicrobial mechanisms of chitosan have been proposed as the interactions between the protonated amino groups (NH_3^+) and negatively charged cell membranes [98], the destruction of the cell wall by chelation of the metals [99], and the formation of a physical barrier preventing the uptake of nutrients into the cell, resulted in cell death due to the alteration of cell permeability [100]. Hence, the functional OH and NH_2 groups in DG and NADG monomers are responsible for exerting the antioxidant and antimicrobial effect in chitosan [97]. However, the polymorphic structures of chitosan consisting of crystallographic region with strong inter-molecular hydrogen bonds and amorphous regions with interactions between polymeric chains can alter the antioxidant and antimicrobial activities of chitosan solution. Regarding to the availability of those functional groups, hydrogen or unpaired electrons can be less available as the result of forming hydrogen bonds and the interactions of polymeric chains in the solution. For these reasons, the antioxidant and antimicrobial activities can be different between α- and β-chitosan since the derived α- and β-chitosan through the deacetylation and depolymerization processes can have different polymorphic structures at similar DDA

and Mw. However, no previous studies have compared the antioxidant and antimicrobial properties between α-and β-chitosan prepared with similar DDA and Mw.

Antioxidant and antimicrobial studies of β-chitosan have been rare in comparison with those of α-chitosan. Huang et al. (2011) investigated the effect of Mw on antioxidant activity of β-chitosan and demonstrated that the radical scavenging activity, the ferrous ion chelation, and the reducing power are higher in 9-13 kDa samples than those in higher Mw of β-chitosan [85]. The same researchers also found that the TEMPO (2,2,6,6-tetramethylpiperidine -1-oxyl radical)-mediated 6-carboxyl β-chitin derivative (T-β-chitin) enhances the radical scavenging activity and ferrous ion chelating ability [101]. On the subject of the antimicrobial activity in β-chitosan, a 220 kDa sample greatly inhibited the growth of *Streptococcus mutans* GS-5 among 10-426 kDa, and the inhibitory effect against various oral *Streptococcus* species was increased at higher DDA of β-chitosan [102]. According to Lin et al. (2009), 90% DDA of chitooligosaccharides (COS, <10 kDa) derived from β-chitosan prevented the growth of all Gram-negative strains, whereas it was only effective to *Staphylococcus xylosus* among various Gram-positive strains [24]. However, Huang et al. (2011) demonstrated the opposite trend in respect to the effects of DDA and Mw versus the findings of Shimojoh et al. (1996) and Lin et al. (2009). They found that antimicrobial activity was enhanced in higher Mw and lower DDA of β-chitosan and *Staphylococcus aureus* (Gram-positive strain) was more susceptible to β-chitosan than *Escherichia coli* (Gram-negative strain).

In respect to the antimicrobial properties, Kim et al. (2007) compared the antibacterial effects of α- and β-chitosan coating solutions. Among 282, 440, 746, and 1,110 kDa of α-chitosan and 577 kDa of β-chitosan, 282 kDa of α-chitosan solubilized in pH 4.5 solution

exerted the highest inhibitory effect against *S. enteritidis* and also preserved the internal quality of eggs [103], whereas β-chitosan showed higher antibacterial effect against *S. aureus, B. cereus,* and *B. subtilis* than α-chitosan. In addition, 50-80 kDa β-chitosan exerted stronger antibacterial activity against *V. parahaemolyticus* than 1200-1700 kDa of β-chitosan [88].

In the application studies, the different characteristics have been found between α- and β-chitosan. To develop water-soluble chitosan by using the Maillard reaction, it was observed that α-chitosan can be more suitable than β-chitosan since the derived water-soluble α-chitosan exerted higher yield and solubility than β-chitosan [104]. However, Oh et al. (2011) indicated that β-chitosan was able to form physical hydrogel unlike α-chitosan since the loose crystallographic β-chitosan was more able to accept water through the lattice and to swell in water than rigid crystallographic α-chitosan [105]. According to Chen et al. (2012), β-chitosan films showed the compatible physicochemical properties and antibacterial activity to α-chitosan films [26].

In summary, the biological properties of α-chitosan are well known. Various factors, including physicochemical properties of chitosan (DDA and Mw), microbial characteristics (microbial species and its hydrophilicity), and environmental conditions (pH and temperatures) have been investigated in association with the biological properties of α-chitosan. However, the biological activities of β-chitosan are rarely investigated and the limited results are controversial. Hence, it is necessary to fully understanding the biological activities of β-chitosan in respect to its structural differences from α-chitosan.

CONCLUSION

Due to the unique chemical composition of squid pens with negligible amounts of mineral and carotenoid, producing β-chitin/chitosan can be cost-effective as the demineralization and decoloration processes are not required in comparison with preparing α-chitosan from crustacean shells. In addition, the polymorphic β-chitin with loose crystallographic structures and lower CI are more susceptible to the alkali deproteinization and deacetylation reactions than the rigid crystallographic α-chitin, thus resulted in different structures and functional properties between derived α- and β-chitosan. Despite the commercial advantages of preparing β-chitin/chitosan and their functional properties, the studies on β-chitin/chitosan have been paid much less attention than the studies on α-chitin/chitosan. Therefore, it is important to investigate the functional properties, especially the antioxidant and antimicrobial activities, in association with the structural properties of β-chitosan.

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Table 2.1 The list of originated nature sources of α- and β-chitin

Researchers	Year	Form of chitin	Nature sources	Species
Dweltz	1960	β-chitin	Squid	*Loligo sp.*
Blackwell	1968	β-chitin	Squid	*Oligobrachia ivanovi*
Minke	1978	α-chitin	Lobster	*Homarus americanus*
Saito et al.	1997	α-chitin	Tubes	*Tevnia jerichonana*
Rhazi et al.	2000	α-chitin	Marbled crab	*Grapsus marmoratus*
			Red crab	*Portunus puber*
			Spider crab	*Maia squinado*
			Lobster	*Homarus vulgaris*
			Locust lobster	*Scyllarus arctus*
			Spiny lobster	*Palinurus vulgaris*
			Crayfish	*Astacus uviatilis*
			Shrimp	*Palñmon fabricius*
			Squilla	*Squilla mantis*
			Cuttlefish	*Sepia officinalis*
		β-chitin	Squid	*Loligo vulgaris*
Noishiki et al.	2003	α-chitin	Centric diatom	*Thalassiosira weissflogii*
Cardenas et al.	2004	α-chitin	Red shrimp	*Pleuroncodes monodon*
Lavall et al.	2007	β-chitin	Squid	*Loligo sp.*
Yen et al.	2008	α-chitin	Snow crab	*Chionoecetes opilio*
Fan et al.	2008	α-chitin	Crab	*Chionoecetes opilio*
			Tubeworm	*Lamellibrachia satsuma*
		β-chitin	Squid	*Todarodes pacificus*
Sagheer et al.	2009	α-chitin	Grooved Tiger Prawn	*Penaeus semisulcatus*
			Jinga Shrimp	*Metapenaeus affinis*
			Blue swimming crab	*Portunus pelagicus*
			Scyllarid Lobster	*Thenus orientalis*
			Cuttlefish	*Sepia sp.*
Palpandi et al.	2009	α-chitin	Shell	*Neritina crepidularia*
Pacheco et al.	2011	α-chitin	Shrimp	*Litopenaeus vanameii*
Delezuk et al.	2011	β-chitin	Squid	*Loligo sp.*
de Andrade et al.	2012	α-chitin	Shrimp	*Litopenaeus vanammei*
			Crab	*Ucides cordatus*
Yeon et al.	2013	β-chitin	Squid	*Todarodes pacifica*

Table 2.2 Comparison of deproteinizing treatments between α- and β-chitin

Forms	Resesarchers	Year	Nature sources (species)	NaOH Cont. (%)	Times (min.)	Temp (℃)	Ratios (g/mL)
α-chitin	No et al.	1989	Crawfish shells (Unidentified)	3.5	120	65	1/10
	Chang et al.	1997	Shrimp shells (*Synuchus sp.*)	10	360	75	1/5
	Percot et al.	2003	Shrimp shells (*Parapenaeopsis sp.)*	4	1440	70	1/15
	Naznin R.	2005	Shrimp shells (*Metapenaeus sp.*)	6	-*	Room	-
	Yen et al.	2009	Crab shells (*Chionoecetes sp.*)	4	180	100	1/10
	Bolat et al.	2010	Crab shells (*Potamon sp.*)	3	10	121	1/10
β-chitin	Tolaimate et al.	2003	Squid pens (*Loligo sp.*)	1.2	1440	Room	1/15
	Chandumpai et al.	2004	Squid pens (*Loligo sp.*)	4	300	50	1/13
	Jang et al.	2004	Squid pens (*Loligo sp.*)	4	1440	10	-

* No regarding information was reported.

Table 2.3 Comparison of the degree of deacetylation (DDA, %) between α- and β-chitin under same deacetylating treatments

Researchers	Year	Treatments	DDA (%)	
			α-chitin	β-chitin
Kurita et al.	1993	30% NaOH, 100 ℃	20	70
Rhazi et al.	2000	50% KOH in 25% ethanol (25 w/w%) and 25% monoethyleneglycol, 120℃ for 24 h	84-97	97-99.5
Tolaimate et al.	2003	50% KOH in 25% ethanol (25 w/w%) and 25% monoethyleneglycol, 120℃ for 2 h	55 & 83.5	95
Sagheer et al.	2009	45% NaOH, 110 ℃ at 2 h	80-85	>90
Santhosh et al.	2010	40% NaOH, room temperature for 7 d	~74	~90
		50% NaOH, room temperature for 7 d	~78	~92

Table 2.4 Comparison of the structural characteristics and physicochemical properties between α- and β-chitin/chitosan

	Structural properties					Physicochemical properties			
	Crystal imperfection	CI*	Hydrogen bonds	Chitosan conformations	Distribution of acetyl groups	DDA**	Reactivity	Solubility	Mw
α-chitin	↓	↑	↑	Rigid	Random	↓	↓	↓	↓
β-chitin	↑	↓	↓	Flexible	Regular	↑	↑	↑	↑
α-chitosan	↓	↑	↑	Rigid	-	↓	↓	↓	↓
β-chitosan	↑	↓	↓	Flexible	-***	↑	↑	↑	↑

* CI: crystallinity index (%)

** DDA: degree of deacetylation (%)

*** No previous study was investigated.

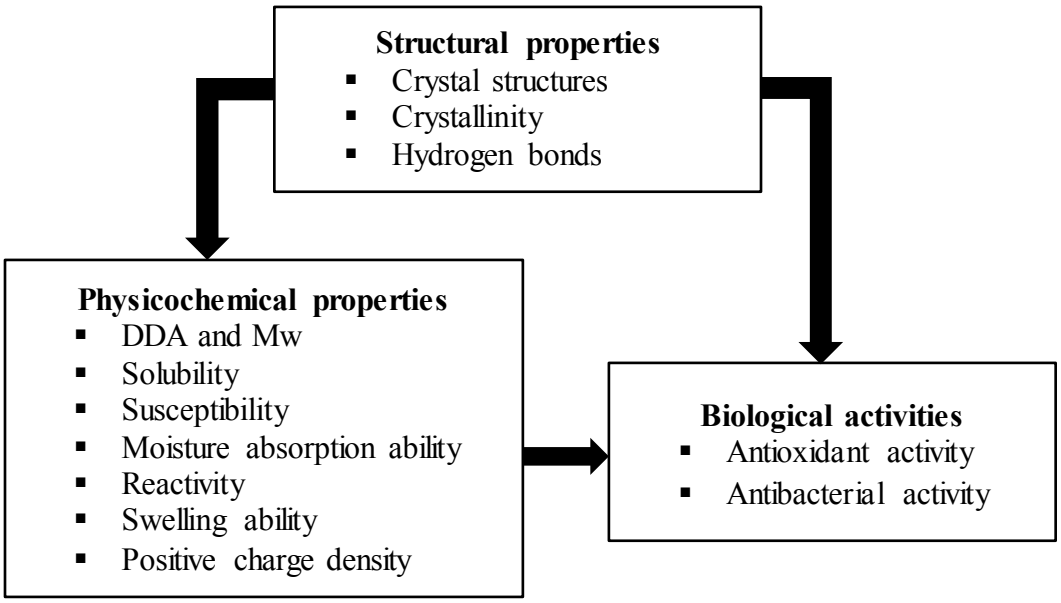

Fig. 2.1 Important structural, physicochemical properties, and biological activities of α- and β-chitin/chitosan

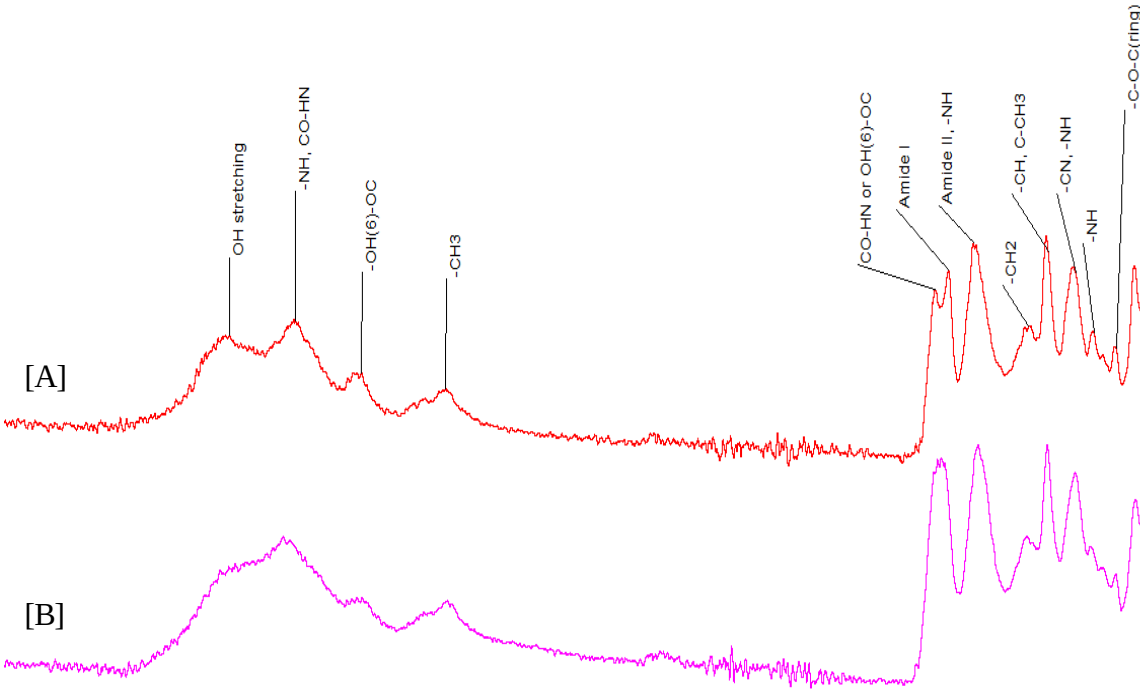

Fig. 2.2 The comparison of FT-IR spectra between α-chitin from shrimp shells (A) and β-chitin from squid pens (B), the identification of FT-IR bands were based on Cárdenas et al. (2004)

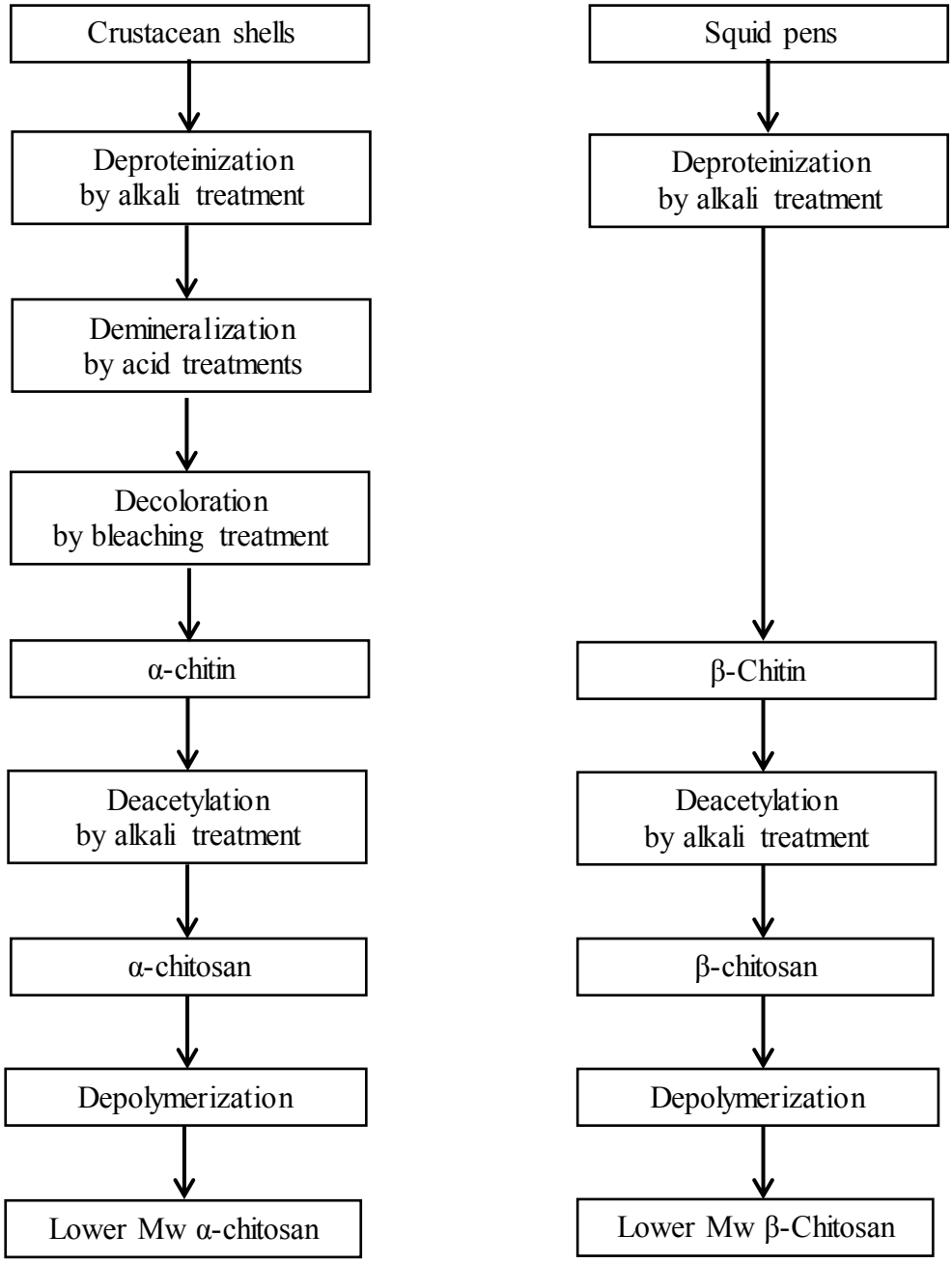

Fig. 2.3 Differences in the extraction procedures between α- and β-chitin/chitosan isolated from crustacean shells and squid pens, respectively.

CHAPTER 3

CHARACTERISTICS OF DEACETYLATION AND DEPOLYMERIZATION OF β-CHITIN FROM JUMBO SQUID PENS (*DOSIDICUS GIGAS*) PENS

Jung, J., Zhao, Y. Published in *Carbohydrate Research* **2011,** 346, 1876-1884.

ABSTRACT

The jumbo squid (*Dosidicus gigas*) pens are good sources of β-chitin. Compared with α-chitin that are usually obtained from shrimp and crab shells, β-chitin is more soluble and reactive to solvent due to its parallel structure and no inter-hydrogen bonding, making it more reactive and affinitive toward solvents, thus more applicable. However, no reported study has investigated the deacetylation and depolymerization characteristics of β-chitin from jumbo squid pens to obtain β-chitosan. This study evaluated the deacetylation process of β-chitin from jumbo squid pens by using strong alkaline reagent of NaOH or KOH. Taguchi design with the orthogonal array was employed to identify the contributing factors on molecular mass (MM) and degree of deacetylation (DDA) of obtained chitosan, including reagent concentration (40, 50%), treatment temperature (60, 90 ℃), time (2, 4, 6 h), and step (1, 2, 3) for NaOH treatment and temperature (90, 120 ℃), time (2, 4, 6 h), and step (1, 2, 3) for KOH treatment. Obtained chitosan was further depolymerized by using cellulase or lysozyme. The optimal treatment conditions for achieving high MM and DDA of chitosan were identified as: using 40% NaOH at 90 ℃ for 6 h with 3 separate steps (2h + 2h + 2h) or 50% NaOH at 90 ℃ for 6 h with 1 step, or using 50% KOH at 90 ℃ for 4 h with 3 steps (1h + 1h + 2h) or 6 h with 1 step. The most contributing factor affecting DDA and MM was temperature and time, respectively. Cellulase resulted in higher degradation ratio, lower relative viscosity, and larger amount of reducing end formations than that of lysozyme due to its higher susceptibility. The fast degradation was observed during the initial 1 ~ 2 h enzymatic treatment. This study demonstrated that the jumbo squid pens are good sources of materials to produce β-chitosan with high DDA and a wide range of MM for various applications.

Key words: jumbo squid pens, β-chitosan, deacetylation, depolymerization

INTRODUCTION

Chitin is a natural polysaccharide with a monomer of 1, 4-linked 2-acetamido-2-deoxy-D-glucan. It has been known as the secondary abundant polymer after cellulose [1]. Chitin exists in three forms as α, β, and γ, in which α-chitin is mainly from shrimp, crab, and krill shells, β-chitin is from squid pens, and γ-chitin is usually from fungi and yeast with its morphology appeared as the combination of α and β structures. The β-chitin is more reactive and affinitive toward solvents due to its parallel structure and no inter-hydrogen bond, making loose binding between the molecules, while α-chitin has anti-parallel structure with inter-hydrogen bonds [2, 3].

Chitin is insoluble in most organic solvents, such as water, alcohol, acetone, hexane, diluted acid, and diluted and concentrated alkaline due to the highly extended hydrogen bonded semi-crystalline structure dominated by the strong CO-HN hydrogen bonds showing the distance about 0.47 nm. [4, 5] For being applicable, the acetyl groups (-COCH3) in chitin need to be removed through a processed called "deacetylation" to obtain chitosan that is soluble in diluted acids. When the majority of the monomers is D-glucosamine (DG) with $-NH_2$ group in C_2 position instead of N-acetyl-D-glucosamine (NAG) taking up of $–NH-CO-CH_3$ group, chitosan becomes highly soluble. Several approaches may be employed to deacetylate chitin, including alkaline deacetylation, [6] intermittent water washing, [7] use of organic solvent, [8] flash treatment, [9] and enzymatic deacetylation. [10] Among them, the alkaline deacetylation using strong alkaline reagents of NaOH or KOH has been mostly used, in which the specific method is usually named by the name of the principal researcher, such as Broussignac method using KOH, [11] and Kurita method using NaOH. [12] Both alkaline could detach acetyl groups from NAG by the nucelophilic addition of hydroxide ions

to carbonyl groups, thus separating it into $-CH_3COO^-$ and NH_2. However, using NaOH or KOH can result in different deacetylating processes, obtaining chitosan with different functional properties. [2,11,12] This may be due to the solubility differences between NaOH and KOH in the organic solvents because of their different dielectric constant. For examples, the dielectric constant of NaOH is 80.1 in water, much higher than that of KOH, which is 25.3 in ethanol and 41.4 in ethylene glycol at 298 K when used for deacetylating chitosan. In the transition state of the deacetylation reaction, the development of ionic charges was initiated by the nucleophilic addition of amide functions on carbonyl groups and favored by the high dielectric constant solvent. [2]

The functionality of chitosan is affected by several factors, including the source of the raw material, molecular mass (MM), degree of deacetylation (DDA), and its physical state (conformation, particle size, etc.). [13-15] Among them, MM and DDA may be mostly critical. Therefore, it is sometimes necessary to depolymerize native chitosan into low molecular mass, a processed called "depolymerization". Low MM chitosan performed better for the drug delivery because of their high solubility. [16] Chitosan depolymerized by different enzymes showed different antimicrobial functions, in which chitinase depolymerized chitosan had stronger inhibition against gram-negative bacteria, while lysozyme depolymerized chitosan was more effective against gram-positive bacteria. [17] In a MM range of $5 \times 10^3 \sim 9.16 \times 10^4$, the antimicrobial activity of chtiosan increased along with increased MM, while in the MM range of $9.16 \times 10^4 \sim 1.08 \times 10^6$, it decreased as MM increased. [18] Different MM of chitosan (1.74, 2.36, and 3.07 g/mol) also showed different antifungal function against *Rhizopus stolonifer*, in which the lower MM was more toward against mycelia growth while the higher MM inhibited the mold germination. [19] Chitosan with a MM

of 71 kDa was less inhibitory against *Bacillus cereus* and *Escherichia coli* than those with MM of 4.74-10 kDa. [20] Therefore, controlling MM of chitosan is necessary for achieving the most effective antimicrobial activity. DDA of chitosan also impacts its properties. Chitosan with 99% DDA showed the highest inhibition against the growth of both gram-negative and gram-positive bacteria, [24] and 90% DDA chitosan had higher reactive oxygen scavenging activity than that of 50% DDA chitosan. [24] In addition, increasing DDA improved mechanical properties (tensile strength, elongation, and young's modulus) of β-chitosan based films. [21, 22, 23]

Depolymerization may be achieved by enzymatic, chemical, and physical method or their combinations. Chemical depolymerization had limited control in the extent of depolymerization due to its harsh condition along with environmental concern of using high concentration of chemical reagents. [24] Physical method, such as ultrasonically assisted treatment, resulted in irregular molecular mass consistently. [25] In contrast, enzymatic method is more applicable due to its controlled extent of reaction. [26]

Previous studies on β-chitosan from squid pens were from squid species of *Loligo lessoniana, Loligo formosana, Loligo vulgaris, Ommasterphes bartrami,* and *Illex argentines*. [2-4, 22, 23] However, the catch of jumbo squid (*Dosidicus gigas*) had increased significantly during 1991 to 2002, and became the third largest amount worldwide in 2002 (406,356 tons, 12.8%) after *Illex argentines* (511,087 tons, 16.1%) and *Todarodes pacificus* (504,438 tons, 15.9%). In spite of its increased production, study using jumbo squid pens as material for producing chitin and chitosan was rarely. As it is well known, the raw materials significantly impact the deacetylation process of chitin and the functionality of resulted chitosan. Therefore, this study aimed to

investigate the optimal deacetylation procedure of β-chitin from jumbo squid pens and the enzymatic depolymerization of obtained β-chitosan to produce a series of low MM chitosan material. Different factors potentially contributing to the deacetylation process of chitosan, including the type and concentration of alkaline, reaction temperature, time, and treatment step were considered statistically. Based on our best knowledge, no study has reported the deacetylaiton and deploymerization characteristics of β-chitin from jumbo squid pens, neither considering all these major contributing factors statistically.

MATERIALS AND METHODS

Materials

The β-chitin from jumbo squid (*Dosidicus gigas*) pens was provided by Dosidicus LLC, USA. Samples were ground into about 18 mesh size by a grinder (Glenmills Inc., USA) and stored inside a desiccator till deacetylation treatment. Sodium hydroxide (NaOH) and potassium hydroxide (KOH) were purchased with pellet type from the Mallinckndt Chemicals Co. (USA). N-acetyl-D-glucosamine and monoethylene glycol were from Sigma Chemical Co. (USA). Toluidine blue indicator and 1/400 potassium polyvinyl sulfate (PVS) were from Wako Chemicals (USA). Hen egg white lysozyme and cellulase from *Aspergillus niger* were obtained from Fordras S.A. (SWISS) and TCI America (USA), respectively. All chemicals were of reagent grade.

Deacetylation of Chitin

In this study, two different alkaline deacetylation processes were carried out: Kurita method using NaOH as reagent and Broussignac method using KOH, named by the names of the primary researchers. Depending on the type of chitin and alkaline reagents employed, the deacetylation process may respond differently. Using NaOH might result in β-chitosan with higher DDA and MM in the moderate treatment condition (40% NaOH at 80 ℃) as compared with the use of KOH.[2,11] However, the opposite behavior might be observed on α-chitin at the same treatment condition.[2] Hence, it is necessary to investigate which alkaline solvent performs more appropriately on β-chitin from jumbo squid pens.

While using Kurita method,[27] NaOH was first diluted to targeted concentrations by dissolving in distilled water. Chitin was then added into NaOH solution at a ratio of 1:20. Four treatment factors, including NaOH concentration (40, 50%), temperature (60, 90 ℃), time (2, 4, 6 h), and treatment step (1, 2, 3 times) were investigated. The typical deacetylation condition for α-chitin from shrimp or squilla shells were using 40-50% NaOH at 80-100 ℃ for 6-12 h.[2] Since the squid pens from the species of *Dosidicus gigas* are much smaller and thinner than other frequently used ones (*Loligo or Illex pens*), the mild treatment condition could be applied. The multiple treatment steps at same treatment time were evaluated for the possible prevention of chitosan degradation during deacetylation.[2] After NaOH treatment under given conditions, samples were washed with distilled water to reach neutral pH and the remained residue was washed out with methanol and acetone. Samples were then dried at 50 ℃ in a dry oven (Precision Scientific Inc., USA) for 24 h.

In Broussignac method,[11] KOH (50% w/w) was dissolved in a mixture of 96% ethanol (25% w/w) and monoethylene glycol (25% w/w) solution. Chitin was then

added into the solution mixture at a ratio of 1:20. According to the previous study, Broussignac process required a minimal treatment condition of 90 ℃ for 2 h to obtain soluble chitosan in 1% acetic acid. [2] Therefore, three treatment factors including temperature (90, 120 ℃), time (2, 4, 6 h), and treatment step (1, 2, 3 times) were considered. After the treatment, same washing and drying procedures as those in the Kurita method were applied.

DDA, intrinsic viscosity, and MM obtained at each treatment condition were determined using the methods described below. Taguchi experimental design with orthogonal arrays was applied for each method (Table 3.1). By using orthogonal arrays, it was expected that the optimal treatment conditions for obtaining chitosan with high DDA and desirable MM values can be identified with minimal treatment combinations (9 in this study) in each method. The most contributed factors and their levels could be identified through the Taguchi design.

Depolymerization of chitosan

High molecular mass (HMM) and low molecular mass (LMM) chitosan prepared from the deacetylation study were depolymerized by using cellulase or lysozyme, known to have different susceptibilities based on the properties of chitosan. Chitosan samples were dissolved in 5% acetic acid solution at a ratio of 2:100 (chitosan: solvent). Cellulase or lysozyme was added into the solution at 1% (w/w) and reacted for up to 7 h. A 20 ml of solution was taken out hourly and then boiled for 10 min to stop the enzymatic reaction. Sodium hydroxide was then added into the solution to reach a final pH 11 for precipitation. Precipitated samples were washed with distilled

water to remove other residues and dried in a 42 ℃ oven for 24 h. MM and DDA of the depolymerized chitosan were measured at each sampling time.

Measurement of Degree of Deacetylation

DDA was measured by using the colloidal titration method. [6] A 50 mg of deacetylated chitosan (0.5%, w/w) was dissolved in 10 ml of 5% (v/v) acetic acid solution, and then transferred into a flask and diluted up to 30 ml with distilled water. After adding 100 µl of toluidine blue indicator, the solution was titrated by the 1/400 potassium polyvinyl sulfate (PPVS) till the solution color changed from blue to violet. DDA was calculated as:

$$DDA\ (\%) = (X/161) / (X/161) + (Y/203) \quad (1)$$

$$X = 1/400*1/1000*F*161*V \quad (2)$$

$$Y = 0.5 * 1/100 - X \quad (3)$$

where X was the weight of D-glucosamine residue, g; F was the factor of 1/400 PVS; V was the volume of consumed PPVS, ml; Y was the weight of N-acetyl-D-glucosamine residue, g; and 161 and 203 in Equ. (1) was the molecular weight of D-glucosamine and N-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucose), respectively.

Determination of Intrinsic Viscosity and Viscosity-Average Mw

Intrinsic viscosity of chitosan was determined by using the Ubbelohde Dilution Viscometer (Cannon instrument Co., USA) that has the capillary size of 0.58 mm in a water bath at 25 ℃. Viscosity-average molecular mass (MM) was then calculated from measured intrinsic viscosity. Solutions used for measuring MM of chitosan

samples from deacetylation and depolymerization treatments were different. This is mainly because chitosan samples obtained from the deacetylation and depolymerization experiments had different DDA, thus required different strengths of acid to completely dissolve for ensuring accurate MM measurement. Our preliminary studies (data not shown) evaluated three types of acid solution that are commonly used for measuring MM in chitosan: 0.1M CH_3COOH, 0.1M CH_3COOH /0.2M NaCl, and 0.3M CH_3COOH /0.2M CH_3COONa, and found that 0.1M acetic acid is able to dissolve all chitosan samples, thus was chosen for measuring MM of chitosan samples in the deacetylation study. The same test was carried out for identifying the solution in measuring MM of depolymerized chitosan by focusing on finding more precise measurement of MM using a commercial chitosan with known MM as an indicator. The 0.1M CH_3COOH/0.2M NaCl solution provided the closest MM value as that reported on the commercial chitosan.

Four different concentrations of chitosan in a range of 0.05% ~ 0.1% were used for measuring the viscosity of the samples. The intrinsic viscosity was measured by the intercept between the Huggins (reduced viscosity, $\eta_{sp}/C \sim C$) and Kraemer (relative viscosity, $\eta_{rel}/C \sim C$) plots when the concentration was 0 [16]. Relative viscosity, reduced viscosity, and intrinsic viscosity were determined as:

$$\eta_{rel} = t/t_0 \quad (4)$$

$$\eta_{sp} = \eta_{rel} - 1, \ \eta_{red} = \eta_{sp}/C \quad (5)$$

$$[\eta] = (\eta_{sp}/C)_{c=0} = (\ln(\eta_{rel})/C)_{c=0} \quad (6)$$

where t was the flow time measured for the sample solution at a given time t; t_0 was the flow time of the solution (0.1M acetic acid and 0.1M acetic acid/0.2M sodium chloride) at t=0; and C was the concentration of chitosan samples in diluted solution.

The viscosity-average molecular mass (MM) of chitosan was calculated by Mark-Houwink-Sakurada (MHS) equation (7):

$[\eta] = K (MM)^{a}$ (7)

where K and a were the constants, $K=1.81 \times 10^{-3}$, a = 0.93 [11]; and [η] was the intrinsic viscosity obtained from the Huggins and Kraemer plots.

Chitosan Hydrolyzing Activity Assay [26]

The extent of hydrolyzing activity in chitosan was measured by investigating the relative viscosity and reducing ends. One percent (w/w) of chitosan solution was prepared in 100 mM sodium acetate buffer with a pH 4.5. Cellulase or lysozyme was added into the solution in a ratio of 1:100 (w/w), respectively, and the reactions continued for 210 min at a 40 ℃ water bath to compare hydrolyzing activity between the two enzymes.

Viscosity of chitosan solutions at different reaction times with the enzymes were measured by using a Brookfield DV-ш + viscometer (Brookfield Inc., USA). Changes of the viscosity were expressed as the relative viscosity with respect to the control, enzyme-free chitosan solution [26].

Relative viscosity (%) = [(Initial viscosity (cp) – Reduced viscosity (cp))/Initial viscosity (cp)] *100

The absorbance of the reducing ends of chitosan was measured using the method of Shales [28] with some modifications [26]. Samples (3 ml) were extracted from the solution at different reaction times, boiled for 10 min to inactivate enzymes, and reacted with 4 ml solution of 0.5 g/l potassium ferricyanide dissolved in 0.5M sodium carbonate. Mixtures were then boiled for 15 min for inducing color changed by the

amount of reducing ends. After cooled down, samples were centrifuged to remove the precipitated chitosan. One ml of distilled water was added into 2 ml of supernatant, and the absorbance of the solution was measured at 420 nm in a UV spectrophotomter (Shimadzu UV 160U, JAPAN).

Experimental Design and Data Analysis

Taguchi design was used in the deacetylation study to identify optimal deacetylation conditions. The orthogonal arrays used in the Taguchi design had nine treatment trials (combinations) for both Kurtia and Broussignac methods as shown in Table 3.1. Each experimental run represented one trial. This array was designed for determining 1) the contribution of individual treatment factor, and 2) the level of each factor. The Taguchi design offered a simple and systematic approach to optimize the experiments, and could significantly reduce numbers of treatment combinations when multiple factors were considered. [29]

In the Taguchi design, two parameters were applied to optimize the treatment conditions. The first parameter (K_{ij}) was the average value of each measured functional parameter in level j (j=1, 2, 3) of each factor i (i=A, B, C, D) and expressed as

$$K_{ij} = \frac{1}{N_i} \sum_{u=1}^{N_i} y_{i,j}$$

where i represented the factor A, B, C, and D; j represented the level 1, 2, and 3; N_i is the number of trials for each factor, and $y_{i,j}$ is the measured values of factor i at level j. This parameter could explain how targeted parameters were changed in different levels of each treatment factor.

The second parameter, R_i, was the difference between the highest and lowest values of K_{ij}, and determined the most contributed factor among all factors. R_i was calculated as

$$R_i = (K_{ij})_{max} - (K_{ij})_{min}$$

where $(K_{ij})_{max}$ and $(K_{ij})_{min}$ indicated the highest and lowest values of the measured parameter in each factor, respectively. As R_i values increased among different factors, the factor which showed the highest value was the most contributed factor in determining the characteristics of the samples. Hence, by using Taguchi design, the levels in each factor and the most contributed factor could be identified by using only 9 treatment combinations.

Deacetylation study was conducted in triplicates and each was considered as a block. A one-way ANOVA was carried out to determine the significant differences among different factors and their levels, and the Tukey test was done for multiple comparisons in Taguchi design (SAS 9.2, SAS Institute, Inc., USA).

A completely randomized 2 x 2 x 8 factorial design was applied in the depolymerization study with two replications. Three independent factors were the type of enzymes (cellulase and lysozyme), the initial MM of chitosan (HMM and LMM), and depolymerized time (0 ~ 7 h). Interactions among the 3 factors were determined. PROC GLM was applied to determine significant difference (P<0.05) among treatment factors using the SAS program (SAS 9.2, SAS Institute, Inc., USA).

RESULTS AND DISCUSSIOS

Deacetylation of Chitin Using Kurita Method [27]

Characteristics of Deacetylation

DDA ranged from 45% to 99% and MM from 5362 kDa to 11684 kDa (Table 3.2). High DDA (>90%) of chitosan were all obtained at 90 ℃ treated with either 40 or 50% NaOH for at least 4 h, in which relatively low MM was observed, indicated that the severe treatment conditions removed more acetyl group from chitin, resulting in higher DDA values and further degradation of chitosan molecules. The MM range obtained in this study was similar to that by Chandumpai et al. (9110 ~ 10240 kDa) in which 1% acetic acid solution was used to measure MM,[3] but highly different from the results by Tolaimate et al. (450 ~ 595 kDa) in which 0.3M acetic acid/0.2M sodium acetate solution was used for measuring MM.[30] As stated in the experimental section, the molecular mass measured in this study was "viscosity-average molecular mass" and highly related to the solubility of chitosan in the solvents, in turn, the type of solvent used for dissolving chitosan samples. Previous studies indicated that MM values measured by dissolving chitosan in acetic acid/sodium chloride and acetic acid/sodium acetate were much lower than that by dissolving in diluted acid solution, [2, 3, 30] probably due to the electrostatic repulsion of chitosan as the polycationic polymer in acidic solvent. The resolving process of chitosan is initiated by the bindings between the hydrogen ions and free amine group to form a cation ion (NH_3^+) when pH is below its pKa. Therefore, the amount of cation ions is important in determining the solubility and viscosity due to their electrostatic repulsions. Viscosity of a solution is increased along with increased amount of NH_3^+ since it made larger spaces between the polymers for water trap, thus longer linear polymer stretching out.[31] However, anion ions, such as Cl^- or CH_3COO^-, in sodium chloride or sodium acetate could block the electrostatic repulsion between cation ions in chitosan, thus decreasing its intrinsic viscosity. [32] The relatively low MM observed in low DDA

samples might also be owing to the rigid crystal structure of chitosan samples, resulting in the lower solubility. [33, 34]

Though there was no statistical difference on MM among nine treatment conditions except the 1st and 9th runs (Table 3.2), MM generally decreased in the severe treatment conditions of using higher NaOH concentration (50%) or longer reaction time at 90 ℃ (Table 3.2). Similarly, Chandumpai reported that MM of chitosan gradually decreased along with increased treatment time from 2 to 8 h in 50% NaOH at 100 ℃. [3] Tolaimate found that β-chitin deacetylated by 40% NaOH at 80 ℃ for 6 h has larger MM than that treated at the same condition for 9 h. [30] Hasegawa also indicated that MM decreases along with increased concentrations of reagents and temperatures. [33] Therefore, the higher concentration of deacetylation reagent and longer reaction time further degraded the polymer. By contrast, Ottey did not show the further polymer degradation by extended reaction time. [354]

Optimal Deacetylation Conditions

Through Taguchi design the average values of three measured parameters (DDA, intrinsic viscosity, and MM) and the rank of each contribution factor on these parameters were obtained (Table 3.2). Since the intrinsic viscosity directly related to MM, it was not discussed separately here. R*i* value of NaOH concentration was the lowest among all tested contributing factors on MM and DDA, R*i* values of temperature and time on DDA were ranked the first and second, and were the second and first on MM, respectively. ANOVA results indicated that NaOH concentration had no significant effect on all measured parameters, but both temperature and time significantly affected DDA, but not MM ($P<0.05$). Therefore, regardless of the

treatment factors and their levels applied in this study while using Kurita method, no polymer degradation (change of MM) occurred in the deacetylation process. Based on this study, it may be concluded that the optimal deacetylation condition to obtain chitosan with DDA values over 95% and without significant polymer degradation is to use 40% NaOH at 90 ℃ for 6 h with three divided steps (2 h +2 h +2 h) or using 50% NaOH at 90 ℃ for a straight 6 h.

Deacetylation of Chitosan using Broussignac Method [11]

Characteristics of Deacetylation

Our preliminary study found that when using Broussignac method samples treated at 60 ℃ were visibly insoluble in 0.1M acetic acid (data not shown), thus increasing reaction temperature to 90 and 120 ℃ was necessary. DDA and MM ranged between 57 - 99% DDA and 34 - 11934 kDa, respectively (Table 3.3). The higher MM chitosan were obtained at 90 ℃ for at least 4 h with 1 or 3 steps (5^{th}, 6^{th} and 8^{th} runs), while MM from treatment at 120 ℃ was significantly ($P<0.05$) lower than those at 90 ℃ except the one for 2 h ($P>0.05$). MM (1797 kDa) obtained at 90 ℃ for 2 h with 2 divided steps was significantly lower than other samples treated at the same temperature ($P>0.05$), probably owning to the lower solubility of chitosan as shown by the low DDA of 57%. Similar to what was observed in Kurita method, the rigid structure with hydrogen bonds in low DDA chitosan might induce lower solubility of chitosan, thus affecting the measurement of MM. MM at 90 ℃ for 4 h with 3 divided steps (1h + 1h + 2h) was significantly ($P<0.05$) higher than that from the same condition with 1 straight step. Similarly, the MM of chitosan deacetylated for 6 h with three divided steps (2h + 2h + 2h) was higher than that with two divided steps. This

might be explained as the multiple-step treatment help prevent the polymer degradation. In the Kurita method, the highest MM was also obtained at 90 ℃ for 4 h. Hence, the treatment condition to obtain the highest MM was at 90 ℃ for 4 h in both methods. Compared with Kurita method, the MM range obtained from the Broussignac method was wider, in which the higher treatment temperature may attribute to the significant degradation of chitosan (decrease in MM).

In respect to DDA, 90 ℃ for 4 h was a minimal condition required to obtain chitosan with DDA over 90%, and the multiple-step helped increase DDA as shown that 4 h with 3 divided steps (1h +1h +2h) gave a 93% DDA verse 87% DDA obtained through a straight 4 h. DDA was over 90% when samples were treated at 120 ℃, and there was no significant difference in DDA in all 120 ^{o}C treated samples regardless of time or multiple-step ($P>0.05$). The lowest DDA (57%) was observed on samples treated at 90 ℃ for 2 h with 2 divided steps. Similar to our results, Tolaimate found that 2 h treatment at 120 ℃ results in DDA over 96%, confirming the important role of temperature, and multi-step resulted in higher DDA compared to the single step at the same treatment time. [2] In this study, sample treated at 120 ℃ for 6 h with 3 divided steps had a 99% DDA, which was close to fully deacetylated chitosan.

Optimal Deacetylation Condition

R*i* value of temperature was the highest among all contributing factors (Table 3.3), and temperature significantly affected MM and DDA ($P<0.05$). This result indicated that while the high temperature treatment increases DDA over 95%, chitosan depolymerization occurred simultaneously. Time was the second contribution factor on DDA, but did not show significant effect on MM. Hence, controlling treatment

time in Broussignac method may provide better control on MM and DDA of chitosan than temperature as it could satisfy our goal to obtain chitosan with high DDA, but less polymer degradation. Multiple-step was the least contributing factor on DDA, and the second on MM. It did not affect DDA ($P>0.05$), but MM, which was different from the result in Kurita method. This result was also inconsistent with the findings by Tolaimate, in which multi-step treatment prevented depolymerization of MM when temperature was at 80 ℃. [2] The difference might be due to the low solubility of some chitosan samples, affecting the accuracy in MM measurement as stated previously. In the future work, solubility of deacetylated chitosan should be considered as a contributing factor in Taguchi design to obtain more accurate result.

Based on the results from this study, it may be concluded that the Broussignac method was more applicable when lower MM and higher DDA chitosan were targeted, while the Kurita method was more suitable for obtaining chitosan with higher MM and higher DDA. This conclusion was similar to that by Tolaimate who compared Kurita and Broussignac methods in the deacetylation of β-chitin from *Loligo vulgaris* squid pens.[2]

Depolymerization of Chitosan by Commercial Enzymes

Enzyme type (ET), initial MM (IM), and depolymerization time (DT) were all significant ($P<0.05$) factors affecting depolymerization of chitosan, and there were significant ($P<0.05$) interactions between ET and DT, and IM and DT ($P<0.05$) (Table 3.4). HMM (2100 KDa) and LMM (594 KDa) chitosan showed about 89% and 56% degradations, respectively by cellulase (EC3.2.1.4) in the first hour, had another 15-20% degradation in the following hour, but no further degradation after 2 h (Fig.

3.1). Lysozyme (EC 3.2.1.17) resulted in about 56% and 68% degradation in HMM chitosan during the first and second hour treatment, but didn't induce further degradation after that, while the reaction on LMM chitosan was significantly slow, no degradation occurred until after 5 h (15% and 47% degradation at 5 h and 6 h, respectively). Therefore, chitosan depolymerization by enzymes was significantly induced during the first 1-2 h of reaction except for lysozyme treatment on LMM chitosan. The initial fast degradation was probably due to the predominant endo-action of cellulase and lysozyme that broke internal bonds (1, 4-glycosidic linkage of polysaccharides) of chitosan. [17] Previous study reported that chitosan depolymerized by cellulase had 83.5% degradation in only 5 min, and 95.3% degradation in 4 h with a final MM of 24 kDa. [36]

The types of enzyme had significantly different impacts on the depolymerization of HMM and LMM chitosan ($P<0.05$). MM values of lysozyme treated samples were significantly ($P<0.05$) higher than that of cellulase treated ones regardless of the difference in the initial MM. According to Lin, lysozyme had less susceptibility than cellulase in terms of chitosan degradation since it only recognizes the site existed 3~5 of N-acetyl-D-glucosamine (NAG), while cellulase can randomly cleave 1, 4-glycosidic linkages of chitosan regardless of the type of monomers. [17] Kurita found that lysozyme had higher susceptibility in 57% DDA chitosan than in higher DDA chitosan. [37] The initial DDA values of HMM and LMM chitosan were about 85% and 95%, respectively, and may explained the less degradation in LMM chitosan when depolymerized by lysozyme.

DDA values were measured during the polymerization process to investigate the potential impacts of the treatment factors (Table 3.6). The initial MM was the only

factor affecting DDA (P<0.05), clearly due to the initial DDA difference in HMM and LMM chitosan. After one hour of deploymerization (Table 3.6), the difference was no longer existed among different treatment conditions. A significant (P<0.05) interaction between enzyme type and initial MM on DDA was observed (Table 3.4). This interaction was probably attributed by the different susceptibility of enzyme to chitosan that had different MM and DDA.

Physicochemical Properties of Depolymerized Chitosan

Changes of relative viscosity and reducing ends formation during depolymerization of chitosan are illustrated in Fig. 3.2 and 3.3, respectively. They are the major physicochemical characteristics changed during the depolymerization of chitosan. Initial viscosity of HMM (2137 kDa) and LMM (594 kDa) chitosan were 2,048 cP and 48 cP, respectively. While MM in HMM chitosan was about 4 times higher than that in LMM, the viscosity of HMM chitosan was about 50 times higher than that of LMM chitosan. Similar to the changes of MM, relative viscosity of HMM chitosan decreased much faster than that of LMM chitosan, approximate 95% reduction in the first 10 min, and no change after that. This was observed on HMM chitosan treated by either cellulose or lysozyme. On the other hand, relative viscosity of LMM chitosan depolymerized by cellulase or lysozyme gradually decreased during the first hour, reached about 73% and 37% reduction at 1 h, respectively, and was stable after that. These observations were similar to previous study that the viscosity change was mostly induced during the initial 10 ~ 20 min as a result of enzymatic action. [25] The reduction of relative viscosity was faster and higher in LMM chitosan

treated by cellulase than that by lysozyme since high DDA in LMM had lower susceptibility on lysozyme treatment.

Theoretically, relative viscosity is proportional to MM, concentration, and the chain entanglement of chitosan, [38] and can be highly increased when MM is higher than a certain critical molecular mass (M_C) of a polymer. The strong dependence of viscosity in high MM can be explained as the effect of chain entanglement in polymer. [39] For example, the viscosity is proportional to $MM^{3.4}$ if $MM>M_C$ and to $MM^{1<a<2.5}$ if $MM<M_C$, and no distinct M_C could be up to MM of 1,000 kDa. [39, 40] Therefore, the relative viscosity of HMM chitosan was significantly higher than that of LLM chitosan due to its stronger dependence on MM. [39] The way how the viscosity was described may also attribute to the high viscosity value in HMM chitosan. Relative viscosity was expressed as the viscosity at sampling time relative to the initial viscosity of chitosan prior to enzyme treatment. The initial viscosity of HMM chitosan was extremely higher than that of LMM chitosan, resulting in higher decreasing rate even though the same amount of viscosity reduction occurred in both HMM and LMM chitosan.

The absorbance value at 420 nm indicates the amount of reducing ends formed by the depolymerization of chitosan (Fig. 3.3). The absorbance value in chitosan depolymerized by cellulase was significantly higher than that by lysozyme in both HMM and LMM chitosan. There was no significant difference in the absorbance between HMM and LMM chitosan ($P<0.05$). The absorbance increased fast in the initial 30 min and then slowly increased after that. The difference in the reducing end formation of chitosan depolymerized by cellulase or lysozyme was probably due to different specificities of enzymes cleaved different active sites of chitosan. [17, 37] As

discussed in the changes of MM and relative viscosity, DDA values of chitosan impacted the specificities of enzymes on the formation of reducing ends, in which were formed by enzymatic depolymerization of polysaccharide. It was known that the active site of cellulase was in 1, 4-glycosidic linkages regardless of the types of monomers, while lysozyme cleaved the active site occupied by the 3 to 5 number of NAG bindings. Initial DDA of chitosan was about 85% in HMM and 96% in LMM. Because of the relatively higher DDA in both HMM and LMM chitosan with less amount of NAG monomers, chitosan depolymerized by lysozyme produced less amount of reducing ends due to lower susceptibility, compared with chitosan depolymerized by cellulase. Cellulase was one of enzymes with the highest chitosan depolymerizing activity. [26] Study in chitosan depolymerized by pepsin, cellulase, lipase A, and chitosanase also showed the fast generation of reducing ends during the first hour.[26] The amount of reducing ends was more dependent on the type of enzymes than the initial properties of chitosan, probably own to the relatively higher DDA in both LMM and HMM chitosan.

CONCLUSION

Under tested treatment temperature and time, the NaOH deactylation of β-chitin obtained from the jumbo squid pens resulted in β-chitosan with a wide range of degree of deacetylation, but no significant change in molecular mass, while KOH treatment produced β-chitosan with high degree of deacetylation (>93%) and huge polymer degradation at 120 ℃. When using NaOH, deacetylation time and temperature were ranked the first and second contributing factor affecting molecular mass and degree of deacetylation, respectively. In KOH deacetylation, temperature

was the most contributing factor impacting both molecular mass and degree of deacetylation of chitosan. Chitosan could be depolymerized by cellulase and lysozyme, in which cellulase had higher susceptibility on chitosan, resulted in higher and faster chitosan degradation than that of lysozyme. Further studies to investigate the antimicrobial and antioxidant functions of β-chitosan from jumbo squid pens are under the way.

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Table 3.1 L9 standard orthogonal array of different contributing factors with different levels applied in Kurita [A] and Broussignac [B] methods [11, 27]

[A] – Kurita method using NaOH as reagent

Experimental Run	Contributing factors (i)			
	Concentration (%) A	Temperature (°C) B	Time (h) C	Multiple-steps D
1	1 (40)	1 (60)	1 (2)	1
2	1 (40)	2 (90)	2 (4)	2
3	1 (40)	2 (90)	3 (6)	3
4	2 (50)	1 (60)	2 (4)	3
5	2 (50)	2 (90)	3 (6)	1
6	2 (50)	1 (60)	1 (2)	2
7	1 (40)	1 (60)	3 (6)	2
8	2 (50)	2 (90)	1 (2)	3
9	1 (40)	2 (90)	2 (4)	1

[B] – Broussignac method using KOH as reagent

Experimental Run	Contributing factors (i)		
	Temperature (°C) A	Time (h) B	Multiple-steps C
1	1 (120)	1 (2)	1
2	1 (120)	2 (4)	2
3	1 (120)	3 (6)	3
4	2 (90)	1 (2)	2
5	2 (90)	2 (4)	3
6	2 (90)	3 (6)	1
7	1 (120)	1 (2)	3
8	2 (90)	2 (4)	1
9	1 (120)	3 (6)	2

Table 3.2 Intrinsic viscosity, molecular mass and degree of deacetylation of deacetylate chitin using Kurita method and estimated parameters from Taguchi design

Run		Measured parameters		
		Intrinsic viscosity (ml/g)	Viscosity-average molecular mass (KDa)	Degree of deacetylation (%)
1		3280.19 $^{b+}$	5362.29 ^{b}	52.72 ^{cd}
2		5708.21 ^{ab}	9730.05 ^{ab}	94.19 ^{ab}
3		4923.07 ^{ab}	8327.55 ^{ab}	98.80 ^{a}
4		3957.25 ^{ab}	6582.98 ^{ab}	62.67 ^{c}
5		4362.08 ^{ab}	7287.50 ^{ab}	95.60 ^{ab}
6		4962.80 ^{ab}	8391.64 ^{ab}	44.95 ^{d}
7		6108.82 ^{a}	10464.37 ^{ab}	80.96 ^{b}
8		5502.29 ^{ab}	9356.56 ^{ab}	89.93 ^{ab}
9		6766.43 ^{a}	11683.76 ^{a}	83.21 ^{ab}
Factors	Levels			
A	K_{A1}	$^{++A}$ 5357	^{A}9114	^{A}81.98
	K_{A2}	^{B} 4696	^{A}7905	^{A}73.29
	R_i^{+++}	661	1209	8.69
B	K_{B1}	^{A}4577	^{A}7700	^{A}60.33
	K_{B2}	^{A}5452	^{A}9277	^{B}92.35
	R_i	875	1577	32.02
C	K_{C1}	^{A}4582	^{A}7703	^{B}62.54
	K_{C2}	^{A}5477	^{A}9332	^{AB}80.02
	K_{C3}	^{A}5131	^{A}8693	^{A}91.79
	R_i	896	1629	29.25
D	K_{D1}	^{A}4803	^{A}8111	^{A}77.18
	K_{D2}	^{A}5593	^{A}9529	^{A}73.37
	K_{D3}	^{A}4794	^{A}8089	^{A}83.80
	R_i	799	1440	10.43
	Rank^{+++} $^{+}$	C>B>D>A	C>B>D>A	B>C>D>A

$^{+}$ Means followed by the lowercase letter in the same row within nine treatments were not significantly different (P>0.05)

$^{++}$ Means preceded by the same capital letter in the same column within each factors were not significantly different (P>0.05)

$^{+++}$ R_i was the difference between the highest and lowest values

$^{++++}$ Ranks were based on the order of Ri values

Table 3.3 Intrinsic viscosity, molecular mass, and degree of deacetylation of deacetylated chitin using Broussignac method and estimated parameters from Taguchi design

Run		Measured parameters		
		Intrinsic viscosity (ml/g)	Viscosity-average molecular mass (KDa)	Degree of deacetylation (%)
1		858.82 $^{cd+}$	1273.31 ^{c}	98.73 ^{a}
2		111.11 ^{d}	143.31 ^{c}	96.06 ^{ab}
3		122.22 ^{d}	157.49 ^{c}	99.14 ^{a}
4		1796.56 ^{c}	2850.47 ^{c}	57.17 ^{c}
5		6902.54 ^{a}	11934.44 ^{a}	93.18 ^{ab}
6		6206.64 ^{ab}	10645.89 ^{ab}	94.31 ^{ab}
7		115.97 ^{d}	148.75 ^{c}	94.03 ^{ab}
8		4788.53 ^{b}	8067.29 ^{b}	87.39 ^{b}
9		29.83 ^{d}	34.64 ^{c}	97.91 ^{a}
Factors	Levels			
A	K_{A1}	^{B}248	^{B}352	^{A}97.17
	K_{A2}	^{A}4924	^{A}8375	^{B}83.01
	$^{+++}R_i$	4676	8023	14.16
B	K_{B1}	^{A}924	A1424	A83.31
	K_{B2}	^{A}3934	A6715	A92.21
	K_{B3}	^{A}2120	A3613	A97.12
	R_i	3010	5291	13.81
C	K_{C1}	^{A}3951	^{A}6662	^{A}93.48
	K_{C2}	^{B}646	^{B}1010	^{A}83.71
	K_{C3}	^{AB}2380	^{AB}4080	^{A}95.45
	R_i	3305	5652	11.74
	$^{++++}$Rank	A>C>B	A>C>B	A>B>C

$^{+}$ Means followed by the lowercase letter in the same row within nine treatments were not significantly different (P>0.05)

$^{++}$ Means preceded by the same capital letter in the same column within each factors were not significantly different (P>0.05)

$^{+++}$ R_i was the difference between the highest and lowest values

$^{++++}$ Ranks were based on the order of Ri value

Table 3.4 Analysis of variance (ANOVA) results (P=0.05) for analyzing independence or interactions among treatment factors during deploymerization of chitosan

Source of Variation	Molecular mass (MM, kDa)			Degree of deacetylation (DDA, %)		
	df	F value	*P* value	df	F value	*P* value
Linear terms						
Enzyme types (ET)	1	111.57	<.0001	1	0.06	0.8158
Initial MM (IM)	1	54.73	<.0001	1	28.42	<.0001
Depolymerized time (DT)	7	56.75	<.0001	7	0.69	0.6759
Interaction terms						
ET X IM	1	3.17	0.0829	1	5.72	0.0217
ET X DT	7	31.09	<.0001	7	0.31	0.9428
IM X DT	7	2.42	0.0368	7	0.92	0.5020
Model	24	33.39	<.0001	24	1.99	0.0274
Error	39			39		
Corrected total	63			63		

Table 3.5 Effect of initial molecular mass of chitosan, type of enzyme, and depolymerization time on the molecular mass of chitosan during depolymerization

			Depolymerization time (h)								*P* value
	Initial MM (IM)	Enzyme types (ET)	0	1	2	3	4	5	6	7	
	HMM+++	Cellulase	$^{+A}2100^{a}$	$^{B}298^{b}$	$^{B}165^{b}$	$^{B}137^{b}$	$^{B}145^{b}$	$^{B}139^{b}$	$^{B}133^{b}$	$^{B}124^{b}$	<.0001
		Lysozyme	$^{A}2100^{a++}$	$^{A}670^{b}$	$^{A}633^{b}$	$^{A}533^{b}$	$^{A}645^{b}$	$^{A}625^{b}$	$^{A}718^{b}$	$^{A}611^{b}$	0.0003
	LMM	Cellulase	$^{B}594^{a}$	$^{B}264^{b}$	$^{B}148^{b}$	$^{B}175^{b}$	$^{B}152^{b}$	$^{B}167^{b}$	$^{B}136^{b}$	$^{B}128^{b}$	<.0001
		Lysozyme	$^{B}594^{a}$	$^{A}686^{a}$	$^{A}604^{a}$	$^{A}553^{a}$	$^{A}584^{a}$	$^{A}505^{ab}$	$^{B}317^{b}$	$^{B}279^{b}$	0.0012
P value			0.0180	0.0036	0.0001	0.001	0.0003	0.0080	0.0012	0.0023	

$^{+}$ Means preceded by the same capital letter in the same column within each treatment were not significantly different ($P>0.05$)
$^{++}$ Means followed by the lowercase letter in the same row within each treatment were not significantly different ($P>0.05$)
$^{+++}$ HMM = higher molecular mass, LMM = lower molecular mass

Table 3.6 Effect of initial molecular mass of chitosan, type of enzyme, and depolymerization time on the degree of deacetylation (DDA) of chitosan during depolymerization

			Depolymerization time (h)								*P* value
	Initial MM[+++] (IM)	Enzyme types (ET)	0	1	2	3	4	5	6	7	
	HMM	Cellulase	$^{B}85.46^{a++}$	$^{A}87.24^{a}$	$^{A}83.08^{a}$	$^{A}88.78^{a}$	$^{A}87.62^{a}$	$^{A}85.01^{a}$	$^{A}87.03^{a}$	$^{A}82.87^{a}$	0.2107
		Lysozyme	$^{B}85.46^{a}$	$^{A}90.63^{a}$	$^{A}89.93^{a}$	$^{A}89.66^{a}$	$^{A}89.67^{a}$	$^{A}86.99^{a}$	$^{A}89.00^{a}$	$^{A}85.30^{a}$	0.1885
	LMM	Cellulase	$^{A}95.11^{a}$	$^{A}92.15^{a}$	$^{A}95.44^{a}$	$^{A}95.34^{a}$	$^{A}91.47^{a}$	$^{A}90.07^{a}$	$^{A}94.16^{a}$	$^{A}93.06^{a}$	0.3451
		Lysozyme	$^{A}95.11^{a}$	$^{A}90.01^{a}$	$^{A}85.20^{a}$	$^{A}90.92^{a}$	$^{A}92.10^{a}$	$^{A}92.15^{a}$	$^{A}90.43^{a}$	$^{A}91.14^{a}$	0.8213
P value			0.0001	0.8167	0.0521	0.1274	0.7391	0.2036	0.2550	0.1969	

[+] Means preceded by the same capital letter in the same column within each treatment were not significantly different ($P>0.05$)
[++] Means followed by the lowercase letter in the same row within each treatment were not significantly different ($P>0.05$)
[+++] HMM = higher molecular mass, LMM = lower molecular mass

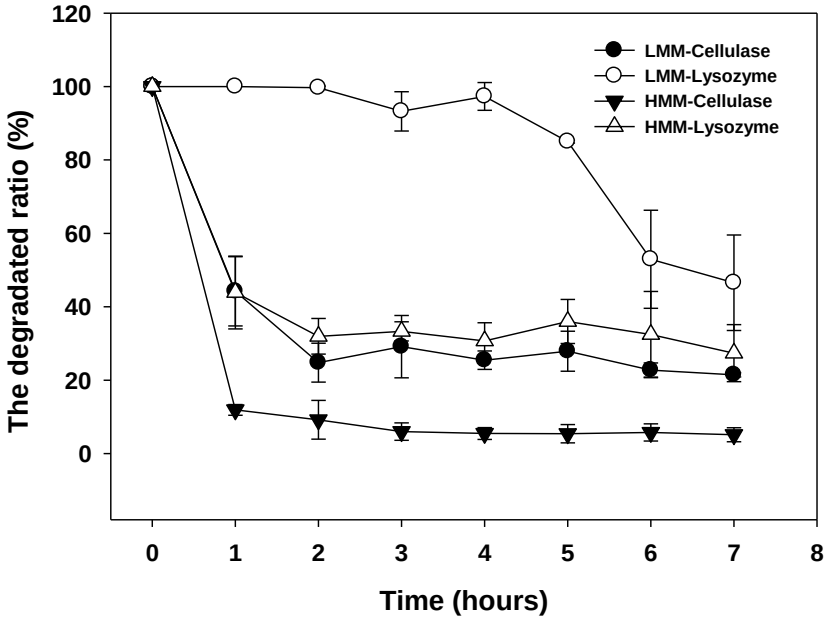

Fig. 3.1 The ratio of depolymerization in chitosan treated with 1% cellulose or lysozyme for 7 h (n=2). Data were mean of two replications. HMM = higher molecular mass; LMM = lower molecular mass.

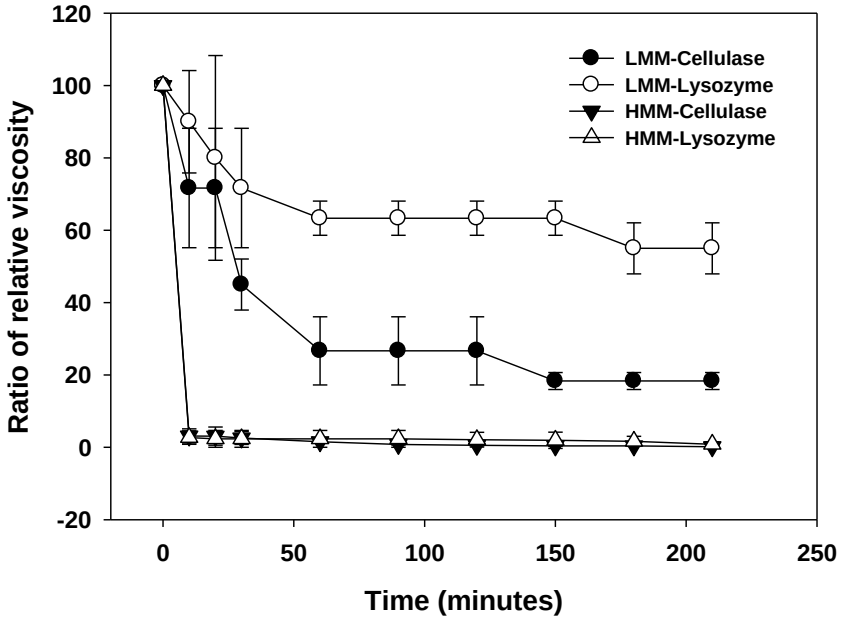

Fig. 3.2 The decreasing ratio of viscosity in 1% (w/v) chitosan solution treated with 1% cellulose or lysozyme for 210 min. The decreasing ratio was expressed as the viscosity at sampling time relative to the initial viscosity of the higher or lower molecular mass chitosan without enzyme treatment (n=2). HHM=higher molecular mass; LMM=lower molecular mass.

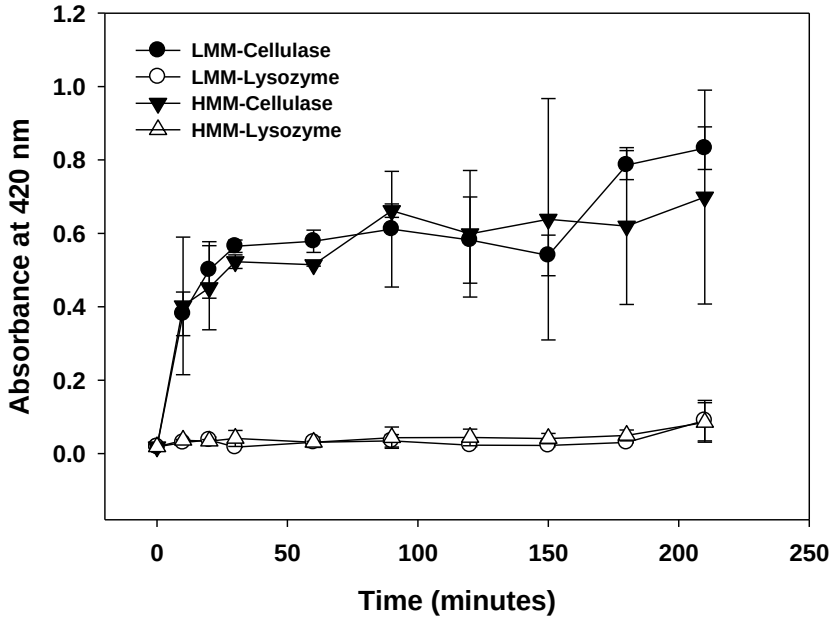

Fig. 3.3 The reducing end formation from 1% chitosan solution treated with 1% cellulose or lysozyme for 210 min (n=2). Ferricyanide absorbance corresponded to the amount of the reducing ends as a function of time. HHM=higher molecular mass; LMM=lower molecular mass.

CHAPTER 4

ALKALI OR ACID INDUCED CHANGES IN THE STRUCTURE, MOISTURE ABSORPTION ABILITY AND DEACETYLATION REACTION OF β-CHITIN EXTRACTED FROM JUMBO SQUID (*DOSIDICUS GIGAS*) PENS IN COMPARISON WITH α-CHITIN FROM SHRIMP SHELLS

Jung, J., Zhao, Y. Submitted to *Food Chemistry*

ABSTRACT

Alkali or acid-induced structural modifications in β-chitin extracted from jumbo squid **(*Dosidicus gigas*)** pens and its moisture absorption ability and deacetylating reaction were investigated and compared with those of native α-chitin from shrimp shells. The β-chitin was converted into α-chitin after 3 h soaking in 40% NaOH or 1-3 h in 40% HCl solution, and the obtained α-chitin had higher moisture absorption ability than that of the native α-chitin from shrimp shells due to the polymorphic destructions by alkali-treatment. In contrast, acid-treated β-chitin had little polymorphic modifications, showing no significant change in its moisture absorption ability. β-chitin was more susceptible to the deacetylating reactions than α-chitin, in which lower concentration of NaOH and shorter time were required to extract β-chitosan. These results demonstrated that alkali or acid-treated β-chitin remained high susceptibility of the native β-chitin toward the solvents in comparison with the native or acid treated α-chitin.

Keywords: α-chitin, β-chitin, jumbo squid pens, crystallinity, moisture absorption ability, deacetylating reaction

INTRODUCTION

The two forms of chitin, α- and β-form, are distinguished in respect to their different structural characteristics [1-3]. Crystallites of α-chitin is tight-packed with inter-sheet hydrogen bonds formed between the antiparallel sheets, whereas that of β-chitin has loose arrangements due to much weaker intermolecular hydrogen bonds by the parallel manner of the polymeric sheets. Moreover, the crystal region (crystallinity) of the semi-crystalline α-chitin is larger than that of β-chitin [4]. These structural differences directly impact their physicochemical properties, in which β-chitin presents much higher solubility and reactivity in alkali solutions during the deacetylation process than that of α-chitin [2, 3]. In addition, extraction of β-chitin from squid pens has shown advantages of unnecessary demineralization and decoloration processes in comparison with extracting α-chitin from shrimp and crab shells due to the negligible amount of mineral and carotenoid in the squid pens [5-7].

Chemical treatments using acid and alkali have been most commonly employed to produce chitin and chitosan. It was generally believed that the demineralization and/or deproteinization processes using lower concentrations of acid and alkali and lower temperatures than those applied in the deacetylation process do not cause significant changes in the molecular weight (Mw) and degree of deacetylation (DDA) of chitin [8]. However, several studies have found that the chemical treatments alter the structural properties of chitin due to swelling, dissociation of hydrogen bonds, and rearrangements of polymeric chains, and different forms of chitin responded differently [9-13]. Feng et al. (2004) and Liu et al. (2008) reported that alkali treatment of α-chitin weakens inter-sheet hydrogen bonds and decreases crystallinity index (CI) along with the polymorphic

modifications as alkali concentration increased. The conversion phenomenon between β- and α-chitin during alkali treatment was also observed by Saito et al. (1997), Li et al. (1999), and Noishiki et al. (2003), in which β-chitin was converted into α-chitin forming inter-sheet hydrogen bonds between C=O and O-H in C_6, similar to the mercerization induced from cellulose I to cellulose II. According to Li et al. [9], alkali or acid caused swelling of crystallites and destruction of the original lateral order, resulted in the rearrangement of those polymeric chains, thus conversion of chitin from one form to another. Hence, alkali or acid-induced conversion of β-chitin to α-chitin with the presence of strong inter-sheet hydrogen bonds might be a concern for preparing chitosan since it may cause the loss of the original functional properties of β-chitin, especially the high reactivity and susceptibility toward solvents. Moreover, it is unclear how the alkali or acid-induced conversion exactly impact their polymorphic structures, in turn the physicochemical properties of resulted chitin, and how the converted form of chitin similar or different from its native form. Such information is critical to fully understand the demineralization, deproteinization, and deacetylation processes in α- and β-chitin, the essential steps in preparing α- and β-chitosan, as well as the functional differences between α- and β- chitosan. Based on our best knowledge, no previous study has systematically reported these conversion phenomena in respect to the polymorphic modifications.

Several previous studies have demonstrated that the functional properties of chitin and chitosan depend on their originated marine sources and species [8, 14]. The jumbo squid (*Dosidicus gigas*) pens are a newly employed source of β-chitin (Jung and Zhao, 2011 and 2012), and have shown some unique properties different from those mostly used β-

chitin extracted from *Loligo* species [5-7, 15]. Therefore, the objectives of this study were to investigate the alkali and acid-induced polymorphic modifications of β-chitin extracted from jumbo squid pens and α-chitin from shrimp shells, and to study the changes in the moisture absorption ability of resulted α- and β-chitin in comparison with their native form. Moreover, the deacetylating reactions of α- and β-chitin under various alkali treatments were also investigated to investigate the impact of structural modifications on the deacetylating process.

MATERIALS AND METHODS

Preparation of Chitin

Dried jumbo squid (*Dosidicus gigas*) pens was donated by Dosidicus LLC (USA) and α-chitin from shrimp shells were purchased from Sigma-Aldrich (USA). Samples were ground into about 18 meshes (Glenmills Inc., USA). Squid pens were deprotenized by 5% NaOH for 3 d at room temperature, washed with distilled water to reach the neutral pH, and then dried at 40 ℃ in an oven (Precision Scientific Inc., USA) for 24 h. Each form of the chitin was treated in 40% HCl or NaOH for 1-4 h under a given condition for studying not only the conversation phenomena from β-chitin to α-chitin, but also the polymorphic properties including crystal characteristics and CI as described below. For deacetylation process, different concentrations of NaOH (40 or 50%), temperatures (60 or 90 ℃), and reaction times (2, 4, or 6 h) were applied.

Viscosity-Average Mw

The viscosity-average Mw of α- and β-chitin were determined by using the Ubbelohde Dilution Viscometer (Cannon instrument Co., USA) with a capillary size of 0.58 nm. Approximate 100 mg of chitin was dissolved in 10 mL of the mixture solution of *N*, *N*-dimethylacetamide (DMAc) containing 5% lithium chloride. The intrinsic viscosity was measured by the intercept between the Huggins (reduced viscosity, $\eta_{sp/C}$~C) and Kraemer (relative viscosity, $\eta_{rel/C}$ ~C) plots when the concentration was 0 [16]. The viscosity-average Mw of chitosan was calculated by Mark-Houwink-Sakurada (MHS) equation: $[\eta] = K (M_W)^a$, where K and a were the constants, $K=2.1 \times 10^{-4}$ and a = 0.88 [17]; and [η] was the intrinsic viscosity obtained from the two plots, Huggins and Kraemer.

Proximate Composition Analysis

Moisture contents of chitin samples were determined by the percentage weight loss of the samples after drying in a forced-air oven at 100 ºC for 24 h. Ash contents were analyzed following AOAC method [18]. Protein contents were measured by Lowry method using bovine serum albumin standards [19]. The Lowry method is sensitive to low protein concentrations (5 – 100 μg/mL). DDA was determined by the colloidal titration method [20].

Moisture Absorption Ability

Functional groups including NH_2 of C_2 or OH of C_3 and C_5 in *N*-acetyl-D-glucosamine or D-glucosamine monomers can trap water penetrating into crystallites of chitin by forming hydrogen bonds. Moreover, these functional groups can be closely

related to the crystal properties or crystallinity index (CI) in the polymorphic structure of chitin as water can access easily to the loose-packed crystallites of β-chitin, compared with the rigid crystallites of α-chitin [21].

Powdered chitin was conditioned in a P_2O_5 added desiccator for 24 h to remove residual moisture [22], and then placed in a self-assembled chamber at 25 ℃ and 80% RH for 40 h. The moisture absorption ability was calculated as the percentage of weight gain of dried samples after 40 h using equation (1):

$$Moisture\ absorptin\ ability\ (\%) = \frac{Wegith\ of\ samples\ after\ 40\ h\ (g) - intial\ weight\ of\ chitin\ (g)}{Initial\ weight\ of\ chitin\ (g)}\ x\ 100 \quad (1)$$

A Fourier-Transform Infrared (FT-IR) Spectroscopic Analysis

A single bound attenuated total reflection (ATR)-FTIR spectrometer (PerkinElmer, USA) was operated by Omnic 7.4 software (Thermo Fisher Inc. USA) under the operating conditions of 32 scans at a 4 cm^{-1} resolution and referenced against air. All spectra were recorded as the absorption mode.

DDA was determined using the method by Sabnis et al. [23] as expressed in equation (2):

$$Degree\ of deacetylation\ (DDA,\%) = 97.67 - [26.486\ x\ (\frac{A_{1655}}{A_{3450}})] \quad (2)$$

Where A_{1655} and A_{3450} were the absorbance at 1655 cm^{-1} indicating the amide I band (a measure of *N*-acetyl group contents) and the absorbance at 3450 cm^{-1} indicating the hydroxyl groups as the reference, respectively.

The partial FT-IR spectra (1300-1800 cm^{-1}) were reported to distinguish the two forms of chitin and the inter-sheet hydrogen bonds. The band around ~1700 cm^{-1} attributed to the stretching vibration of C=O in amide [12], which could be split by the inter-sheet hydrogen bond with neighboring O-H of C_6 in α-chitin, whereas β-chitin was shifted to a single peak indicating no inter-sheet hydrogen bonds and much weaker intermolecular hydrogen bonds [24].

X-Ray Diffraction (XRD)

X-ray diffraction patterns were recorded using a XRG 3100 x-ray diffractometer (Philips, U.S.) with a Cu Kα (1.54 Å) at a voltage of 40 kV and a current of 30 mA. A typical scan range was from 5° to 40° (2θ) at scanning speed of 0.025°/sec.

The CI was determined by equation (3):

$$Crystallinity\ (CI, \%) = \frac{I_{110} - I_{am}}{I_{110}}\ x\ 100 \qquad (3)$$

Where I_{110} was the maximum intensity of the (110) plane at 2θ = ~19° and I_{am} was the intensity of the amorphous regions at 2θ = ~12.6° [25, 26].

Among various types of crystal lattices found in the polymeric structure of chitin including 020, 110, 120, 101, or 130 planes, the *d*-spacing and apparent crystal size (D_{ap}) of (020) and (110) planes were reported as both were appeared in the native and the processed α- and β-chitin. The *d*-spacing was computed using Bragg's law (4) [13]:

$$d\ (\text{Å}) = \frac{\lambda}{2 \sin \theta} \qquad (4)$$

Where *d* was plane spacing; λ was 1.54 Å, wavelength of Cu Kα radiation; and θ was one-half angle of reflections.

The apparent crystal size (D_{ap}) was calculated with the aid of Scherrer equation (5) [25, 27]:

$$D_{ap}(\text{Å}) = \frac{k\lambda}{\beta_0 \cos\theta} \quad (5)$$

Where β_0 (in radians) was the half-width of the reflection; k was a constant indicating the crystallite perfection with a value of 0.9; λ was 1.54 Å, the wavelength of Cu Kα radiation; and θ was one-half angle of reflections.

Experimental Design and Statistical Analysis

The native and processed α- and β-chitin samples were tested using a completely randomized design (CRD). Moisture, protein, and ash contents, and the moisture absorption ability were all determined in duplicate, and data were analyzed for statistical significance via least significant difference (LSD) post hoc testing as appropriate using statistical software (SAS v9.2, The SAS Institute, USA). Results were considered to be significantly different if $P<0.05$.

RESULTS AND DISCUSSION

Proximate Composition and Polymeric Structure of β-Chitin Extracted from Jumbo Squid Pens

The proximate compositions of α- and β-chitin are reported in Table 4.1. Moisture content of β-chitin extracted from jumbo squid pens was significantly higher than that of the commercial α-chitin from shrimp shells. This might be because the crystallites of β-

chitin are less tight due to much weaker intermolecular hydrogen bonds than that of α-chitin, thus moisture accessed easily to crystallites of β-chitin and was more able to form hydrogen bonds with NH_2 or OH. Similarly, Kurita et al. (1993) reported higher retention of absorbed water in β-chitin than that in α-chitin. Mw of β-chitin was almost as twice higher as that of α-chitin at similar DDA (Table 4.1). According to Tolaimate et al. (2003), Mw of β-chitin was 2-3 times higher than that of α-chitin at the same DDA, which was consistent with our result. Protein and ash contents in both α- and β-chitin were negligible, thus no further deprotenization and demineralization procedures were applied on samples used in this study. The ash (mineral) content of β-chitin was below 1% prior to acid treatment (so-called demineralization), lower than that reported in the previous study on *Loligo vulgaris* (1.7%) [15].

Crystal property of β-chitin was distinguished from that of α-chitin based upon partial FT-IR spectra (Fig. 4.1). The C=O band in amide (~1700 cm^{-1} indicated by dash lines) was split by inter-sheet hydrogen bonds in α-chitin due to antiparallel manner between the polymeric sheets, whereas β-chitin was shifted to a single peak without inter-sheet hydrogen bonds and much weaker intermolecular hydrogen bonds due to the parallel manner, similar to the previous finding by Cardenas et al. [24]. XRD patterns of α- and β-chitin were in the range of 5-40 º(2θ) (Figs. 4.2 and 4.3). Five crystalline planes (020, 110, 120, 101, and 130) at reflections of 9.4, 12.9, 19.4, 21.0, 23.8, and 26.5 were observed in α-chitin, whereas only two crystalline planes (020 and 110) at reflections of 8.9 and 19.7 were appeared in β-chitin (Figs. 4.2 and 4.3). Moreover, the peaks in α-chitin were sharper than those in β-chitin, indicating that crystal structure of α-chitin was more rigid and stable than that of β-chitin (Figs. 4.2 and 4.3). Table 4.2 shows CI,

relative intensities (RI, %), *d*-spacing, and D_{ap} of each crystal plane (020 and 110) commonly appeared in both α- and β-chitin. CI of β-chitin was ~8% lower than that of α-chitin similar to XRD patterns showing lower intensities and broader shapes of the peaks in β-chitin. The *d*-spacing of (020) plane was relatively larger in β-chitin, indicating that space distances between aligned polymeric chains were wider than those of α-chitin. Furthermore, D_{ap} of (020) and (110) planes in β-chitin were smaller than those of α-chitin. Hence, β-chitin extracted from jumbo squid pens had loose crystallites and lower CI, thus higher reactivity toward solvents, more swelling, and higher solubility than α-chitin. These results were similar to the previous findings on β-chitin extracted from *Ommastrephes bartramii* and *Loligo* species [2, 5, 7, 28].

Alkali or Acid Induced Conversion between α- and β-Chitin

Alkali or acid induced conversion of chitin from β-form to α-form was compared with the mercerization of cellulose where the parallel chains of cellulose I were converted into the antiparallel manner of cellulose II [29, 30]. The different forms of chitin showing antiparallel or parallel manner could be distinguished by FT-IR spectra in C=O band depending on the presence of inter-sheet hydrogen bonds between C=O and O-H in C_6.

Figs. 4.1 and 4.4 represent the partial FT-IR spectra (~1300-1800 cm^{-1}) of alkali or acid treated α- and β-chitin for 1-4 h, respectively. In alkali treated α-chitin, the split peak in C=O band shifted to a single peak after 2 h treatment due to the dissociation of inter-sheet hydrogen bonds. Similarly, Feng et al. (2004) observed the dissociation of hydrogen bonds along with the polymorphic changes in α-chitin after alkali-freezing treatment. Liu et al. (2008) reported similar FT-IR spectra of α-chitin after 40% alkali

treatment for 4 h, shown β-chitin with a single peak in C=O band. According to Li et al. (1999), however, α-chitin processed in 40% NaOH at 3 ℃ for 3 h remained its native form with strong hydrogen bonds due to the favorable packing nature of the crystallites. In alkali processed β-chitin, C=O band was slightly split at 1 and 2 h, and clear split was appeared at 3 h indicating the conversion β-chitin into α-chitin, but shifted to a single peak at 4 h. Therefore, the conversion phenomenon in β-chitin depended on the reaction time (1-4 h). According to Noishiki et al. (2003), the conversion was occurred in 30% NaOH after 1 h. In acid processed chitin, C=O band in α-chitin was all split at 1-4 h indicating that α-form remained with the presence of inter-sheet hydrogen bonds unlike alkali treatments, whereas the conversion from β-form into α-form was appeared at 1-3 h. A single peak of C=O band in β-chitin was observed at 4 h similar to the alkali treatment. According to Saito et al. (1994), the conversion from β-form to α-form was appeared by 7-8 N HCl treatment for 30 min. Hence, acid was able to induce the conversion of β-chitin to α-chitin after 1-3 h treatment, whereas α-chitin still presented the antiparallel polymeric sheets with inter-sheet hydrogen bonds after 1-4 h acid treatment.

Alkali or Acid Induced Structural Changes in α- and β-Chitin

Alkali or acid induced conversion in α- and β-chitin may impact their structural properties, such as crystal properties and CI of the polymorphic chitin. Hence, the structural properties in alkali or acid treated α- and β-chitin after various reaction times were analyzed for interpreting how the conversion phenomenon may impact the structural changes.

XRD patterns of the alkali or acid treated α- and β-chitin are illustrated in Figs. 4.2 and 4.3, respectively. For alkali treatment, the intensities of the peaks were significantly decreased in both α- and β-chitin and the peaks of (020) and (110) planes of β-chitin were shifted, meaning the destruction of crystallite by alkali treatment. After 2-3 h alkali treatment, α-chitin displayed sharp peaks with high intensities at ~32° and the peaks of (130) plane at ~26° was also observed in alkali treated β-chitin (Fig. 4.2), assuming that alkali induced the formation of some crystallites. However, alkali treated β-chitin showing the conversion into α-form exerted much less rigid crystallites and lower CI, presenting broader peaks and lower intensities in comparison with native α-chitin or β-chitin. For acid treated samples, the intensities of the peaks were not significantly decreased in α-chitin, showing higher intensities and sharper peaks in (020), (110), and (130) planes, whereas β-chitin had shifted peaks to lower angles in (020) and (110) planes at 3-4 h, interpreting that crystallites of acid treated β-chitin were destroyed and the space distance in each crystal plane became larger. Hence, the α-chitin converted from β-chitin by acid treatment had less rigid crystallites than that of the native α-chitin from shrimp shells.

Table 4.2 reports CI (%), RI (%), *d*-spacing (Å), and D_{ap} (Å) of (020) and (110) planes in alkali or acid treated α- and β-chitin, respectively. In alkali treatment, CI of α-chitin was more decreased than that of β-chitin. RI of (020) and (110) planes in α-chitin was lower than those in β-chitin after 1-2 h treatment. Moreover, D_{ap} of (110) plane was also decreased at 3-4 h in α-chitin. These results were consistent with the XRD patterns showing lower intensities in alkali treated α-chitin due to the destruction of crystallites and the FT-IR spectra presenting that inter-sheet hydrogen bonds of α-chitin were

dissociated by alkali treatments for 1-4 h. In contrast, D_{ap} of (020) and (110) planes increased in β-chitin, compared with native β-chitin, but RI of (110) plane decreased at 3-4 h where the conversion into α-form was appeared. Hence, alkali processed β-chitin remained loose crystal structure with lower CI similar to the XRD patterns representing decreased intensities and shifting of the peaks. In acid treatment, CI of α-chitin slightly decreased but no significant changes in *d*-spacing and D_{ap}, indicating that there was no significant destruction of crystallites, whereas *d*-spacing and D_{ap} of β-chitin were similar to those of native β-chitin along with higher *d*-spacing of (020) plane indicating larger space distances between the polymeric chains. Hence, crystallites of acid treated β-chitin were less rigid than the native or acid treated α-chitin.

In summary, alkali treated α- and β-chitin exerted significant destruction of crystallites and lower CI than their native chitin, and α-chitin converted from β-chitin as a result of alkali treatment had loose-packed crystallites and lower CI than native α-chitin. In contrast, acid had relatively less impact on the structural properties of α- and β-chitin.

Moisture Absorption Ability of Alkali or Acid Treated Chitin in Relation to their Structural Properties

Moisture absorption ability (MAA, %) of the native and alkali or acid treated α- and β-chitin is reported in Table 4.3. The native β-chitin had significantly higher MAA (~7.0%) than that of the native α-chitin (~-0.8%). This result was consistent with the previous studies reporting higher reactivity, swelling, and retention ability of absorbed water in β-chitin in comparison with α-chitin [1-3].

In alkali treatment, MAA of α-chitin significantly increased after 1 h, and that of β-chitin was increased after 1-2 h and had the highest MAA at 3-4 h with MAA of ~23-27%, which was significantly higher than those of α-chitin at 3-4 h. Similarly, Kurita et al. (1993) found that the crystal structure of β-chitin is destroyed easily by high concentration of alkali treatment than that of α-chitin, showing much higher hygroscopicity and the retention of the absorbed water in β-chitosan than that in α-chitosan. Likewise, Wada and Saito [31] reported that β-chitin readily expanded along the b-axis direction (no inter-sheet hydrogen bonds between stacked sheets) by heat and water was easily swollen along the b-axis direction. Based upon the XRD patterns in this study, the intensities of the peaks were significantly decreased in alkali treated α- and β-chitin, but the peak of (110) plane was shifted to lower angles in alkali treated β-chitin at 2-4 h, indicating the destruction of crystallites. Moreover, RI of alkali treated β-chitin at 3-4 h was lower than that of native α-and β-chitin. In spite of the conversion of β-chitin into α-chitin at 3 h, MAA was significantly higher probably due to the destruction of crystallites and the lower CI than those of the native α- and β-chitin. Hence, moisture absorption ability in alkali treated α- and β-chitin was significantly higher than that of native α- and β- chitin due to the destruction of crystallites. The α-chitin converted from β-chitin exerted significantly higher moisture absorption ability than that of the native α-chitin, assuming that its reactivity and swelling ability were higher than native α-chitin even with the presence of inter-sheet hydrogen bonds within the crystallites.

In acid treatment, moisture absorption ability of α-chitin was significantly increased at 1 h, whereas that of β-chitin remained similar to that of the native chitin. Increase of the moisture absorption ability of acid treated α-chitin at 1 h was probably due to the

decreased CI in comparison with native α-chitin. In contrast, there was no significant decrease of CI in acid treated β-chitin in comparison with native β-chitin and the conversion into α-form was appeared at 1-3 h, but the peaks of (020) and (110) planes were shifted to lower angles indicating the destruction of crystallites. Hence, moisture absorption ability of acid treated β-chitin was not significantly changed by the complex structural modification. Compared with alkali treatment, acid induced less polymorphic changes in α- and β-chitin.

In summary, α-chitin converted from β-chitin showed enhanced moisture absorption ability in comparison with the native α- and β-chitin due to the polymorphic destruction by alkali treatments, remaining higher swelling susceptibility of native β-chitin toward solutions. This finding demonstrated that α-chitin originated from β-chitin is more susceptible toward deacetylation and depolymerization in comparison with the native α- and β-chitin,

Comparison in Deacetylation in α- and β-Chitin under the Various Alkali Treatments

DDA of α- and β-chitin subjected to various alkali treatments are shown in Table 4.4. DDA of α-chitosan was ~40-89% and that of β-chitosan was ~63-92% under the same deacetylating treatment conditions, exhibiting relatively higher DDA in β-chitosan. Different deacetylating treatments were required for obtaining same DDA of α- and β-chitosan, in which for obtaining ~60% DDA, 40% NaOH at 90 ℃ for 6 h and 40% NaOH at 60℃ for 2 h were applied for α- and β-chitosan, respectively, while for obtaining ~75% DDA, α- and β-chitin were deacetylated by 50% NaOH at 60 ℃ for 6 h and 50% NaOH

at 60 ℃ for 2 h, respectively. Hence, relatively milder deacetylation treatments with lower temperature or shorter reaction time were required to extract 60 and 75% DDA of β-chitosan than those required for obtaining α-chitosan. This result may be interpreted by the different polymorphic structure between α- and β-chitin, in which α-chitin with strong inter-sheet hydrogen bonds in tight-packed crystal structures with higher CI was less reactive toward alkali treatment during the deacetylation process than β-chitin did, consistent with previous finding showing higher reactivity and swelling ability of β-chitin in alkali solutions than that of α-chitin [1, 3, 5, 15]. Hence, producing β-chitosan from squid pens resulted in low production cost by using lower concentrations of reagents and shorter reactions times than that for α-chitin.

CONCLUSION

In comparison with α-chitin from shrimp shells, β-chitin from jumbo squid pens had loose arrangements of polymeric chains, thus lower CI, which led to the higher moisture absorption ability than that of α-chitin. β-chitin could be converted into α-form after 3 h or 1-3 h treatment in alkali or acid solution, respectively. Alkali treatment resulted in polymorphic destructions in both α- and β-chitin, exhibiting higher moisture absorption ability than their native form. Moreover, moisture absorption ability of the α-chitin converted from β-chitin was significantly higher than that of the native α-chitin due to the destruction of crystallites and decrease of CI as a result of alkali treatment. Acid treated α-chitin retained its inter-sheet hydrogen bonds, but its moisture absorption ability was also significantly increased after 1 h treatment in comparison with the native α-chitin due to reduced CI. Acid induced less destruction of crystallites in β-chitin than alkali showing

no significant change in its moisture absorption ability. Therefore, the exact impact of alkali and acid treatment on the structural property and moisture absorption ability of chitin depended on the form of chitin and the reaction time, and alkali treated β-chitin was able to retain higher reactivity even after converted into α-form. In addition, the mild deacetylating treatment was required for β-chitin than that for α-chitin when preparing similar DDA of β- and α-chitosan. These results implicated that producing β-chitosan from squid pens can be more cost effective owning to β-chitin's loose crystallites and high reactivity toward solvent.

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Table 4.1 The proximate compositions of α- and β-chitin.

	α-chitin	β-chitin
Moisture contents (%)	1.17 ± 0.64	2.20 ± 0.30
Protein (μg/ml)	70.43 ± 20.98	40.87 ± 5.32
Viscosity-average molecular weight (kDa)	266,858	441,737
Degree of deacetylation (DDA, %)*	54.05 ± 0.95	58.66 ± 3.25
Ash contents (%)	0.68 ± 0.11	0.84 ± 0.34

* DDA was determined by FT-IR.

Table 4.2 Comparison of crystallinity index (CI, %), relative intensities (RI, %), *d*-spacing, and apparent crystal size (D_{ap}) in (020) and (110) planes of α- and β-chitin treated by 40% NaOH or HCl for 1-4 h.

	40% NaOH							40% HCl						
		(020)			(110)				(020)			(110)		
Treatment time (h)	CI (%)	RI (%)	*d* (Å)	D_{ap} (Å)	RI (%)	*d* (Å)	D_{ap} (Å)	CI (%)	RI (%)	*d* (Å)	D_{ap} (Å)	RI (%)	*d* (Å)	D_{ap} (Å)
	α-chitin							α-chitin						
0	75.45	45.0	9.4	198.95	100	4.6	190.67	75.45	45.0	9.4	198.95	100	4.6	190.67
1	59.45	29.9	9.2	176.89	54.3	4.6	109.80	71.01	51.4	9.4	206.69	100	4.6	198.90
2	60.49	29.8	9.5	213.37	61.8	4.6	84.50	66.29	41.3	9.3	221.04	100	4.6	208.48
3	64.50	46.7	9.2	196.03	100	4.6	92.10	67.66	48.8	9.4	178.66	100	4.6	192.73
4	60.59	52.6	9.3	197.73	100	4.5	104.01	66.60	45.4	9.3	173.79	100	4.6	201.67
	β-chitin							β-chitin						
0	67.33	47.7	10.0	113.92	100	4.5	73.09	67.33	47.7	10.0	113.92	100	4.5	73.09
1	59.28	49.3	9.3	127.16	100	4.6	114.66	65.06	36.9	10.1	123.47	100	4.4	74.64
2	63.29	43.6	9.8	195.98	100	4.6	126.31	67.96	40.6	10.4	128.23	100	4.4	70.43
3	62.09	41.1	9.5	204.54	80.1	4.6	132.33	67.96	58.5	11.0	105.31	100	4.7	72.06
4	58.33	46.2	9.4	201.73	95.7	4.5	130.13	68.75	42.2	10.6	113.04	100	4.5	73.70

Table 4.3 Changes of moisture absorption ability (%) of α- and β-chitin treated by 40% NaOH or 40% HCl.

	Reagent	40% NaOH		40% HCl	
	Forms of chitin	α-chitin	β-chitin	α-chitin	β-chitin
Treatment time (h)	0	^{*B} -0.835 ^{b**}	^{C} 6.969 ^{a}	^{C} -0.835 ^{b}	^{AB} 6.969 ^{a}
	1	^{A} 15.709 ^{ab}	^{B} 16.515 ^{a}	^{A} 8.392 ^{ab}	^{AB} 7.098 ^{b}
	2	^{A} 11.024 ^{a}	^{BC} 10.187 ^{ab}	^{B} 3.971 ^{c}	^{B} 4.890 ^{bc}
	3	^{A} 8.616 ^{b}	^{A} 27.449 ^{a}	^{AB} 5.193 ^{b}	^{AB} 6.220 ^{b}
	4	^{A} 8.203 ^{b}	^{A} 23.274 ^{a}	^{AB} 5.533 ^{b}	^{A} 8.920 ^{b}

*Means proceeded by the same capital letter in the same column within the form of chitin were not significantly different (P>0.05).

** Means preceded by the same small letter in the same raw were not significantly different (P>0.05).

Table 4.4 Degree of deacetylation (DDA) and molecular weight (Mw) of α- and β-chitin when subjected to deacetylating treatments under different concentrations of NaOH, temperatures, and reaction times.

	Factors			α-chitin		β-chitin	
Trials	NaOH (%)	Temp. (°C)	Time (h)	DDA (%)	Mw (kDa)	DDA (%)	Mw (kDa)
1	40	60	2	49.16	ND[+]	62.61	ND
2			4	63.24	ND	77.43	4538
3			6	66.52	ND	78.09	5235
4		90	2	39.71	ND	84.29	4364
5			4	54.14	ND	86.36	3500
6			6	60.57	ND	89.07	2587
7	50	60	2	67.03	ND	75.98	5132
8			4	80.23	2310	79.50	4610
9			6	77.34	2175	80.76	4381
10		90	2	79.62	2163	85.52	4613
11			4	82.71	2037	84.92	3557
12			6	88.84	1773	91.65	3182

[+] ND: Mw was undetectable since prepared chitosan was unable to be solubilized in the acid solution.

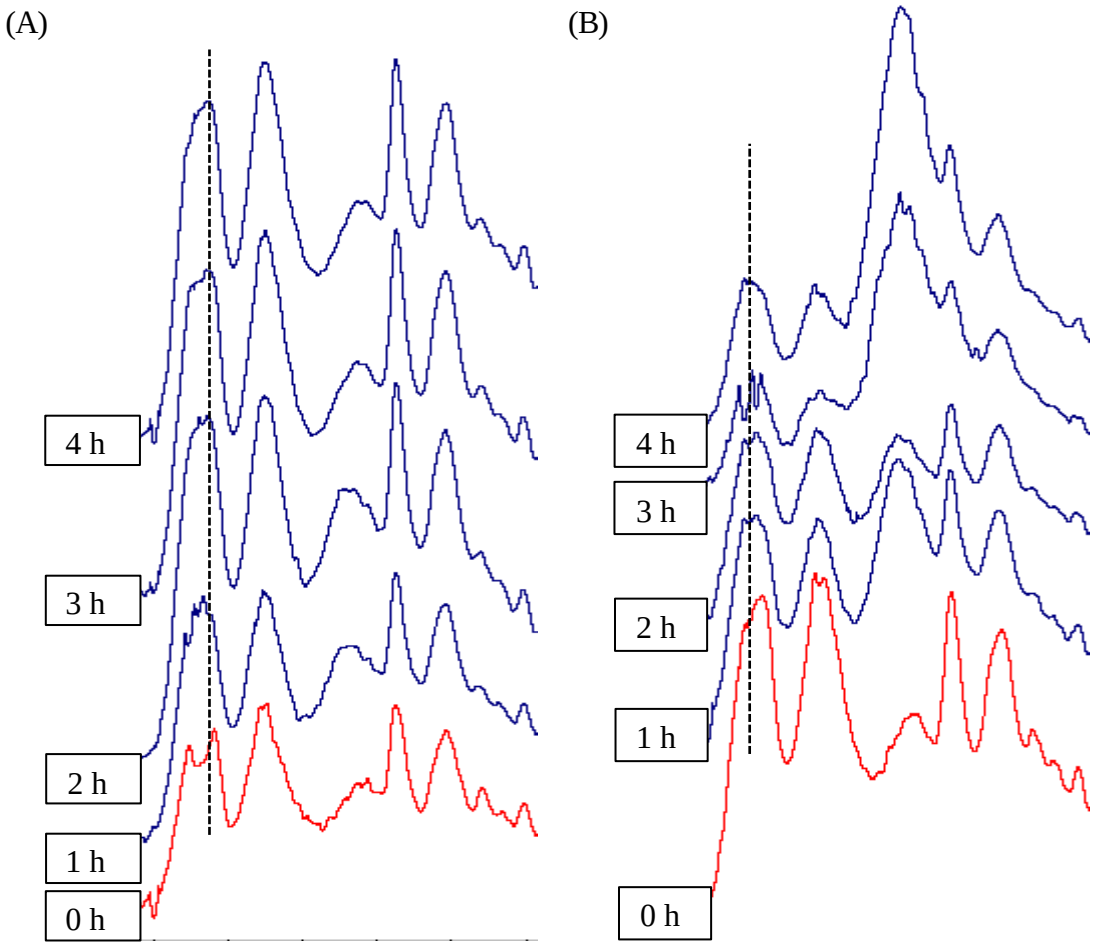

Fig. 4.1 The comparison of FT-IR spectra between native α- (A) and β-chitin (B) and chitin treated by 40% NaOH for 1-4 h. Dash lines indicate C=O band in amide to distinguish the form of chitin since C=O band was split by inter-sheet hydrogen bonds formed with hydroxyl groups in α-chitin.

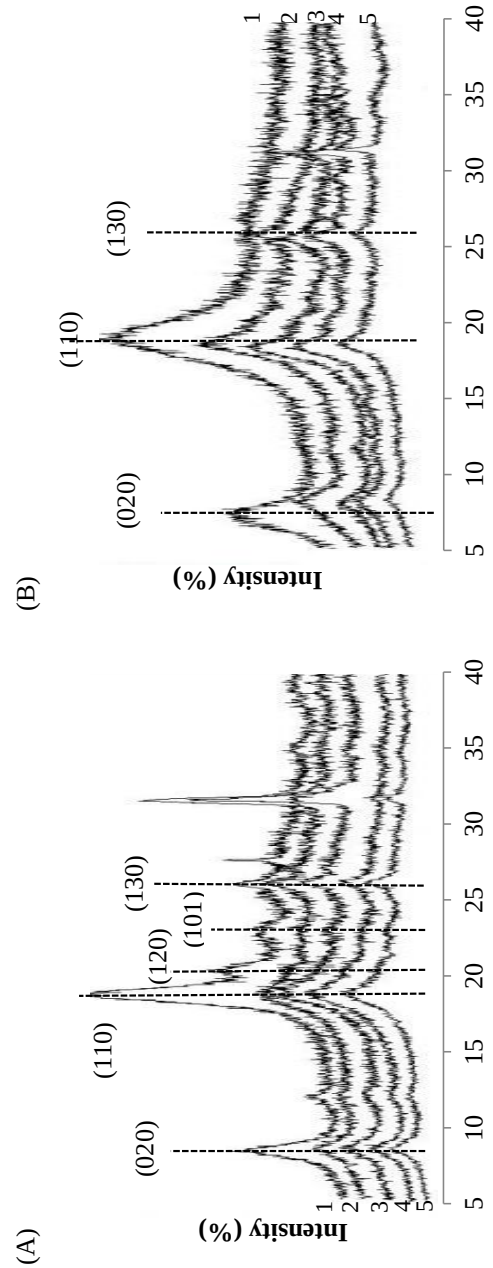

Fig. 4.2 Comparison of X-ray diffraction patterns of α- (A) and β- (B) chitin treated by 40% NaOH. 1: original chitin; 2, 3, 4, 5: chitin treated in 40% NaOH for 1, 2, 3, or 4 h, respectively.

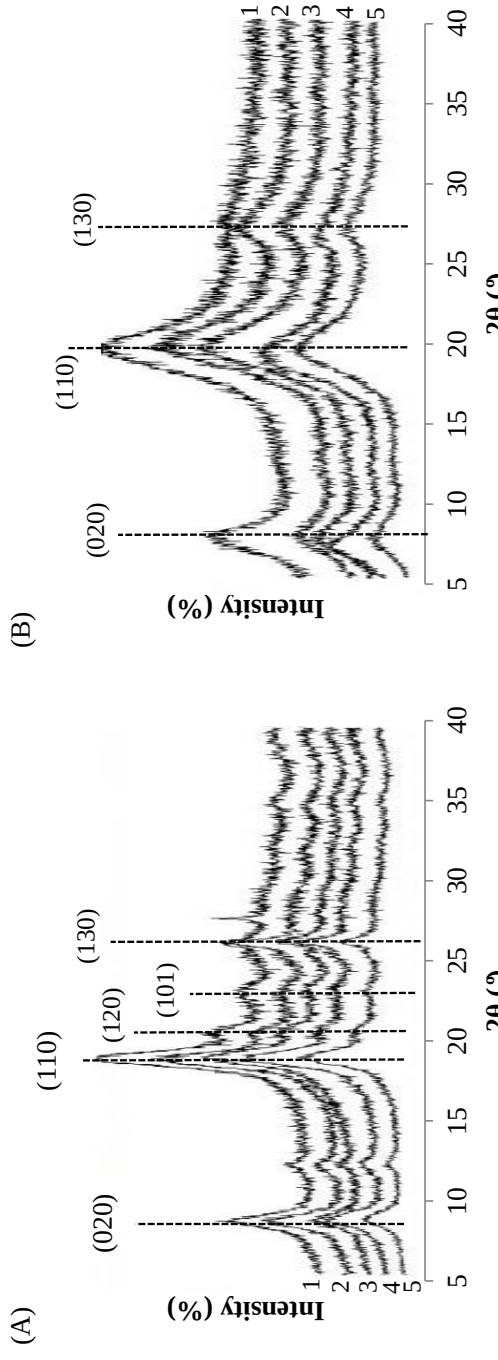

Fig. 4.3 Comparison of X-ray diffraction patterns of α- (A) and β- (B) chitin treated by 40% HCl. 1: original chitin; 2, 3, 4, 5: chitin soaked in 40% NaOH for 1, 2, 3, or 4 h, respectively.

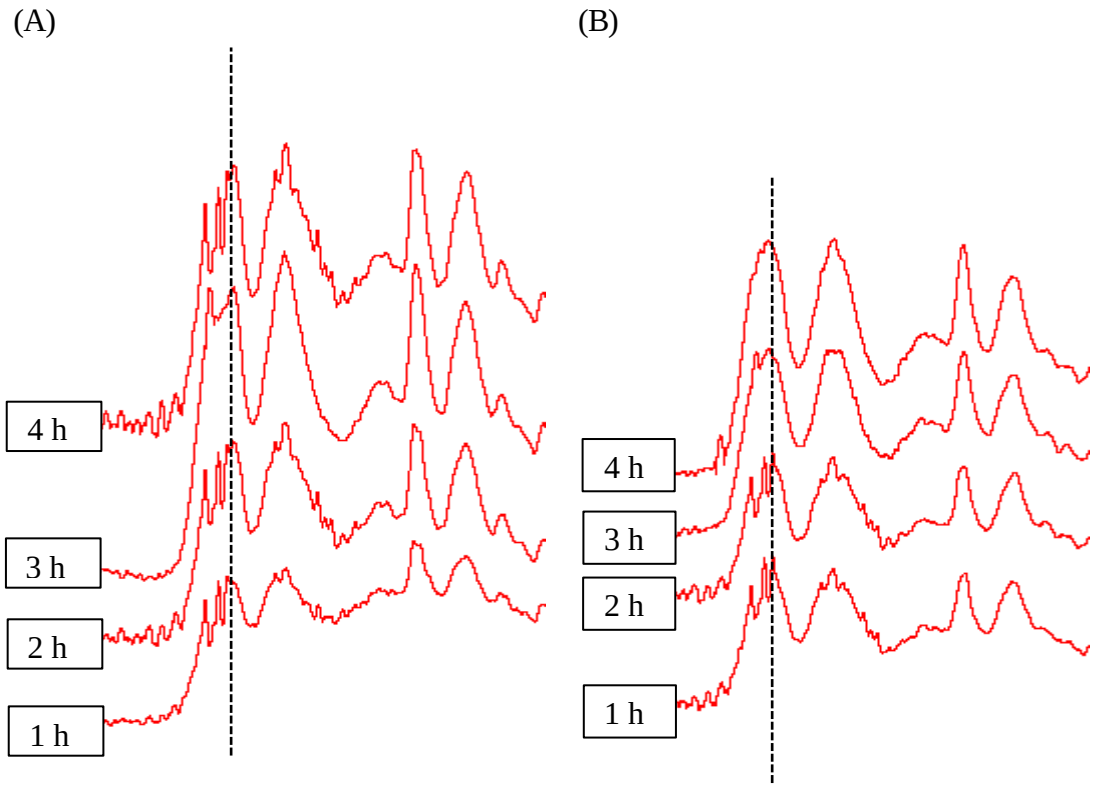

Fig. 4.4 The comparison of FT-IR spectra of α- (A) and β-chitin (B) treated by 40% HCl for 1-4 h; Dash lines indicate C=O band in amide to distinguish the form of chitin since C=O band was split by inter-sheet hydrogen bonds formed with hydroxyl groups in α-chitin.

CHAPTER 5

COMPARISON IN ANTIOXIDANT ACTION BETWEEN α-CHITOSAN and β-CHITOSAN AT A WIDE RANGE OF MOLECULAR WEIGHT AND CHITOSAN CONCENTRATION

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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.

Keywords: β-chitosan, jumbo squid pens, α-chitosan, antioxidant activity, molecular weight

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 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 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.

MATERIALS AND METHODS

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.

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 ℃ oven (Thermo Fisher Scientific Inc., USA) for 24 h. β-chitin

was deacetylated by two treatment conditions: 1) 50% NaOH at 90 ℃ for 6 h to obtain 97% DDA; and 2) 40% NaOH at 90 ℃ 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 ℃ 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 ℃ 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 8,000 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 ℃ 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.

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).

Determination of Viscosity-Average Mw

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 0.1M CH_3COOH and 0.2M NaCl. The intrinsic viscosity was measured by the intercept between the Huggins (reduced viscosity, $\eta sp/C \sim C$) and Kraemer (relative viscosity, $\eta rel/C \sim C$) plots when the concentration was 0. [22] The viscosity-average molecular weight of chitosan was calculated by Mark-Houwink-Sakurada (MHS) equation:

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

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.

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., U.S.) 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] x 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.

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 ℃ 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]

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 (0.1mL, 2mM). 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] x 100. EC_{50} value (mg/mL) was determined in the concentration at which chelating activity reached 50% effect.

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 (10mM, 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 ℃ 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 ℃ for 20 min. Absorbance was measured at 532 nm in a spectrophotometer (Shimadzu, Japan). Hydroxyl radical scavenging activity was calculated as: $[1-(A_a - A_b)/A_c] \times 100$, 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%.

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.

RESULTS

Solubility

Changes of transmittance (T, %) in different Mw of α- and β-chitosan solutions at the pH range of 3 to 11 are reported in Fig. 5.1, 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 to 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.

Antioxidant Activity

Mw and coli-overlap concentration of α- and β-chitosan samples are shown in Table 5.1. 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- (88%) and β (86%)-chitosan 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 5.2. 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 5.3. 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 5.4 along with R^2 values, the coordination between EC_{50} and CS. In 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. 5.2). 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.

DISCUSSION

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

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.

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.

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. [34] 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, 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, 35] 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.

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 5.4). Unlike other measurements, EC_{50} of hydroxyl radical scavenging activity in β-chitosan was poorly coordinated with CS and less dependent on Mw (Table 5.4). As shown by the multiple comparison results in Table 5.3, 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 5.4), 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, similarl 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 to 281 kDa. [31] This result indicated antioxidant mechanisms can be exerted differently in chitosan depending on Mw.

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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Table 5.1 Differences in molecular weight (Mw), and coil-overlap concentration (C*) between α- and β-chitosan

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*, coil-overlap concentrations is the lowest concentrations of chitosan getting entangled in solutions, obtained from 1/[η]

Table 5.2 Analysis of variance (ANOVA) table (P=0.05) for analyzing main effect or interactions among tested factors including the form of chitosan, molecular weight, and chitosan concentration

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 x Mw	3	14.00	<.0001	3	23.17	<.0001	3	5.83	0.0016	3	9.42	<.0001
Mw x CS	12	4.38	<.0001	12	2.00	0.0434	12	7.06	<.0001	12	1.48	0.1635
FC x 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		

Table 5.3 The multiple comparison using Tukey's Studentized Range (HSD) Test among main 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 5.4 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	N.D.[*]	-	N.D.	-	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.

[*] N.D. indicated that EC_{50} was negative value.

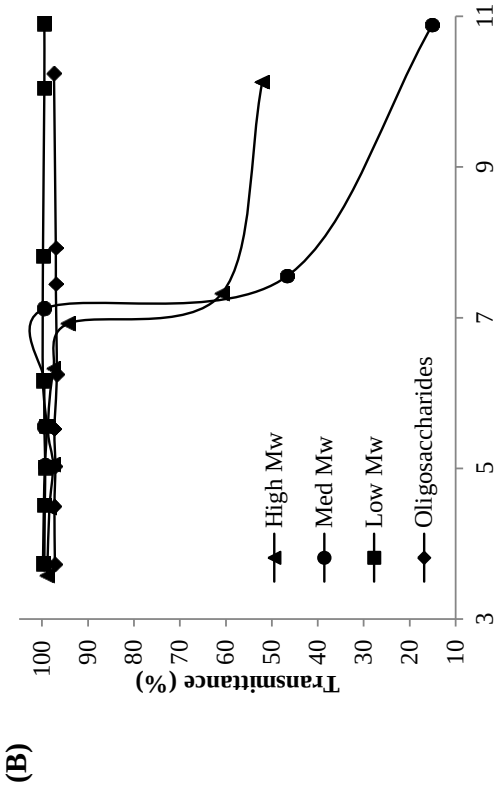

Fig. 5.1 Transmittance (T, %) of different molecular weights of α-chitosan (88% DDA) and β-chitosan (97% DDA, plus additional sample with 86% DDA) solutions at pH range of 3-11

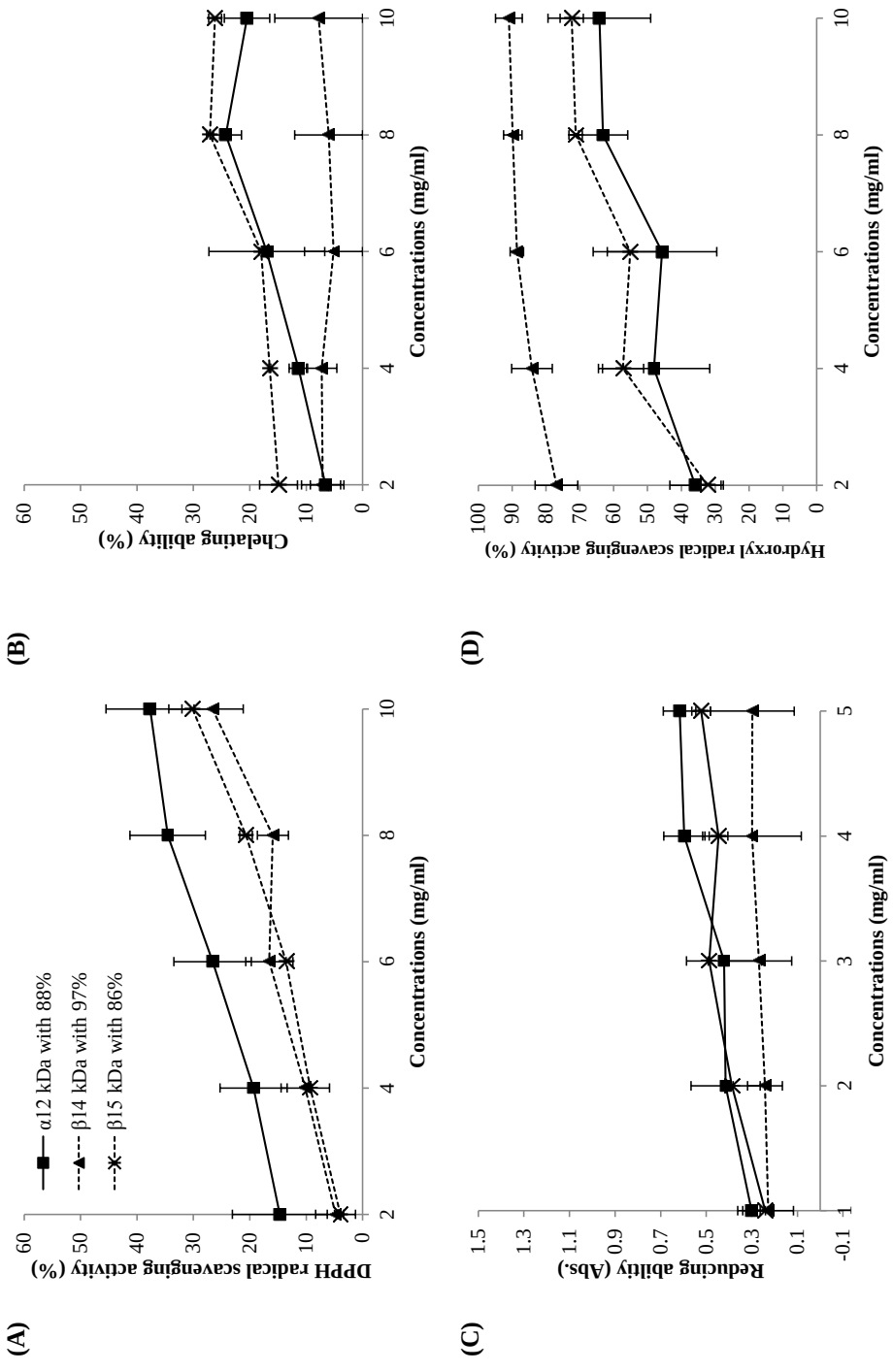

Fig. 5.2 Effect of DDA on measured antioxidant activity of α- and β-chitosan at low Mw of 20-30 kDa

CHAPTER 6

IMPACT OF THE STRUCTURAL DIFFERENCES BETWEEN α- AND β-CHITOSAN ON THEIR DEPOLYMERIZING REACTION AND ANTIBACTERIAL ACTIVITY

Jung, J., Zhao, Y. Submitted to *Journal of Agricultural and Food Chemistry*

ABSTRACT

The polymeric structure characteristics of β-chitosan from jumbo squid (*Dosidicus gigas*) pens and α-chitosan from shrimp shells during deploymerization by cellulase hydrolysis at different degrees of deacetylation (DDA) (60, 75 and 90%) were investigated by using Fourier Transform Infrared spectroscopy and X-ray Diffraction. Antibacterial activity of β-chitosan against *E. coli* and *L. innocua* was compared with that of α-chitosan at similar Mw and DDA by studying inhibition ratio and minimal inhibition concentration (MIC) and was coordinated with the structural characteristics of the two forms of chitosan. β-chitosan is more affirmative to cellulase hydrolysis than α-chitosan due to its relatively lower crystallinity (CI) and loose crystal property, and the 75% DDA chitosan was more susceptible to cellulase than the 90% DDA ones with the 75% DDA of β-chitosan mostly reactive. Both forms of chitosan showed more inhibition against *E. coli* than against *L. innocua*, no difference against *L. innocua* between the two forms of chitosan was observed, but the two forms of chitosan exhibited different antibacterial activity against *E. coli*, in which 75% DDA/31 kDa β-chitosan showed significantly higher inhibition (lower MIC) than that of 75% DDA/31 kDa α-chitosan, whereas 90% DDA/74-76 kDa α-chitosan had higher inhibition ratio than that of 90% DDA/74-76 kDa of β-chitosan. This result may be explained by the impact of the different structural properties between α- and β-chitosan on chitosan conformations in the solution. This study provided new information about the biological activities of β-chitosan, a bioactive compound with unique functionalities and great potential for food and other applications.

Keywords: α- and β-chitosan, jumbo squid pens, depolymerization, antibacterial activity, structural properties

INTRODUCTION

Antibacterial activity of β-chitosan might be different from that of α-chitosan due to several reasons. First, they are obtained from different marine sources, in which β-chitin is mainly obtained from squid pens, while α-chitin is mostly extracted from shrimp or crab shells [1, 2]. Several studies have demonstrated that the functional properties of chitin or chitosan depend on the originated marine sources and species [3-6]. Secondly, different intra- and inter-molecular structures of α- and β-chitosan influences the conformations of the chitosan solutions, thus altering their antibacterial mechanisms, such as the interactions between the protonated amino groups (NH_3^+) of chitosan solubilized in acids and the negatively charged bacterial cell membranes [7]. Since the surface phenomenon between chitosan and bacterial cells plays critical role in the antibacterial action of chitosan, the chitosan conformation in the solution needs to be flexible to increase the contact with bacterial cells in the suspension along with enhanced electrostatic interaction. However, polymorphic chitosan with strong intra- or inter-molecular hydrogen bonds can induce the rigid conformation with lower flexibility in the soluble status. Based on the previous findings, the structural properties of β-chitosan were different from those of α-chitosan after the deacetylation process as β-chitin exhibits higher solubility, reactivity, and swelling ability toward solvents, led to more structural modifications of β-chitin than that of α-chitin after alkali treatments [3, 6, 8-10]. Hence, the different intra- or inter-molecular behaviors between α- and β-chitosan could alter the chitosan conformations in the solution, which in turn impacts their antibacterial activity. Moreover, the degree of deacetylation (DDA) and molecular weight (Mw) of chitosan strongly impact its

structural properties as well. Kumar et al. (2004) reported the reduced crystallinity index (CI) in lower Mw of α-chitosan [11], whereas opposite result was observed by Ogawa et al. (1991) [12]. Liu et al. (2006) also found increased CI in low Mw and high DDA of α-chitosan [13].

So far, the antibacterial studies on chitosan have been focused on readily accessible α-chitosan extracted from crustacean shells [14-17], but little was reported on β-chitosan from squid pens [18, 19]. No previous study has compared the antibacterial activity between α- and β-chitosan based on their polymeric structural differences. In addition, it is unclear how α- and β-chitosan respond differently to the enzymatic depolymerization in association with their polymorphic structures.

Our previous studies have demonstrated that β-chitin obtained from jumbo squid (*Dosidicus gigas*) pens, a newly employed source of β-chitin, has unique deacetylation and depolymerization characteristics along with significantly different antioxidant activity from α-chitosan [20, 21]. Therefore, the objectives of this study were to investigate the depolymerizing reaction of β-chitosan prepared with ~60%, 75%, and 90% DDA in comparison with α-chitosan, and to compare the antibacterial activity against *L. innocua* (Gram-positive) and *E. coli* (Gram-negative) between the two forms of chitosan at a wide range of DDA and Mw in association with their structure properties. Inhibition ratio (IR, %) and minimum inhibitory concentrations (MIC, %) of chitosan, as well as the hydrophilicity and power of negative charge of each bacterium, were studied to represent and interpret the antibacterial activity of chitosan. Meanwhile, the polymorphic characteristics of both forms of chitosan at different DDA and Mw were investigated by using the Fourier-transform infrared (FT-IR) spectroscopy and X-ray diffraction (XRD).

MATERIALS AND METHODS

Chitosan Preparation

Dried jumbo squid (*Dosidicus gigas*) pens were provided by Dosidicus LLC (USA), and α-chitin from shrimp shells was purchased from Sigma-Aldrich (USA). After grinding into about 18 meshes (Glenmills Inc., USA), squid pens were deproteinized by treating in 5% NaOH for 3 d at room temperature, washed with distilled water, and then dried at 40 ℃ oven (Precision Scientific Inc., USA) for 24 h. For preparing chitosan with different DDAs (~60, 75, and 90%), chitin was deacetylated by using the following conditions as described in our previous study: NaOH concentrations (40 or 50%), temperatures (60 or 90 ℃), and reaction times (2, 4, or 6 h) [20].

Depolymerization of Chitosan

α-chitosan at ~75% DDA/2175 kDa and ~90% DDA/1773 kDa, and β-chitosan at ~60% DDA (Mw was undetectable), ~75% DDA/4610 kDa, and ~90% DDA/3182 kDa were depolymerized by using cellulase to investigate their depolymerizing reactions [20]. Briefly, chitosan solutions were prepared at a ratio of 1:100 (chitosan: 2% acetic acid), and adjusted to pH 5 by 10% NaOH. Cellulase was added at the same weight of chitosan in the solutions and reacted for 1-4 h. The hydrolyzates were boiled for 10 min to inactivate enzyme and centrifuged at 8,000 g for 30 min to remove denatured enzyme. A 10% NaOH was added into the solution until about pH 9 for precipitation. Precipitated samples were washed with distilled water and dried at 40 ℃ oven for 24 h. The Mw of depolymerized β-chitosan were analyzed and

compared with that of α-chitosan at similar DDA of ~60, 75, and 90%. Since it's impossible to get exact same Mw between the two forms of chitosan at same DDA, they were classified as High, Med, and Low. Specifically, High/Med/Low Mw were 133/45/31 kDa in 75% DDA α-chitosan, 111/74/27 kDa in 90% DDA α-chitosan, 72/31/20 kDa in 75% DDA β-chitosan, and 76/40/17 kDa 90% DDA β-chitosan.

Viscosity-average Mw and DDA

The viscosity-average Mw of α- and β-chitosan was determined by using the Ubbelohde Dilution Viscometer (Cannon instrument Co., USA) with a capillary size of 0.58 nm. Approximate 100 mg of chitosan was dissolved in 10 mL of the mixture solution of 0.1M CH_3COOH and 0.2M NaCl [20]. The intrinsic viscosity was measured by the intercept between the Huggins (reduced viscosity) and Kraemer (relative viscosity) plots when the concentration was 0 [22]. The viscosity-average Mw of chitosan was calculated by using Mark-Houwink-Sakurada (MHS) equation (1) [23]:

$$[\eta] = K (M_W)^a \quad (1)$$

where K and a were constants, $K=1.81 \times 10^{-3}$ and a = 0.93; and [η] was the intrinsic viscosity. DDA was determined by the colloidal titration method [24].

Antibacterial Activity

Cultures of *Escherichia coli* ATCC 25922 and *Listeria innocua* ATCC 51742 (American Type Culture Collection) were stored in appropriate solid media (*E. coli* on tryptic soy agar (TSA) (Becton, Dickinson and Co., USA) and *L. innocua* on brain heart infusion (BHA) agar (Becton, Dickinson and Co., USA)) under refrigeration (4 ℃)

during the course of the study. Prior to a given microbiological assay, a single typical colony of each bacterium was inoculated in appropriate broth (*E. coli* in tryptic soy broth (TSB) (EMD Chemicals, Inc., USA) and *L. innocua* in brain heart infusion (BHI) broth (Becton, Dickenson and Co., USA)) and enriched at 37 ℃ for 24 h.

For determining the minimum inhibitory concentration (MIC, %) of chitosan, 1% chitosan solubilized in 1% of acetic acid solution or 1% acetic acid solution alone was used to produce a series of serially diluted tubes ranging from 0.5% to 0.02%. The aliquot (0.5 mL) of enriched *E. coli* and *L. innocua* was inoculated into prepared test tubes under the aseptic condition. After incubating for 24 h at 37 ℃, the optical density (OD) of each test tube was determined at 620 nm using the spectrophotometer (Shimadzu, Japan). MIC was the lowest concentration among the tested ranges of concentrations, in where OD of the cultured tubes treated by chitosan solution (treatment) was lower than the OD of the cultured tubes treated by acetic acid alone (control) [25]. Inhibition ratio (IR, %) was further investigated to compare the relative antibacterial activity of different chitosan samples. IR was calculated as Eq. (2):

$$Inhibition\ ratio\ (IR,\%) = \frac{OD\ of\ cell\ suspension\ treated\ with\ control - OD\ of\ cell\ suspension\ treated\ with\ chitosan\ solution}{OD\ of\ cell\ suspension\ treated\ with\ control}\ x\ 100 \quad (2)$$

Hydrophillicity of Bacterial Cell

Hydrophilicity of the bacteria was determined by adding each bacterium into the mixture of n-hexane (hydrophobic) and water (hydrophilic) prepared with different

ratios of n-hexane and water (v/v, 0:5, 1:4, 2:3, 3:2, and 4:1), and then measuring the OD of cell suspension in water phase. Enriched bacteria (~1 x 10^6 CFU/mL for *E. coli* and ~1 x 10^5 CFU/mL for *L. innocua*) were prepared after incubation for 1 d. A 5 mL of cell suspension was added into 5 mL of the two-phase mixture of n-hexane and water at different ratios. The mixtures were stirred for 3 min and allowed to settle for 5 min. OD of the lower part (water phase) was determined at 600 nm using UV spectrophotometer (Shimadzu, Japan). Hydrophillicity (%) of each bacterium was calculated as Eq. (3) [26]:

$$Hydrophillicity\ (\%) = \frac{O.D.of\ water\ phase\ at\ different\ ratios\ in\ the\ mixture}{O.D.of\ water\ phase\ without\ hexane\ in\ the\ mixture} x100 \quad (3)$$

Power of Negative Charge in Bacterial Cell Wall

The power of negative charge was determined by using anion exchange resin, Dowex 1x8 [26]. Dowex 1x8 was washed five times with distilled water and balanced with 0.1 M HCl in the test tube for 6 h. After adding 5 mL of enriched bacteria (~1x10^6 for *E. coli* and ~1x10^5 for *L. innocua*) in Dowex 1x8, the OD of the water phase was determined at 660 nm using UV spectrophotometer (Shimadzu, Japan). The control was OD of enriched bacteria without Dowex 1x8 treatment. Relative cell density (RCD) was calculated as Eq. (4):

$$RCD(\%) = \frac{O.D.of\ bacterium\ suspended\ in\ water\ phase\ after\ Dowex\ 1x8\ treatment}{O.D.of\ bacterium\ suspended\ in\ water\ phase\ without\ Dowex\ 1x8\ treatment} x100 \quad (4)$$

The power of the negative charge for each bacterium was defined as subtracted RCD (%) from 100%.

A Fourier-Transform Infrared (FTIR) Spectroscopic Analysis

A single bound attenuated total reflection (ATR)-FTIR spectrometer (PerkinElmer, USA) was operated by Omnic 7.4 software (Thermo Fisher Inc. USA) under the operating condition of 32 scans at a 4 cm^{-1} resolution and referenced against air. All spectra were recorded as the absorption mode. Partial FT-IR spectra (2200-3700 cm^{-1}) were reported to investigate the intra-sheet or inter-sheet hydrogen bonds and crystal characteristics of prepared chitosan samples. Five assigned bands around 3480 cm^{-1}, ~3420 cm^{-1}, 3290 cm^{-1}, 2920 cm^{-1}, and 2880 cm^{-1} attributed to vibrations of OH, NH, and CH stretching, respectively.

X-ray Diffraction (XRD)

X-ray diffraction patterns were recorded using a XRG 3100 x-ray diffractometer (Philips, U.S.) with a Cu Kα (1.54 Å) at a voltage of 40 kV and a current of 30 mA. A typical scan range was from 5 º to 40 º (2θ) at scanning speed of 0.025 º/sec. The CI was determined as Eq. (5):

$$Crystallinity\ (CI, \%) = \frac{I_{110} - I_{am}}{I_{110}}\ x\ 100 \quad (5)$$

Where I_{110} is the maximum intensity of the (110) plane at 2θ = ~19 º and I_{am} was the intensity of the amorphous regions at 2θ = ~12.6 º [27, 28].

The *d*-spacing and relative intensity (%) were reported for various crystal planes (020, 110, 120, 101, or 130) appeared in polymorphic structures of chitin. The *d*-spacing was computed using Bragg's law (6) [17]:

$$d\ (\text{Å}) = \frac{\lambda}{2 \sin \theta}$$

(6)

Where d was plane spacing; λ was 1.54 Å, wavelength of Cu Kα radiation; and θ was one-half angle of reflections.

Experimental Design and Statistical Analysis

The depolymerizing reaction of β-chitosan was investigated and compared with that of α-chitosan prepared at the similar DDA (~60, 75, and 90%) through a completely randomized design (CRD). To investigate the antibacterial activity of chitosan related to DDA, Mw, and the chitosan form, a completely randomized factorial design was applied with total 14 chitosan samples: 75% DDA of α-chitosan with Mw of 133, 45 and 31 kDa, 90% DDA of α-chitosan with Mw of 111, 74 and 27 kDa, 60% DDA of β-chitosan with Mw of 73 kDa, 75% DDA of β-chitosan with Mw of 72, 31 and 20 kDa, and 90% DDA of β-chitosan with Mw of 76, 40, and 17 kDa, whereas acetic acid was applied as a control. Mw (kDa), IR (%), and MIC (%) were all determined in duplicate, and the results were analyzed for statistical significance via least significant difference (LSD) post hoc testing as appropriate using statistical software (SAS v9.2, The SAS Institute, USA). Results were considered to be significantly different if $P<0.05$.

RESULTS AND DISCUSSION

Structural Properties of α- and β-Chitosan Prepared at a Wide Range of DDA and Mw

Figs. 6.1 and 6.2 present partial FT-IR spectra (2200-3700 cm^{-1}) that illustrate the intra- or inter-molecular hydrogen bonds and CH stretching in α- and β-chitosan at a wide range of DDA and Mw, respectively. The spectrum region between 3000 and 3600 cm^{-1} attributed to the vibration of either OH or NH, indicating the hydrogen bonds appeared in C(6)OH···O=C, C(3)OH···O, C(6)OH···OHC(3), C(2)NH···O=C, and C(6)HO···HNC(2). The corresponding region between 2800 and 2900 cm^{-1} attributed to the vibration of CH stretching, assuming the free hydroxymethyl (CH_2OH) groups dissociated from hydrogen bonds. These structural properties of chitosan in the solid status can be associated with the antibacterial mechanisms of chitosan as they could impact chitosan conformations in the soluble status. Additionally, *d*-spacing and the relative intensities (%) of each crystal plane are presented to interpret the crystal properties of polymorphic chitosan (Table 6.2).

In 75% DDA of α-chitosan, the peak intensities of OH and NH_2 strengthened as Mw decreased, indicating that OH and NH_2 were more readily to form hydrogen bonds in lower Mw (≤ 31 kDa). The peak intensity of CH stretching was the highest in 45 kDa chitosan, and decreased in other Mw samples (Fig. 6.1A), assuming that the hydrogen bonds might be weaker in 75% DDA/45 kDa α-chitosan. Crystallites of (120), (101), and (130) planes appeared as Mw decreased (≤ 31 kDa) along with increasing CI (Table 6.2). In 90% DDA of α-chitosan, no NH bands distinguished and the OH bands had no significant difference among 20-111 kDa chitosan (Fig. 6.1B). However, the peak intensities of CH stretching in higher Mw (74 and 111 kDa) were relatively intense than those in lower Mw (27 kDa) (Fig. 6.1B). Crystallites of (120) planes were observed in lower Mw (≤ 27 kDa) and CI increased as Mw decreased

(Table 6.2). Hence, the hydrogen bonds in 90% DDA/74 and 111 kDa α-chitosan were weaker than those in 27 kDa sample.

For 60% and 75% DDA of β-chitosan, the peak of OH bands were more intense in 60% DDA/73 kDa and 75% DDA/72 kDa β-chitosan than in 75% DDA/20 and 31 kDa β-chitosan, whereas no significant difference in CH stretching bands was observed among samples with different DDA or Mw (Fig. 6.2A). Similar to 75% or 90% DDA of α-chitosan, crystallites of (020), (120), and (101) planes were formed and CI was relatively higher in lower DDA and Mw samples (Table 6.2). Hence, the hydrogen bonds associated with OH could be weaker in 75% DDA/20-31 kDa of β-chitosan. In 90% DDA of β-chitosan, no NH bands were distinguished and the peak intensities of OH bands had no significant difference among 17-76 kDa, similar to the 90% DDA of α-chitosan (Fig. 6.2B). However, the bands of CH stretching strengthened in lower Mw sample (≤ 40 kDa) (Fig. 6.2B), which had no significant difference in CI and crystal formation in comparison with 76 kDa sample (Table 6.2). Hence, the hydrogen bonds associated with OH or NH_2 could be weaker in 90% DDA/17-40 kDa β-chitosan.

In summary, α- and β-chitosan had different structural properties in association with DDA and Mw. The intra- and inter-molecular behaviors and crystal properties could alter the flexibility of chitosan conformation along with the degree of solubility, the major factors impacting the antibacterial activity of chitosan, thus the different structural characteristics of α- and β-chitosan may lead to their different antibacterial action based on the surface phenomenon with the negatively charged bacterial cells, which were reported and discussed below.

Depolymerization Reaction of α- and β-Chitosan

Table 6.1 shows the depolymerizing reaction of β-chitosan during 1-4 h in comparison with α-chitosan at the similar DDAs. The 60% DDA of α-chitosan was unable to be depolymerizd by cellulase, probably due to its higher CI (51%) and relative intensity (RI, 47.5%) of the crystal plane (020) than that of 60% DDA of β-chitosan (Table 6.2). In contrast, the depolymerization was occurred in 60% DDA of β-chitosan as it was solubilized in the acidic solution, but no further degradation was observed after 1 h (Table 6.1). Similarly, Lin et al., (2009) demonstrated that the degradation of 80% DDA chitosan is limited in comparison with 92% DDA chitosan due to its lower solubility [29]. Mw of 75% DDA/72 kDa and 90% DDA/67 kDa β-chitosan was significantly lower than 75% DDA/133 kDa and 90% DDA/111 kDa DDA of α-chitosan at the first 1 h, indicating β-chitosan was more affirmative to cellulase than α-chitosan. Based on the structural properties of native 75% and 90% DDA of α- and β-chitosan prior to the depolymerization process (Table 6.2), the crystallite of (020) plane was appeared in 75% DDA of α-chitosan, and the CI of 75% and 90% DDA of β-chitosan was slightly lower than that of 90% DDA of α-chitosan even though Mw of β-chitosan was significantly higher than that of α-chitosan (Table 6.2). Hence, polymorphic β-chitosan with lower CI and less crystallites was more susceptible to cellulase than α-chitosan regardless of the initial Mw.

In respect to the influence of DDA on the depolymerizing reaction in α- and β-chitosan, Mw of 75% DDA α- and β-chitosan at 4 h was significantly lower than that of 90% DDA samples (Table 6.1). Zhang et al. (2001) indicated that the degradation rate decreases as DDA increases since the higher DDA chitosan has a lower affinity

to the enzyme [30]. Although it has been known that cellulase randomly cleaves β-1,4-glycosidic bond along chitosan polymeric chains [29], this study found that the presence of a certain amount of acetyl groups may enhance the depolymerization of chitosan. Moreover, 75% DDA of β-chitosan had Mw < 10 kDa at 4 h, assumed to be the oligosaccharide since the hydrolyzed solution was unable to be precipitated under the alkaline pH. Hence, β-chitosan with 75% DDA was mostly susceptible to cellulase depolymerization among all tested chitosan samples.

Antibacterial Activity of α- and β-Chitosan Related to DDA and Mw

Table 6.3 presents the antibacterial activity (IR or MIC) of α- and β-chitosan against *L. innocua* and *E. coli* at different DDA and Mw. Overall, the inhibition against *E. coli* was higher than against *L. innocua*. This difference could be interpreted by the different powers of negative charges between *E. coli* and *L. innocua*. The negative charge of *E. coli* (~60%) was stronger than that of *L. innocua* (~42%), which led to enhanced electrostatic interactions with the cationic amino groups in chitosan solutions (Fig. 6.3). Similarly, Chung et al. (2004) reported that the cellular adsorptive amount of chitosan is higher onto Gram-negative strain with higher electrostatic interaction in comparison with Gram-positive strain [26]. Hence, the protonated amino groups in chitosan solubilized in acidic solution were more able to bind with stronger negative charged *E. coli*, making the cytoplasm to flow out of the bacterial cells. Although the hydrophilicity of *L. innocua* at the hexane:water ratio of 2:1, 3:1, or 4:1 was higher than that of *E. coli*, no significant difference in hydrophilicity between the two strains was observed in more polar solutions at hexane:water ratio of 0:1 or 1:1 (Fig. 6.3). Since the actual antibacterial experiments

were carried in the polar solutions, the higher hydrophilicity of *L. innocua* had less influence on the antibacterial activity of chitosan than the stronger negative charged *E. coli*.

For *L. innocua*, MIC of 75% DDA β-chitosan (0.03) was lower than that of 90% DDA β-chitosan (0.06) at Mw of 17-20 kDa (Table 6.3). This result was contradicted with the previous report, in which the antimicrobial activity enhanced in higher DDA chitosan [31]. This difference was probably associated with the tested range of Mw in different studies as well as the distribution of acetyl groups. In 75% DDA of β-chitosan, the IR of Low Mw (95.1%) and Med Mw (96.2%) were significantly higher than that of High Mw (84.4%).

For *E. coli*, MIC of 90% DDA α-chitosan (0.02) was lower than that of 75% DDA α-chitosan (0.03) at Mw of 27-31 kDa, whereas IR of 75% DDA β-chitosan (72.7%) was significantly higher than that of 60% DDA (47.8%) and 90% DDA β-chitosan (43.3%) at Mw of 72-76 kDa (Table 6.3). Med Mw of chitosan showed enhanced inhibition against *E. coli* than Low and High Mw of 75% and 90% DDA of α-chitosan and 90% DDA of β-chitosan, respectively. According to Eaton et al. (2008), higher Mw chitosan could exert higher inhibitory effect as it formed an impermeable layer around the cell wall to block the transportation of essential nutrients into the cells [32]. However, the electrostatic interaction between the cationic amino groups of chitosan and the anionic bacterial cell membrane can be decreased with increased Mw due to the electrostatic repulsion between extensively charged amino groups in high Mw chitosan. Similarly, Uchida et al. (1989) stated that the antibacterial activity of slightly hydrolyzed chitosan is higher than that of native chitosan or chitosan

oligomers. [33] Hence, the Med Mw of 75% DDA and 90% DDA of α- and β-chitosan had stronger antibacterial activity.

Antibacterial Activity of α- and β-Chitosan in Association with Their Structure Properties

To carry the comparative study of the antibacterial activity between α- and β-chitosan, DDA and Mw were retained close between the two forms of chitosan as these two parameters significantly affect the antibacterial activity (Table 6.3). As stated in previous studies and confirmed in this study, chitosan extracted from α- and β-chitin have different structures and interactions between the polymeric chains since the different forms of chitin responded differently to the deacetylation and depoymerization processes [8, 10]. These different intra- or inter-molecular behaviors between α- and β-chitosan impact the flexibility of chitosan conformations, thus altering the contact of protonated amino groups of chitosan with negatively charged bacterial cells in the suspension, which could result in different antibacterial activities [34, 35].

For *L. innocua*, no significant difference in the antibacterial activity was observed between α- and β-chitosan (Table 6.3). This result might be understood from the characteristics of *L. innocua*. The electrostatic interaction with strongly charged bacterial cells could be associated with the structural properties, and resulted in different antibacterial activity between the two forms of chitosan. However, the electrostatic interaction between α- and β-chitosan was difficult to be differentiated against *L. innocua* since its power of negative charges was significantly weaker than *E. coli* (Fig. 6.3). In contrast, the different antibacterial activity against *E. coli* between α- and β-chitosan

could be due to its negative charge strongly associated with the structural properties of α- and β-chitosan (Fig. 6.3).

MIC of 75% DDA/31 kDa β-chitosan (0.02) against *E. coli* was lower than that of α-chitosan (0.03) (Table 6.3). In 75% DDA/31 kDa, α-chitosan had higher CI with the presence of the crystal formation in (120), (101), and (130) planes in comparison with β-chitosan (Table 6.2). In addition, the band of CH stretching in β-chitosan was slightly intense than that in α-chitosan (Figs. 6.1 and 6.2). Hence, α-chitosan conformations in the solution could be more rigid due to strong intra- or inter-molecular hydrogen bonds, thus decreasing the contacts to the bacterial cells in the suspension than β-chitosan conformations. Reversely, 90% DDA/74-76 kDa α-chitosan exerted significantly higher IR (58.4%) than β-chitosan (43.3%) against *E. coli* (Table 6.3). Although CI of α-chitosan was relatively higher than that of β-chitosan (Table 6.2), the band of CH stretching in α-chitosan was intense in comparison with that in β-chitosan, it thus could be assumed that hydrogen bonds was weaker in α-chitosan. Hence, the flexible conformation of α-chitosan could enhance the electrostatic interaction between cationic chitosan and anionic bacterial cells, increasing antibacterial activity. Therefore, α- and β-chitosan showed different antibacterial activity against *E. coli* due to their structural difference at similar DDA and Mw.

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Table 6.1 Mw of different degrees of deacetylation (DDA, %) of α- and β-chitosan by cellulase hydrolysis at different reaction times (h)

Reaction time (h)	α-chitosan		β-chitosan		
	~75% DDA	~90% DDA	~60% DDA	~75% DDA	~90% DDA
0	2175	1773	ND*	4610	3182
1	$^{A}133^{a}$	$^{AB}111^{a}$	$^{B}53^{b}$	$^{B}72^{a}$	$^{B}67^{a}$
2	$^{C}45^{b}$	$^{A}74^{b}$	$^{B}57^{ab}$	$^{D}31^{b}$	$^{A}76^{a}$
3	$^{BC}31^{c}$	$^{C}20^{c}$	$^{A}73^{a}$	$^{C}20^{c}$	$^{B}40^{b}$
4	$^{B}11^{d}$	$^{A}27^{c}$	--***	ND**	$^{B}17^{c}$

Means preceded by the same capital letter in the same raw were not significantly different (P>0.05)

Means proceeded by the same small letter in the same column were not significantly different (P>0.05)

*ND: non-detected since prepared chitosan was unable to be solubilized in the solution for measuring viscosity-average Mw.

** ND: non-detected as Mw was significantly lower than 10 kDa.

*** --: no further experiment was carried out due to non-significant degradation.

Table 6.2 Crystallinity (CI, %), *d*-spacing (*d*, Å), and relative intensity (RI, %) of various planes (020, 110, 120, 101, and 120) appeared in α- and β-chitosan at different degrees of deacetylation (DDA) and molecular weights (Mw)

Chitosan samples		(020)		(110)		(120)		(101)		(130)	
Form (DDA, Mw)	CI (%)*	*d* (Å)**	RI (%)	*d* (Å)	RI (%)	*d* (Å)	RI (%)	*d* (Å)	RI (%)	*d* (Å)	RI (%)
α (60%, ND+)	51	10.0	47.5	4.4	100	-	-	-	-	-	-
α (75%, 11 kDa)	56	-	-	4.5	100	4.0	61.8	3.0	60.5	-	-
α (75%, 31 kDa)	54	-	-	4.5	100	3.9	73.9	3.0	66.6	2.5	51.6
α (75%, 45 kDa)	57	-	-	4.4	100	-	-	-	-	-	-
α (75%, 133 kDa)	37	-	-	4.3	100	-	-	-	-	-	-
α (75%, 2175 kDa)	46	10.0	33.0	4.4	100	-	-	-	-	-	-
α (90%, 20 kDa)	66	-	-	4.4	100	4.0	56.9	-	-	-	-
α (90%, 74 kDa)	61	-	-	4.4	100	-	-	-	-	-	-
α (90%, 111 kDa)	41	-	-	4.4	100	-	-	-	-	-	-
α (90%, 1773 kDa)	51	-	-	4.4	100	-	-	-	-	-	
β (60%, 72 kDa)	38	10.6	60.4	4.5	100	-	-	3.0	67.9	-	-
β (60%, ND)	45	-	-	4.4	100	-	-	-	-	-	-
β (75%, 20 kDa)	44	9.8	44.6	4.4	100	3.9	68.7	3.0	72.9	-	-
β (75%, 31 kDa)	29	9.8	40.3	4.4	100	3.9	73.3	3.0	76.2	-	-
β (75%, 72 kDa)	29	-	-	4.5	100	-	-	3.0	66.0	-	-
β (75%, 4610 kDa)	47	-	-	4.4	100	-	-	3.4	64.8	-	-
β (90%, 17 kDa)	45	-	-	4.4	100	-	-	-	-	-	-
β (90%, 40 kDa)	49	-	-	4.4	100	-	-	-	-	-	-
β (90%, 76 kDa)	47	-	-	4.4	100	-	-	-	-	-	-
β (90%, 3182 kDa)	48	-	-	4.4	100	-	-	-	-	-	-

* Crystallinity was determined by X-ray diffraction (XRD) using the equation: $(I_{110}-I_{am})/I_{110}*100$, where I_{110} was the maximum intensity of the reflection (110) at $2\theta = 19$ ° and I_{am} was the intensity of the amorphous diffraction in the same unit at $2\theta = 12.6$ °.
** *d* (Å) = $\lambda/\sin\theta$, where *d* was plane spacing; λ is 1.54 Å , wavelength of Cu Kα radiation; and θ was one-half angle of reflections.
+ ND: Mw was undetectable since prepared chitosan was unable to be solubilized in the solution for measuring viscosity-average Mw.

Table 6.3 Inhibition ratio (IR, %) and minimum inhibitory concentration (MIC, %) of α- and β-chitosan against *L. innocua* or *E. coli* at different degrees of deacetylation (DDA, %) and molecular weights (Mw, kDa)

	L. innocua						*E. coli*					
	Degree of deacetylation (DDA, %)											
	60		75		90		60		75		90	
Form (Mw)	IR	MIC	IR	MIC	IR	MIC	IR	MIC	IR	MIC	IR	MIC
α (27-31 kDa)	*		98.5 [a]	0.03	86.6 [a]	0.03			-	0.03	-	0.02
β (17-20 kDa)			-**	0.03	-	0.06			61.9 [a]	0.02	64.1 [a]	0.02
β (72-76 kDa)	95.7 [a]	0.03	84.4 [a]	0.03	90.3 [a]	0.03	47.8 [b]	0.02	72.7 [a]	0.02	43.3 [b]	0.02
	Molecular weight (Mw, kDa)											
	High[+]		Med[++]		Low[+++]		High[+]		Med[++]		Low[+++]	
Form (DDA)	IR	MIC	IR	MIC	IR	MIC	IR	MIC	IR	MIC	IR	MIC
α (75%)	95.2 [a]	0.03	96.3 [a]	0.03	98.5 [a]	0.03	-	0.03	-	0.02	-	0.03
α (90%)	92.9 [a]	0.03	96.8 [a]	0.03	91.4 [a]	0.03	58.4 [b]	0.02	75.7 [a]	0.02	62.2 [b]	0.02
β (75%)	84.4 [b]	0.03	96.2 [a]	0.03	95.1 [a]	0.03	72.7 [a]	0.02	64.0 [a]	0.02	61.9 [a]	0.02
β (90%)	91.7 [a]	0.03	94.6 [a]	0.03	95.1 [a]	0.03	54.1 [b]	0.02	92.3 [a]	0.02	64.1 [ab]	0.02
	Chitosan form (CF)											
	α		β				α		β			
DDA, Mw	IR	MIC	IR	MIC			IR	MIC	IR	MIC		
75%, 31 kDa	98.5 [a]	0.03	96.2 [a]	0.03			-	0.03	-	0.02		
90%, 17-20 kDa	91.4 [a]	0.03	95.1 [a]	0.03			75.4 [a]	0.02	64.1 [a]	0.02		
90%, 74-76 kDa	96.8 [a]	0.03	90.3 [a]	0.03			58.4 [a]	0.02	43.3 [b]	0.02		

Means preceded by the same letter in the same raw within each bacterium were not significantly different (P>0.05).

[+] Mw of α (75% DDA), α (90% DDA), β (75% DDA), and β (90% DDA) were 133 kDa, 111 kDa, 72 kDa, and 76 kDa, respectively.

++ Mw of α (75% DDA), α (90% DDA), β (75% DDA), and β (90% DDA) were 45 kDa, 74 kDa, 31 kDa, and 40 kDa, respectively.

+++ Mw of α (75% DDA), α (90% DDA), β (75% DDA), and β (90% DDA) were 31 kDa, 27 kDa, 20 kDa, and 17 kDa, respectively.

* The blank cell meant that 60% DDA of α- and β-chitosan were unable to be hydrolyzed to lower Mw.

** Since MIC was significantly different among the samples, no IR needed to be reported.

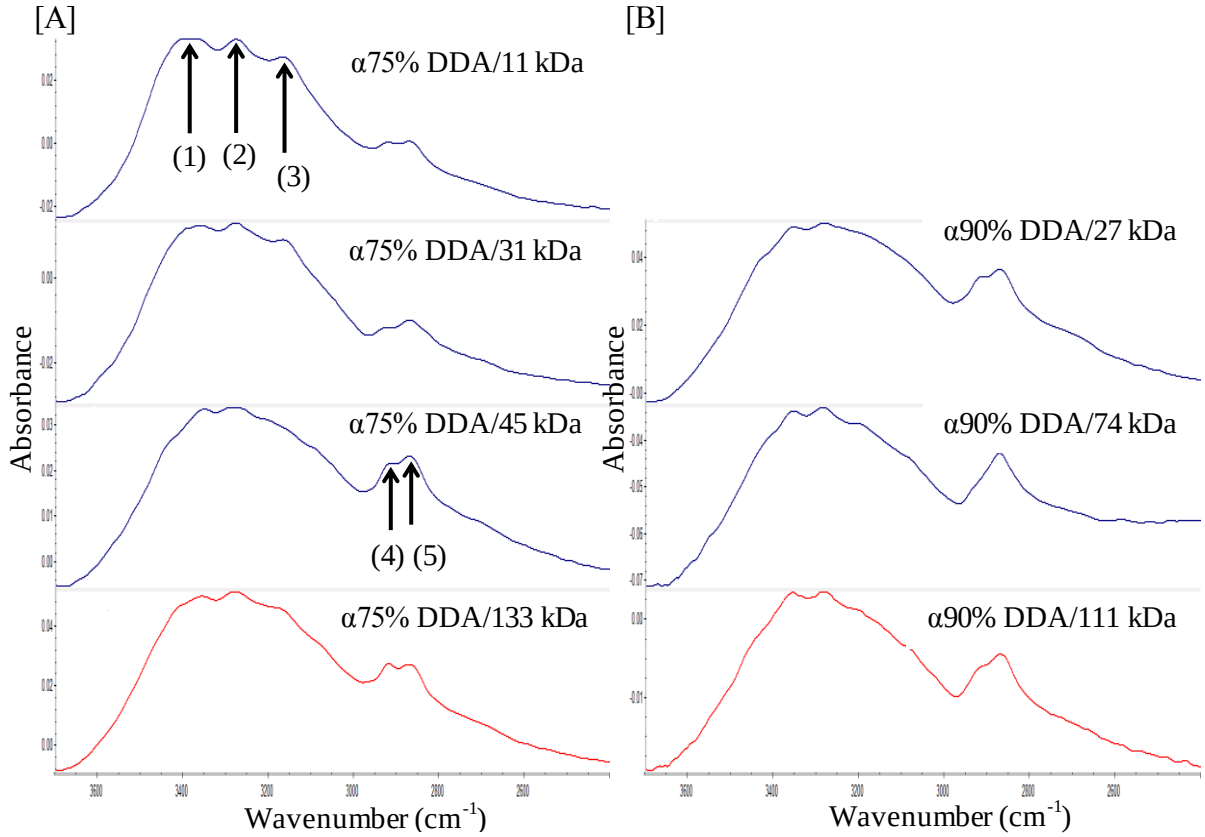

Fig. 6.1 Partial FT-IR spectra (2400-3700 cm^{-1}) of 75% DDA of α-chitosan (A) and 90% DDA of α-chitosan (B) at different molecular weights (Mw); (1) and (2) attributed to the vibration of OH; (3) attributed to the vibration of NH; (4) and (5) attributed to the vibration of CH stretching.

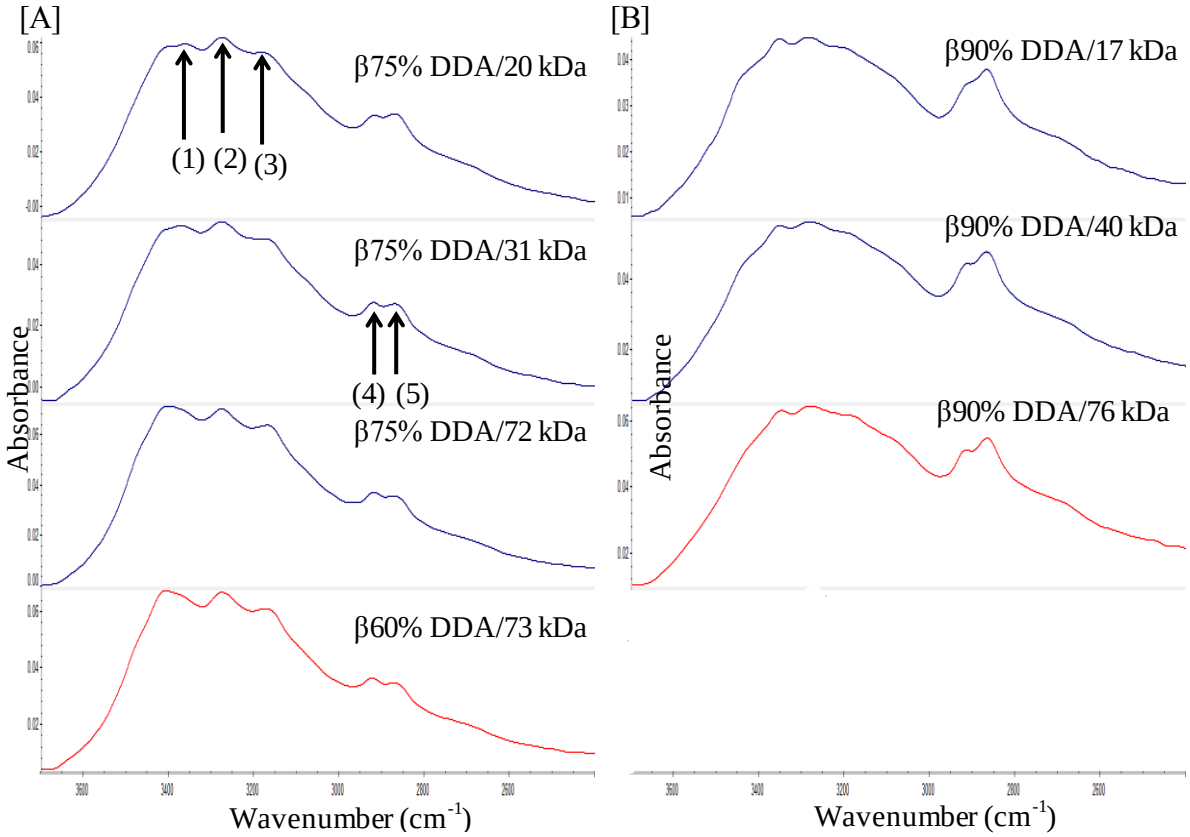

Fig. 6.2 Partial FT-IR spectra (2400-3700 cm^{-1}) of 60% DDA and 75% DDA of β-chitosan (A) and 90% DDA of β-chitosan (B) at different molecular weights (Mw); (1) and (2) attributed to the vibration of OH; (3) attributed to the vibration of NH; (4) and (5) attributed to the vibration of CH stretching.

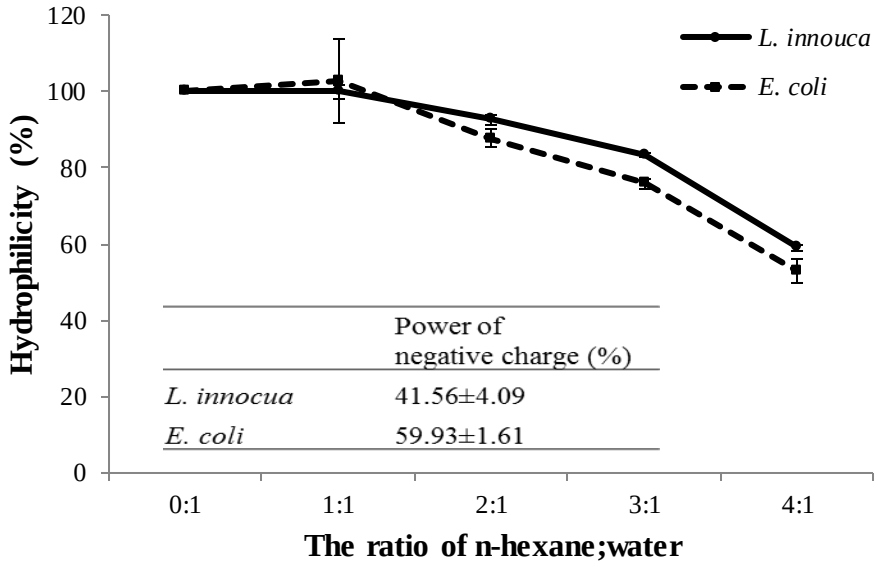

Fig. 6.3 The hydrophillicity of *L. innocua* and *E. coli* at different ratios of n-hexane and water and the power of negative charges of *L. innocua* and *E. coli*; Power of negative charge (%) =100-relative cell density (RCD), RCD = O.D. of cell suspension treated with Dowex 1x8/O.D. of cell suspension without Dowex 1x8 *100.

CHAPTER 7

OVERALL CONCLUSIONS

The feasibility of employing jumbo squid pens as the raw material for preparing β-chitin and chitosan was evaluated by comparing its structures, physicochemcial properties, and biological activities with commercially utilized α-chitin and chitosan extracted from shrimp shells. When extracting β-chitin from jumbo squid pens, the demineralization and depigmentation processes required for preparing α-chitin from shrimp shells could be omitted since the mineral and pigment contents in jumbo squid pens were negligible, and the resulted β-chitin had signficiantly higher moisture content and molecular weight (Mw) in comparison with α-chitin. The optimal deacetylation and depolymerization conditions using NaOH and cellulase were further developed to prepare β-chitosan with a wide range of degree of deacetylation (DDA) and Mw.

β-chitin has unique polymorphic structure with less inter-molecular hydrogen bonds and lower crystallinity (CI), thus exerting higher reactivity toward solvents than α-chitin. β-chitin could convert to α-chitin when subjected to alklai or acid treatment at certain reaction time, and the converted α-form showed higher moisture absorption ability than that of the native α-chitin as the result of polymorphic destruction. Hence, β-chitin could retain its high reactivity toward solvents even after forming rigid-packed α-form as demonstrated in its deacetylating and depolymerizing reactions, where lower NaOH concentration and shorter reaction time were needed for β-chitin than for α-chitin when preparing similar DDA of chitosan and β-chitosan was more susceptible to cellulase than α-chitosan. These differences might be explained by the unique structural property of β-

chitin and chitosan, in which lower CI and less crystallite in β-chitosan might increase its solubility and affinity to cellulase.

Different antioxidant and antibacterial activities between α- and β-chitosan under similar DDA and Mw were also observed. At high Mw of 300-320 kDa, β-chitosan exhibited significantly higher DPPH radical scavenging activity and reducing ability than those of α-chitosan. The 75% DDA/31 kDa of β-chitosan showed significantly higher antibacterial activity against *E. coli* than the same DDA and Mw of α-chitosan, whereas α-chitosan had higher inhibition ratio (%) against *E. coli* than β-chitosan at 90% DDA/74-76 kDa. These differences might be attributed by the different intra- or inter-molecular behaviors between α- and β-chitosan that impacted the flexibility of chitosan conformations, thus altering the antioxidant and antibacterial mechanisms of chitosan in solutions.

For fully understanding the differences between β- and α-chitosan, especially their antioxidant and antibacterial activities, the conformational properties of β- and α-chitosan solutions should be further investigated by using NMR spectroscopy and by coordinating with their structural properties in the solid form. In addition, future studies to evaluate the antifungal activity, develop β-chitosan deriatives and/or nano-β-chitosan with improved functionality, and apply β-chitosan in various food and non-food systems are all important to fully utilize the abundant source of squid pens and the unique properties of β-chitosan.

APPENDIX I

THE CONTRIBUTION OF ACIDULANT TO THE ANTIBACTRERIAL ACTIVITY OF ACID SOLUBLE α- AND β-CHITOSAN SOLUTIONS AND THEIR FILMS

Jung, J., Cavender, G., and Zhao, Y. Submitted to *International Journal of Food Microbiology*

ABSTRACT

This study evaluated individual contributions of dissolving acids (acetic acid, lactic acid, and hydrochloric acid) or acid solubilized chitosan (α- and β-forms) to the antibacterial activity against *L. innocua* and *E. coli* as solutions and dried films. The presence of chitosan significantly ($P<0.05$) altered percentage of inhibition (PI, %) of acid solubilized chitosan solutions against *L. innocua* and *E. coli*, thus the additional inhibition (AI, %) of chitosan in comparison with acid alone was further investigated to quantify the antibacterial activity of chitosan alone. AI of 300-320 kDa α- and β-chitosan reported positive values varying (~17-65%) in all types of acid against *L. innocua* and *E. coli*, indicating that the chitosan itself had offered additional antibacterial activity compared with the acid solution, and was higher than that of 4-5 kDa α- and β-chitosan. Higher Mw showed significant higher adsorptive ratios (%) than lower Mw, suggesting that the increased inhibition was the result of surface phenomena. AI, adsorptive ratio, and cellular leakage depended on chitosan forms, acid types, Mw, and bacterial species. In chitosan films, the contribution of acids to antibacterial activity of acid solubilized chitosan was assumed by comparing PI between non-rinsed and rinsed films. PI against *E. coli* was shown to be affected, not only by acid types and rinsing of films, but also by the interaction between chitosan forms and rinsing of films. PI of rinsed β-chitosan acetate and hydrochloride films was ~26% and ~28% lower than those of non-rinsed films. This decrease after rinsing suggested that part of the antibacterial activity of chitosan films is due to the presence of soluble acid compounds and/or other active fragments. The structural characteristics of the films also impacted their antibacterial activity. Thus, the

contribution of acidulant to the antibacterial activity of acid solubilized α- and β-chitosan could be numerically estimated by AI calculations for coating solutions and rinsing of films for films.

Keywords: α- and β-chitosan, chitosan salt film, coating solution, antibacterial activity

INTRODUCTION

While chitin, a biopolymer found in the structural components of marine invertebrates or crustacean shells, some fungi and certain bacteria, is widespread in nature, it has limited industrial use. This lack of use is primarily due to its compact structure and lack of appreciable solubility in aqueous solutions. By utilizing simple chemical and enzymatic methods, chitin can be transformed into chitosan, a compound that not only has greater solubility in acidic aqueous solutions, but also shows potent antibacterial ability. While the underlying mechanisms behind this activity are unclear, several have been suggested, from the alteration of cell permeability due to interaction between protonated amino groups (NH_3^+) and negatively charged cell membranes [1], to the destruction of the cell wall by chelation of the metals present therein [2], to the formation of a physical barrier preventing the uptake of nutrients into the cell, resulting in cell death [3]. Despite the lack of consensus on the reason behind the activity, it has been observed in multiple studies [4-10] that the antibacterial activity of chitosan is influenced by various factors, including molecular weight (Mw), degree of deacetylation (DDA), pH and inhibition time, as well as bacterial species and Gram-stain group. Gerasimenko et al. [11] examined chitosan with molecular weights from 5-27 kDa, and reported that antibacterial activity against *E. coli* increases as Mw decreased. Similarly, Kim et al. [12] found that chitosan treated with lipase to reduce Mw provided higher antibacterial activity across several bacterial species compared with unaltered chitosan. The relationship between Mw and antibacterial is not cut and dry, as No et al. [5] found that chitosan with a Mw in excess of 59 kDa exerts higher antibacterial activity against *B. megaterium, B. cereus, and E. sakazaki* than that with a lower Mw (less than 22 kDa). DDA also plays a role in the

antibacterial effect of chitosan, with Lin et al. [13] finding that lower Mw chitosan (~ 26 kDa) with higher DDA exerted higher antibacterial effect than those with lower DDA. The pH was also one of factors affecting antibacterial activity of chitosan, with Tsai et al. [1] reporting that acidic pH increased the bactericidal effects of chitosan, and No et al. [14] finding higher antibacterial activity in lower pH among the tested range of pH (4.5-5.9). Finally, with respect to the effect of Gram-stain group, there seems to be little consensus. Chung et al. [4] reported that antibacterial activity of chitosan was stronger against Gram-negative bacteria than Gram-positive bacteria; however, Zhong et al. [6] observed the opposite trend. The sometimes conflicting results of these studies, along with the differing effects of the various factors highlight the need for additional research, particularly as it related to potential interactions between the factors.

Chitosan is insoluble in water, but is acid-soluble and therefore may be dissolved in acidic solutions for applications such as coating and dried films. Both organic acids and inorganic acids can be used as a solvent, provided that the pH of the solution is below the pKa of the amino groups (~6.3). That said, organic acids, such as acetic acid and lactic acid, possess strong antibacterial activity by themselves and even the inorganic acids show some activity [15, 16]. Thus, while chitosan has its own antibacterial activity as mentioned above, the contributions of chitosan to the antibacterial activity of applications made with acid-soluble chitosan are difficult to isolate from the antibacterial effects of the acid in the solutions. Further, as in all multi-factored systems, there exists the possibility of interactive effect between one or more factors. Therefore, the aim of this study was to evaluate the antibacterial activity of solutions and films made with acid-

soluble α- and β-chitosan, as well as to determine the contribution of different factors, such as Mw, the chitosan forms (CF), and the acid type (AT).

MATERIALS AND METHODS

Preparation of Chitosan, Chitosan Solutions and Films

Dried jumbo squid (*Dosidicus gigas*) pens were obtained from Dosidicus LLC, USA and used to prepare β-chitosan after Jung and Zhao (2011). Briefly, dried squid pens were ground into about 18 mesh (ASTM) and then deproteinized and deacetylated by 5% NaOH and 50% NaOH, respectively. Commercially available α-chitosan from shrimp shells was purchased from Primax (Iceland). Both α- and β-chitosan were depolymerized using cellulase in order to reach the desired molecular weight. After the inactivation and removal of the enzyme, filtered samples were precipitated using 10% NaOH. These precipitated samples were then washed with distilled water and ethanol to remove any residues and dried at 40 ℃ for 24 h. Mw and DDA of both types of chitosan were determined using the methods described below.

Chitosan solutions were prepared by dissolving powdered chitosan (1% w/v) in a 1% aqueous solution of acetic, lactic, or hydrochloric acid. Films were prepared by transferring 20 mL of a given chitosan solution into a 60 mm diameter petri dish and allowing it to dry at 40 ℃ for 48 h. Due to the inability of low Mw chitosan to form films, only the higher Mw samples (300-320 kDa) were used in the film production.

Determination of Mw and DDA

Mw of 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 0.1M CH_3COOH and 0.2M NaCl [17]. The intrinsic viscosity was measured by the intercept between the Huggins (reduced viscosity) and Kraemer (relative viscosity) plots when the concentration was 0 [18]. The viscosity-average molecular weight of chitosan was calculated by using Mark-Houwink-Sakurada (MHS) equation (1) [19]:

$$[\Gamma] = K (M_W)^a \quad (1)$$

where K and a were the constants, $K=1.81 \times 10^{-3}$ and a = 0.93; and [Γ] was the intrinsic viscosity obtained from the Huggins and Kraemer plots. DDA was determined by the colloidal titration method [20]. Mw and DDA of the commercial α-chitosan and β-chitosan prepared in this study were 300 kDa with 88% DDA and 320 kDa with 86% DDA, respectively, while the Mw and DDA of α- and β-chitosan oligosaccharides were 5 kDa with 94% DDA and 4 kDa with 95% DDA, respectively.

Preparation of Microbial Cultures

Escherichia coli ATCC 25922 and *Listeria innocua* ATCC 51742 were obtained from the American Type Culture Collection. Each organism was cultured on appropriate solid media (*E. coli* on tryptic soy agar (TSA) (Becton, Dickinson and Co., USA) and *L. innocua* on brain heart infusion agar (BHA) (Becton, Dickinson and Co., USA)) and stored under refrigeration (4 ℃) during the course of the study. Prior to a given microbiological assay, samples of a single typical colony were inoculated in tubes of appropriate broth (*E. coli* in tryptic soy broth (TSB) (EMD Chemicals, Inc., USA) and *L.*

innocua in brain heart infusion (BHI) (Becton, Dickenson and Co., USA)) and incubated at 37 ℃ for 24 h.

The Effect of Acids on Antibacterial Activity of Chitosan Solution and Films

This study investigated two potential applications for chitosan, namely solutions and dry films, and determined parameters based on the applications. For the study of solutions, percentage of inhibition (PI, %) against test bacteria, cellular adsorption, and bacterial cellular leakage were determined. For dried films, PI and Fourier-transform infrared (FT-IR) spectra with crystallinity (CI) were compared between non-rinsed film and rinsed film.

PI (%) was determined using a slightly modified broth dilution MIC (minimum inhibitory concentration) method [6]. Aliquots (5 mL) of appropriate broth (TSB or BHI) were transferred into 16 mm test tubes prior to steam sterilization. For chitosan solution study, either a 1% chitosan solution dissolved in 1% of a given acid or a 1% solution of the acid alone was used to produce a series of serially diluted tubes ranging from 0.5% to 0.03125% of chitosan and acid. The resultant series of five tubes plus an additional tube serving as a control were then inoculated with 0.5 ml of cultured *E. coli* or *L. innoccua* under aseptic conditions. For the films, identically sized samples (3 x 3 cm) of each type of film were added to 5 mL of broth, and then inoculated with 0.5 mL of cultured bacterium. In addition, samples of each film were also tested after being rinsed for 1 min in the appropriate broth (BHI for *L. innocua* or TSB for *E. coli)* prior to testing in order to remove residual acids and active fragments which may have been formed during drying. Inoculated tubes were incubated for 24 h at 37 ℃. Optical density (OD) of cultured tubes

was determined by spectrophotometer (620 nm) and used to calculate PI (%) using Eq. (2):

$$PI(\%) = \frac{\begin{matrix}OD\ of\ enriched\ bacterium - \\ OD\ of\ enriched\ bacterium\ treated\ with\ chitosan\ solubilized\ in\ acids, acid\ alone, or\ films\end{matrix}}{OD\ of\ enriched\ bacterium} x100$$

(2)

Resultant data were analyzed using ANOVA to compare the effect of the presence of chitosan (PC), acid type (AT), and concentration (CS)of chitosan solutions and AT, chitosan form (CF), and rinsing of film (RF) of films.

To aid in comparison, "additional inhibition (AI)" was used to attempt isolation of the antibacterial activity of the chitosan itself from that of the acids, and was calculated using Eq. (3):

$$AI\ (\%) = \frac{\begin{matrix}(OD\ of\ cell\ suspension\ treated\ by\ acids - \\ OD\ of\ cell\ suspension\ treated\ by\ chitosan\ solution\ solubilized\ in\ acids)\end{matrix}}{OD\ of\ cell\ suspension\ treated\ by\ acids}\ \text{x}\ 100 \qquad (3)$$

Measurements of Transmittance (%) in Rinsing Solution

To investigate the release of residual acids or active fragments during the rinsing of films as well as the relationship between antibacterial activity and rinsing, transmittance (T %, measured at 620 nm) of the rinsing solutions were measured and compared to those of BHI broth and TSB solutions which had not been used to rinse the films.

A Fourier-Transform Infrared (FT-IR) Spectroscopic Analysis of Dried Chitosan Films

A single bound attenuated total reflection (ATR) FT-IR spectrometer (PerkinElmer, USA), operated by Omnic 7.4 software (Thermo Fisher Inc., USA) was used to examine chitosan films with different formulations and the effect of rinsing. Twenty scans were performed at a resolution 4 cm^{-1}, referenced against air, and all spectra were recorded in absorbance mode. Additionally, crystallinity index (*CI*) was determined from the spectra, using Eq. (4) originally developed by Focher et al. [21].

$$\mathrm{CI} = \frac{A_{1382}}{A_{2920}} \quad (4)$$

Adsorptive Characteristics of Acid-Soluble Chitosan onto Bacterial Cell Wall

The cellular adsorption of acid-soluble chitosan with different Mw (4-5 kDa or 300-320 kDa) was determined using a modified method after Chung et al. (2004). Gerasimenko et al. [11] reported that antibacterial activity against *E. coli* increases as Mw decreased within 5-27 kDa. In contrast, No et al. [14] reported that chitosan at Mw > 28 kDa provides higher antibacterial activity than chitosan at Mw < 22 kDa. According to Tsai [22], 12 kDa has the strongest activity against many pathogens. Hence, two ranges of Mw chitosan, oligosaccharides < 10 kDa and higher Mw > 300 kDa, were selected because antibacterial activity in these two Mw can be significantly different based upon previous studies. A 4 mL aliquot of chitosan solution (0.1% chitosan in acetic, lactic, or hydrochloric acid) was added to 4 mL of PBS buffer containing an enriched cell suspension. The mixture was shaken and allowed to settle for 10 min, and then the upper solution was collected at 3, 4, and 5 h. The residual chitosan was analyzed by 3-methyl-2-

benzothiazolinone hydrazine hydrochloride hydrate (MBTH) method [23]. The adsorptive ratio (%) of chitosan on each bacterium was determined as:

$$Adsorptive\ ratio(\%) = \frac{Initial\ amount\ of\ chitosan\ (mM) - residual\ amount\ of\ chitosan\ (mM) after\ 3\ h, 4h, and\ 5h}{Initial\ amount\ of\ chitosan\ (mM)} x100$$

(6)

Bacterial Cellular Leakage by β-Galactosidase Assay

Increase in the levels of the enzyme, β-galactosidase, which has been shown to correlate with cellular leakage, was determined using the spectroscopic methodology described by Sudarshan et al. [24]. After culturing in lactose media for 24 h, 1 mL of cell suspension (*E. coli* or *L. innocua*) was transferred to BHI broth or TSB, and enriched for 24 h at 37 ℃. Enriched cells were then centrifuged and decanted, the resultant pellet was resuspended in the same volume of chilled Z buffer prepared according to Sudarshan et al. (1992), and 1.0 mL of this cell suspension was added into 1.0 mL of either 0.1% chitosan in 1% acid, 1% chitosan in 1% acid or a control solution consisting of 1% acid without chitosan. To prepared solution, 0.5 mL of Z buffer and 0.5 mL of o-nitrophenol-β-D-galactopyranoside (ONPG) (Tokyo Chemcial Industry, Japan) (4 mg/mL) were added and incubated at 37 ℃ for 24 h. Samples were then analyzed using a spectrophotometer (Model UV160U, Shimadzu Corporation, Japan) at a wavelength of 420 nm.

Statistical Analysis

The two applications investigated in this study (chitosan solution and dried film) were tested separately using a completely randomized factorial design. In the solution study,

three treatment factors were tested, including the presence of chitosan (PC; 5 levels: acid alone, 4-5 kDa and 300-320 kDa of α-chitosan, and 4-5 kDa and 300-320 kDa of β-chitosan), acid type (AT; 3 levels: acetic acid, lactic acid, and hydrochloric acid), and concentrations of chitosan (CS; 5 levels: 0.031, 0.062, 0.125, 0.250, and 0.500%). Treatment factors for dried films were chitosan forms (CF; 2 levels: α and β-form), acid type (AT; 3 levels: acetic acid, lactic acid, and hydrochloric acid), and rinse of films (RF; 2 levels: non-rinsed or rinsed). All experiments were performed in duplicate, and the resultant data were analyzed for statistical significance via multi-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc testing as appropriate using statistical software (SAS v9.2, The SAS Institute, USA). Results were considered to be different if $\alpha<0.05$.

RESULTS AND DISCUSSIONS

Chitosan Salt Solution

Percentage of Inhibition and Additional Inhibition of Chitosan and Acid Solutions

PC significantly impacted PI against *E. coli*, and interacted with AT and CS against *E. coli* ($P<0.05$). However, there was no main PC effect ($P>0.05$), but significantly interactive with AT and CS for *L. innocua* (Table 7.1). AT significantly impacted *L. innocua* and CS also had significant effect against both *L. innocua* and *E. coli* ($P<0.05$). The observed differences in PI between chitosan solubilized in acids and acid alone suggest that the chitosan itself contributes to the antibacterial activity of those solutions.

As previously mentioned, AI is intended to estimate the additional antibacterial activity of chitosan compared with the effect of the acid alone. AI for *L. innocua* and *E.*

coli are presented in Figs. 7.1A and 7.1B, respectively. It should be noted that these results include only the data from the lowest concentration (0.03125%) of chitosan solution, due to the limitations of the methodology - briefly, chitosan has been shown to have the tendency to self-aggregate in the aqueous solution in a concentration and molecular weight- dependent manner [25]. These aggregates greatly increase the OD of the solutions, preventing meaningful measurement of cell density related OD in our higher concentration and higher Mw chitosan solutions. Of course, this limitation is relatively minor as previous research indicated that the antibacterial activity of chitosan solutions is concentration dependent, with 0.01% solutions having a greater effect than those of 0.2 or even 0.5% (Sudarshan et al. [24]. Due to this inverse relationship between concentration and antibacterial effect, AI of chitosan salt solution was calculated based upon comparison of our most dilute samples, 0.03125% chitosan in 0.03125% of each type of acids and 0.03125% acid alone. Positive AI indicated that the chitosan itself offers additional antibacterial activity compared with the acid alone, thus allowing the contribution of chitosan to the antibacterial to be estimated. For *L. innocua*, the AI of 300-320 kDa α- and β-chitosan were ~50-65% and ~19~26, respectively, whereas the AI of 4-5 kDa β-chitosan varied greatly based upon the solubilizing acid, ranging from no AI for acetic acid and AI of ~9-26% for lactic and hydrochloric acids. For *E. coli*, the AI of 300-320 kDa α- and β-chitosan ranged between ~17 and 54%, respectively, and among the 4-5 kDa samples, positive AI (~21%) was only found for lactic acid. Overall, it was found that AI was greater in the higher Mw samples (300-320 kDa) compared with the lower ones (4-5 kDa) and varied based on AT, CF, and bacterial species.

Antibacterial Mechanisms and Their Relationship with Antibacterial Activity

While the exact nature of the antimicrobial activity of chitosan is still not fully understood, two of the most common mechanisms suggested are the cellular adsorption of chitosan and cellular leakage caused by the penetration or permeation of the bacterial cell wall by chitosan. In this study, both phenomena were examined, and the results are presented in Fig. 7.2 and Table 7.2, respectively. In general, 300-320 kDa chitosan showed significantly higher adsorptive ratios compared with the 4-5 kDa chitosan. This was true for all acid types against *E. coli* and both acetic and hydrochloric acids against *L. innocua*. This activity difference based upon Mw can be explained due to simple structural properties in different Mw chitosan. Chitosan molecules become polycationic when solubilized (gaining hydrogen from solution) allowing it to better interact with the negatively charged bacterial cell surface [26, 27]. Since higher Mw chitosan molecules contain more amino groups which can become cationic, they are better able to interact with the bacterial cells. However, electrostatic interaction between chitosan and bacterial cell wall can be decreased with increasing Mw higher than a certain range of Mw due to electrostatic repulsions between extensively charged chitosan polymers. Similarly, Uchida et al. [28] reported higher antibacterial activity in slightly hydrolyzed chitosan than native chitosan or chitosan oligomers. Therefore, cellular adsorption is closely related to antibacterial activity depending on Mw, possibly presenting the highest ability in a certain range of Mw.

Cellular leakage data showed an interesting pattern, with the amount of leakage generally being higher in 0.1% of chitosan solutions compared with those at 1.0%, and *E. coli* showing greater leakage than *L. innocua*. This was not the first time such a

phenomena had been observed, as Sudarshan et al. [24] found similar trends regarding concentrations of chitosan solutions, reported that the maximum reduction of bacteria occurred at the lowest concentrations (0.1 mg/mL) in comparison with chitosan concentrations of 2.0 or 5.0 mg/mL, and suggested that the large number of positive charges in the solutions may impart a net positive charge, helping to keep the bacteria in suspension. Another possibility for the observed relationship between concentrations and cellular leakage is that the higher concentration of chitosan salt solutions could allow for the formation of an impenetrable layer of chitosan outside cell membranes, thus lessening the detection of cellular leakage. This was similar to the findings by Fernandes et al. [29] who reported that the high Mw chitosan polymer could form a mechanical barrier thereby preventing the uptake of nutrients into the vegetative cell. However, regarding the effect of Mw against *L. innocua*, 0.1% of 5 kDa α-chitosan lactic acid and HCl was significantly higher than chitosan in acetic acid, while no difference in acid type for 4 kDa β-chitosan (Table 7.2). Unlikely for *E. coli*, there was no significant difference among Mw. The differences between the two bacterial species could be explained either by the differences in cell wall composition between Gram-positive and Gram-negative cells or more simply due to the vast size differences between *L. innocua* and *E. coli,* with the large size of the latter offering more potential sites for permeation and the small size of the former making it easier to form an impenetrable layer around the bacterial cell wall.

Chitosan Salt Films

Structural Characteristics of Chitosan Salt Films

Crystallinity (CI) and FT-IR spectra are shown in Fig. 7.3 for α-chitosan and Fig. 7.4 for β-chitosan. CF altered CI among various types of films, with α- chitosan showing a higher CI than β-chitosan in general. In particular, non-rinsed β-chitosan acetate and hydrochloride films showed lower CI than those films made of α-chitosan. This result is likely related to the structural difference between the two chitosan forms, as Lima and Airoldi [30] had also reported that β-chitosan had lower CI compared to α-chitosan. Similarly, other studies have shown that the CF impacts the ability of chitosan to form a hydrogel, with β-chitosan having the ability as it easily accepts water into the lattice due to lower CI and less hydrogen bonds [31]. The rinsing of the films changed the CI of films in a non-uniform manner. While the CI of rinsed α- and β-chitosan acetate films was increased compared with non-rinsed samples, the CI of α- and β-chitosan lactate and hydrochloride film was decreased. In the previous studies, Kawada et al. [32] reported that crystallization degree of chitosan acetate is higher than that of chitosan lactate, and chitosan lactate films also had lower strength due to the presence of the larger lactate counter ion in the structural matrix [33]. Therefore, rinsing away of these large ions could have a more profound effect on the CI of lactate films, compared with those of chitosan acetate films.

Rinsing of film (RF) and chitosan form (CF) also impacted FT-IR spectra, particularly in the range of 3000-3500 cm^{-1}, the region associated with the formation of salts and/or hydrogen bonds by OH or NH functional groups in the chitosan film complex. The intensity of these peaks was reduced in rinsed films compared with non-rinsed films (Figs. 7.3 and 7.4). Further, in β-chitosan acetate and hydrochloride films, rinsing resulted in much larger decreases in peak intensity compared with the decreases seen

from rinsing similar α-chitosan films (Figs. 7.3 and 7.4). Whether this effect is due to the release of entrapped salts and any residual acids or the dissociation of hydrogen bonds between chitosan molecules was unknown. In respect to the effect of CF, the structural differences, particularly the increased number of chitosan-hydrogen bonds in α-chitosan, are the likely cause of the differences between the FT-IR spectra among similar chitosan salt films.

Antibacterial Activity of Chitosan Salt Films Depending on AT, CF, and RF

As shown in Table 7.1, for *L. innocua*, no difference in PI for any of the treatments, either alone or in combination ($P>0.05$). In contrast, the PI against *E. coli* was affected not only by AT and RF, but also by the interaction between CF and RF ($P<0.05$).

Individual means testing of the *E.* coli data using the LSD method showed an interesting pattern of effect (Table 7.3). For non-rinsed films, β-chitosan hydrochloride film showing the highest PI (92.85%) was significantly higher than all other films. For rinsed films, PI of α- and β-chitosan hydrochloride film was significantly higher than β-chitosan acetate film. Several previous studies had investigated the effect of acidulant type on bacterial species. Buchanan and Edelson [34] looked at the effects of citric, malic, lactic, hydrochloric and acetic acids on cell viability of *E. coli* O157:H7 at an acid concentration of 0.5% (w/v). For nine strains evaluated, lactic acid was the most effective for reducing the viable cell population, and HCl was the least effective, with the order of acetic, citric, and malic acid sensitivity varying in a strain-dependent manner. Chung et al. [4] examined the effect of chitosan on two major waterborne pathogens, *E. coli* and *S. aureus*, and showed that organic acids with low carbon number were better solvents for

chitosan than inorganic acids as it induced higher inhibition versus those bacteria. These previous results, however, were inconsistent with our study, which might be due to different types of applications applied in each study, as solubility of chitosan salt films varied directly impacting their antibacterial activity in the aqueous solution. As mentioned in the structural characteristics based upon CI and FT-IR, CF and RF altered CI or FT-IR among various types of chitosan salt films, which can be related to the antibacterial activity. CI of non-rinsed β-chitosan acetate and hydrochloride films were relatively lower than that of non-rinsed α-chitosan acetate and hydrochloride films, which was consistent with the result of PI showing higher PI in β-chitosan acetate and hydrochloride films than other chitosan salt films since lower CI has been shown to increase the solubility of chitosan films in aqueous solution [35, 36]. This lead to the release of active fragments (e.g. protonated glucosamine) from the chitosan salt films, which were thought to interact with the cell membrane in the suspended cells, resulting in cell death [3, 37].

Most forms of chitosan films showed no significant change in PI against *E. coli* after rinsing (Table 7.3). However, β-chitosan acetate and hydrochloride films both showed significant reductions in PI after rinsing. This result could be related to the structural changes, as rinsed films also showed a lower intensity FT-IR peaks in the broad bands associated with OH or NH groups compared with those of non-rinsed films (Fig. 7.4). One explanation would be the hydrogen bonds formed with OH or NH functional groups in chitosan film complex becoming dissociated during rinsing, which should in turn release active fragments from the chitosan salt film complex into the rinsing solution. Also, intensities of the peaks of β-chitosan salt films were significantly lower than those

of α-chitosan salt films after rinsing and drying (Fig. 7.3 and 7.4), indicating that structural characteristics of films were altered depending on CF. Furthermore, as the release of residual acid compounds and/or active fragments are one of the proposed mechanisms suggested to explain the antibacterial activity of cast films, it follows that films which have a less ordered structure would be more likely to release said compounds/fragments. This seems to be the case, as the β-chitosan acetate and hydrochloride films showed the greatest reduction in activity due to rinsing as well as the lowest initial CI.

The transmittance (T, %) of rinse solutions provided further insights about the effect of RF on antibacterial activity (Table 7.3). Decreases in transmittance were most likely caused by increases in suspended compounds/fragments released from the film. Being insoluble, these compounds would increase the turbidity of the rinse solution, thereby lowering T(%), and as these compounds are thought to contribute to antibacterial activity, a rinse solution which showed a significantly low T (%), should mean that the film which was rinsed therein would have a much lower PI. This seems to be borne out, as the β-chitosan hydrochloride film rinse solution showed lowest T (15.55%) and significant decreases in PI in rinsed films in comparison with non-rinsed film. Hence, the contribution of acid to antibacterial activity of films can be estimated by significantly decrease of PI in rinsed acetate and hydrochloride films than non-rinsed films due to significant loss of active compounds (i.e. protonated glucosamine) or any residual acids after rinsing of films.

CONCLUSIONS

In this study, we attempted to isolate and estimate the contribution of chitosan from the contribution of acidulant in acidic solutions of α- and β-chitosan and films formed from the solutions. We also sought to gain further insight into the various factors that influence the antibacterial activity of chitosan solutions and films. Comparing the antibacterial activity of chitosan solutions with acid solutions by additional inhibition (AI) calculations allowed us to quantify the contributions of both components, revealing a complex interplay between chitosan form, acid type, Mw, and bacterial species. The separation of the contribution of acid to the antibacterial activity of films was accomplished by comparing PI between cast films and films which had been rinsed to remove any residual acids in the film complex. The phenomena involved in the antibacterial activity of both films and solutions were quite complex and depended not only on a series of factors, but also upon the interactions between them. In chitosan solutions, it was found that while the dissolving acid provided some antibacterial effect, the chitosan itself also contributed. It was also found that the differing forms (α vs. β) and molecular weights of chitosan had differing activities, with lower molecular weights tending to have lower antibacterial effects, as has been shown in various earlier studies. The actual mechanism involved in this inactivation appears to be quite complex, but related to both the cellular adsorption of chitosan and the induction of cellular leakage, and influenced by the properties of the bacteria, chitosan forms, and the acid types used to solubilize it. In general, higher Mw corresponded with greater levels of adsorption and *E. coli* was more prone to cellular leakage. The mechanisms involved in the antibacterial activity of cast films proved even more complex. While the antibacterial activity against *L. innocua* did not differ significantly across the various factors, the activity against *E.*

coli varied depending on acid types and whether or not the film had been rinsed. The effects of rinsing also varied depending on whether α or β chitosan were used to prepare the film. Investigation into the structural and physical changes imparted by rinsing revealed notable changes in the structure of the film due to rinsing in general, with some combinations of chitosan and acid being more pronounced than others.

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Table 7.1 Analysis of variance (ANOVA) (*P*=0.05) for main and interaction effect on percentage of inhibition (PI, %) against tested bacteria in chitosan solutions and dried chitosan films.

Chitosan solutions					Chitosan films				
Source of Variation	*L. innocua*		*E. coli*		Source of Variation	*L. innocua*		*E. coli*	
	df	*P* value	df	*P* value		df	*P* value	df	*P* value
Main effects					Main effects				
Presence of chitosan (PC)+	4	0.0700	2	0.0267	Acid types (AT)*	2	0.1223	2	0.0058
Acid types (AT)++	2	<.0001	2	0.0077	Chitosan forms (CF)**	1	0.0664	1	0.4052
Concentrations (CS)+++	5	<.0001	5	<.0001	Rinse of films (RF)***	1	0.7048	1	0.0044
Interaction effects					Interaction effects				
PC x AT	8	0.0020	4	<.0001	AT x FC	2	0.0614	2	0.0612
AT x CS	10	<.0001	10	<.0001	FC x RF	1	0.2309	1	0.0170
PC x CS	20	<.0001	10	<.0001	AT x RF	2	0.3175	2	0.6996
Model	33	<.0001	33	<.0001	Model	9	0.0882	9	0.0050
Error	74		74		Error	14		14	
Corrected total	107		107		Corrected total	23		23	

+ Factors include 4-5 or 300-320 kDa α- and β-chitosan salt solutions

++ Factors include acetic acid, lactic acid, and hydrochloric acid

+++ Factors include 0.015, 0.031, 0.062, 0.125, 0.250, and 0.500% of chitosan salt solution or acid alone

* Factors include acetic acid, lactic acid, and hydrochloric acid

** Factors include α- and β-chitosan

*** Factors include film and rinsed film

Table 7.2 Assessment of bacterial cellular leakage by β-galactosidase assay in 0.1 or 1.0% of 4-5 kDa or 300-320 kDa α- and β-chitosan acetate, lactate, and hydrochloride solutions compared with acid alone.

		L. innocua			*E. coli*		
		Type of acid			Type of acid		
Treatments	Concentrations of acid or chitosan (%)	Acetic acid	Lactic acid	HCl	Acetic acid	Lactic acid	HCl
Control *	0.1	**A 0.071 a***	C 0.061 a	B 0.074 a	B 0.940 a	A 0.881 a	A 0.880 a
α-300 kDa	0.1	A 0.083 a	C 0.061 a	B 0.082 a	C 0.831 a	A 0.850 a	AB 0.802 a
β-320 kDa	0.1	A 0.050 b	C 0.046 b	B 0.087 a	A 1.202 a	A 0.880 b	B 0.684 c
α-5 kDa	0.1	A 0.190 c	A 1.636 a	A 1.132 b	BC 0.898 a	A 0.937 a	A 0.891 a
β-4 kDa	0.1	A 0.271 a	B 0.148 a	B 0.223 a	BC 0.851 a	A 0.824 a	A 0.846 a
Control	1.0	A 0.185 a	C 0.067 b	B 0.080 ab	B 0.494 a	C 0.580 a	A 0.539 a
α-300 kDa	1.0	B 0.040 b	C 0.081 a	B 0.051 ab	B 0.481 b	A 0.871 a	A 0.512 b
β-320 kDa	1.0	B 0.064 a	C 0.071 a	B 0.047 a	AB 0.585 b	AB 0.760 a	A 0.639 ab
α-5 kDa	1.0	B 0.089 b	B 0.698 a	B 0.069 b	A 0.664 a	AB 0.749 a	A 0.719 a
β-4 kDa	1.0	A 0.193 c	A 0.987 a	A 0.474 b	AB 0.555 a	BC 0.683 a	A 0.535 a

* Control indicates acid alone.

** Means proceeded by the same capital letter in the same column within the same concentration were not significantly different (P>0.05).

*** Means preceded by the same small letter in the same raw within bacterial species were not significantly different (P>0.05).

Table 7.3 Percentage of inhibition (PI, %) of chitosan films against *E. coli* and transmittance (T, %) of the rinsing solution.

		PI (%)		T (%)
Chitosan forms	Type of acid	Non-rinsed Film	Rinsed film	[+] Rinsing solution
α	Acetic acid	[*B] 63.24 [a]**	[AB] 60.20 [a]	[B] 88.95
	Lactic acid	[B] 60.42 [a]	[AB] 51.41 [a]	[A] 93.20
	Hydrochloric acid	[B] 60.56 [a]	[A] 65.51 [a]	[C] 51.15
β	Acetic acid	[B] 70.86 [a]	[B] 43.13 [b]	[A] 93.50
	Lactic acid	[B] 57.49 [a]	[AB] 50.23 [a]	[D] 40.25
	Hydrochloric acid	[A] 92.85 [a]	[A] 64.57 [b]	[E] 15.55

* Means proceeded by the same capital letter in the same column were not significantly different ($P>0.05$)

** Means preceded by the same small letter in the same row within the parameter of PI (%) were not significantly different ($P>0.05$)

[+] Films were rinsed in sterile tryptic soy broth (TSB) for 1 min.

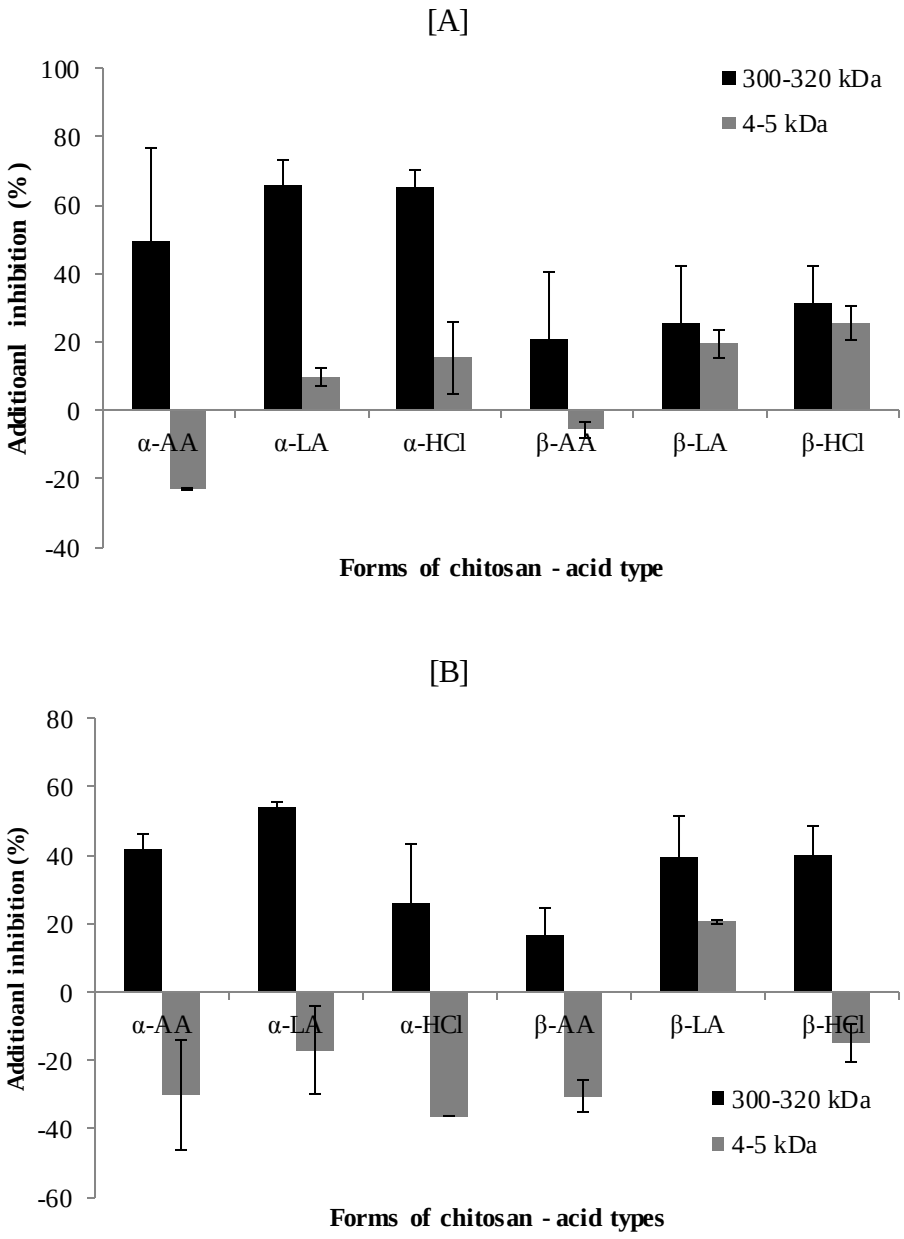

Fig. 7.1 The additioanl inhibition (%) in 0.03125% of 4-5 kDa and 300-320 kDa α- and β-chitosan solutions solublized in 0.03125% each type of acids verse 0.03125% of acid alone against *L. innocua* (A) and *E. coli* (B). Additional inhibition (%) = (OD of cell suspension treated by acids – OD of cell suspension treated by chitosan solution solubilized in acids)/OD of cell suspension treated by acids.

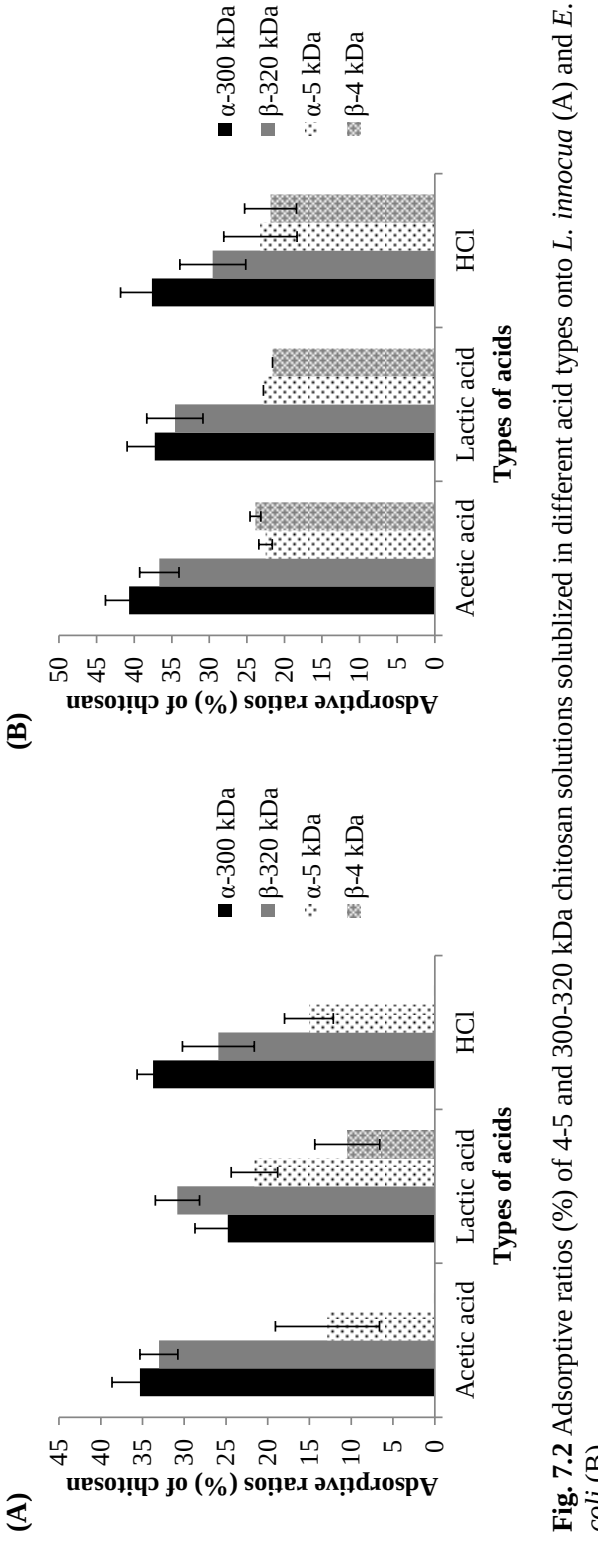

Fig. 7.2 Adsorptive ratios (%) of 4-5 and 300-320 kDa chitosan solutions solublized in different acid types onto *L. innocua* (A) and *E. coli* (B).

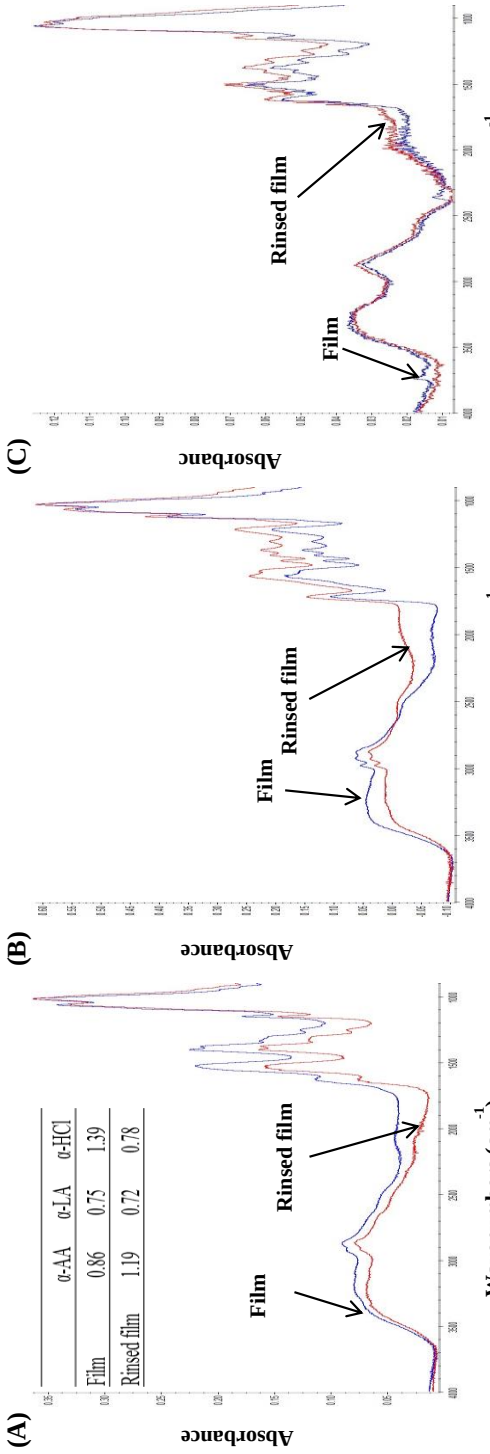

Fig. 7.3 Comparison of FT-IR spectra and crystallinity index (CI) between non-rinsed film (film) and rinsed film. (A) α-chitosan acetate film, (B) α-chitosan lactate film, and (C) α-chitosan hydrochloride film. CI = A_{1382}/A_{2920} cm^{-1}. Rinsed film was prepared by rinsing in media for 5 min and drying at 40 ℃ for 1 d.

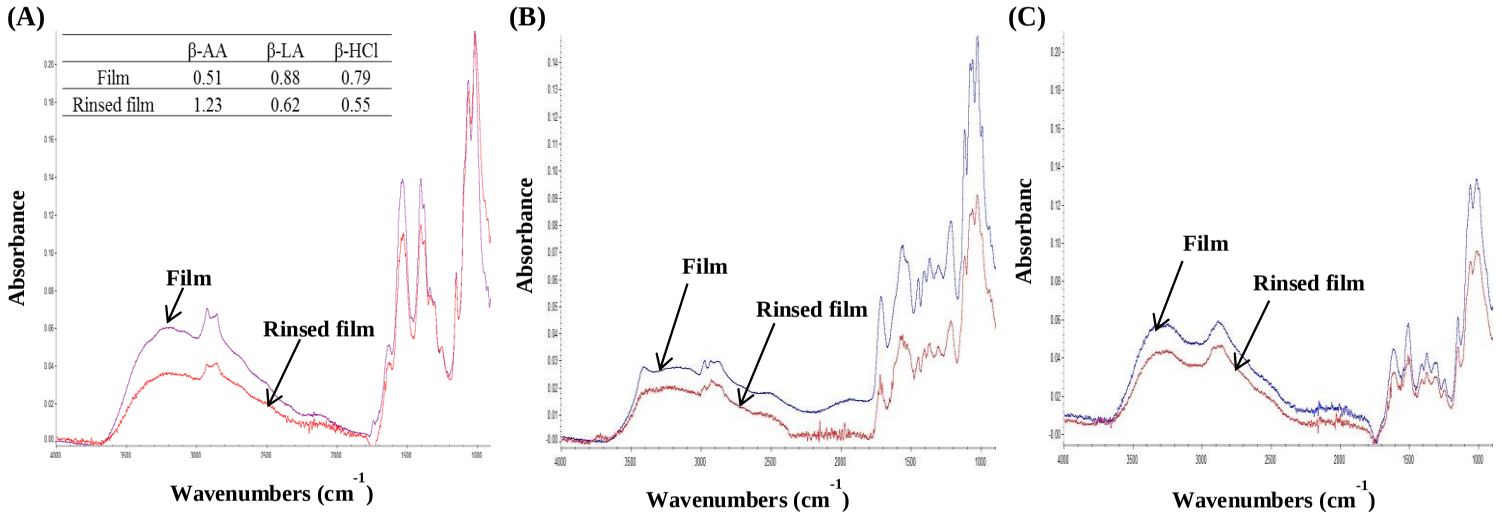

Fig. 7.4 Comparison of FT-IR spectra and crystallinity index (CI) between non-rinsed film (film) and rinsed film. (A) β-chitosan acetate film, (B) β-chitosan lactate film, and (C) β-chitosan hydrochloride film; CI = A_{1382}/A_{2920} cm^{-1}; Rinsed film was prepared by rinsing in media for 5 min and drying at 40 ℃ for 1 d.