Impact of the Structural Differences between α- and β-Chitosan on Their Depolymerizing Reaction and Antibacterial Activity

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ABSTRACT: The polymeric structure characteristics of β-chitosan from jumbo squid (*Dosidicus gigas*) pens and α-chitosan from shrimp shells during depolymerization 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 *Escherichia coli* and *Listeria innocua* was compared with that of α-chitosan at similar M_w and degrees of deacetylation (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 was more reactive 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*, and no difference against *L. innocua* between the two forms of chitosan was observed. However, the two forms of chitosan exhibited different levels of antibacterial activity against *E. coli*, in which 75% DDA/31 kDa β-chitosan demonstrated significantly higher inhibition (lower MIC) than that of 75% DDA/31 kDa α-chitosan, whereas 90% DDA/74−76 kDa α-chitosan had a 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-chitosan, β-chitosan, jumbo squid pens, depolymerization, antibacterial activity, structural properties*

INTRODUCTION

Antibacterial activity of β-chitosan might be different from that of α-chitosan because of 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] Second, the different intra- and intermolecular structures of α- and β-chitosan influence 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 intermolecular hydrogen bonds can induce the rigid conformation with lower flexibility in the soluble status. On the basis of 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, leading to more structural modifications of β-chitin than that of α-chitin after alkali treatments.[3,6,8−10] Hence, the different intra- or intermolecular 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 (M_w) of chitosan strongly impact its structural properties as well. Kumar et al. (2004) reported the reduced crystallinity index (CI) in lower M_w of α-chitosan,[11] whereas the opposite result was observed by Ogawa et al. (1991).[12] Liu et al. (2006) also found increased CI in low M_w 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 *Listeria innocua* (Gram-positive) and *Escherichia coli* (Gram-negative) between the two forms of

chitosan at a wide range of DDA and M_w 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 M_w 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 them into about 18 meshes (1.0 mm, Glenmills Inc., USA), squid pens were deproteinized by treating in 5% NaOH for 3 days at room temperature, washed with distilled water, and then dried at 40 °C oven (Precision Scientific Inc., USA) for 24 h. No further demineralization process was applied for squid pens as the mineral content was very low (~1.70%).[22] 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 °C), 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 (M_w was undetectable), ~75% DDA/4610 kDa, and ~90% DDA/3182 kDa were depolymerized by using cellulase obtained from *Aspergillus niger* (TCI America, USA).[20] Briefly, chitosan solutions were prepared at a ratio of 1:100 (chitosan: 2% acetic acid) and adjusted to pH 5 by 10% NaOH under a constant stirring. Cellulase was added at the same weight of chitosan in the solutions and reacted for 1–4 h at 50 °C. The hydrolyzates were boiled for 10 min to inactivate enzyme and centrifuged at 8000*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 °C oven for 24 h. The M_w of depolymerized β-chitosan were analyzed and compared with that of α-chitosan at similar DDA of ~60, 75, and 90%. Since it was impossible to get exact same M_w between the two forms of chitosan at the same DDAs, they were classified as High, Med, and Low. Specifically, High/Med/Low M_w 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 in 90% DDA β-chitosan along with 73 kDa in 60% DDA β-chitosan.

Viscosity-Average M_w and DDA. The viscosity-average M_w 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. Approximately 100 mg of chitosan was dissolved in 10 mL of the mixture solution of 0.1 M CH_3COOH and 0.2 M 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.[23] The viscosity-average M_w of chitosan was calculated by using Mark−Houwink−Sakurada (MHS):[24]

$$[\eta] = K(M_w)^a \tag{1}$$

where K and a are constants, $K = 1.81 \times 10^{-3}$ and $a = 0.93$, and $[\eta]$ is the intrinsic viscosity. DDA was determined by the colloidal titration method.[25]

Antibacterial Activity. Cultures of *E. coli* ATCC 25922 and *L. 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 °C) 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 °C for 24 h.

For determining the minimum inhibitory concentration (MIC, %) of chitosan, 1% chitosan solubilized in 1% 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 aseptic conditions. After incubation at 37 °C for 24 h, the optical density (OD) of each test tube was determined at 620 nm using a spectrophotometer (Shimadzu, Japan). MIC was the lowest concentration among the tested ranges of concentrations, in which the 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).[26] The inhibition ratio (IR, %) was further investigated to compare the relative antibacterial activity of different chitosan samples. IR was calculated as

inhibition ratio (IR, %)

= [OD of cell suspension treated with control

− OD of cell suspension treated with chitosan solution]

/[OD of cell suspension treated with control] × 100　　(2)

Hydrophilicity 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 × 10^6 CFU/mL for *E. coli* and ~1 × 10^5 CFU/mL for *L. innocua*) were prepared after incubation for 1 day. 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). Hydrophilicity (%) of each bacterium was calculated:[27]

hydrophilicity (%)

= [OD of water phase at different ratios in the mixture]

/[OD of water phase without hexane in the mixture]

× 100　　(3)

Power of Negative Charge in Bacterial Cell Wall. The power of negative charge was determined by using anion exchange resin, Dowex 1X8.[27] Dowex 1X8 was washed five times with distilled water and balanced with 0.1 M HCl in a test tube for 6 h. After adding 5 mL of enriched bacteria (~1 × 10^6 for *E. coli* and ~1 × 10^5 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

RCB (%)

= [OD of bacterium suspended in water phase after Dowex 1

X8 treatment]

/[OD of bacterium suspended in water phase without Dowex 1

X8 treatment] × 100　　(4)

The power of the negative charge forO 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 (Perkin Elmer, 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 intrasheet or intersheet 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° to 40° (2θ) at scanning speed of 0.025°/s. The CI was determined as

$$\text{crystallinity (CI, \%)} = \frac{I_{110} - I_{am}}{I_{110}} \times 100 \tag{5}$$

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°.[28,29]

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

$$d(\text{Å}) = \frac{\lambda}{2 \sin \theta} \tag{6}$$

where d is plane spacing; λ is 1.54 Å, wavelength of Cu Kα radiation; and θ is 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 a similar DDA (~60, 75, and 90%) through a completely randomized design (CRD). To investigate the antibacterial activity of chitosan related to DDA, M_w, and the chitosan form, a completely randomized factorial design was applied with total 14 chitosan samples: 75% DDA of α-chitosan with M_w of 133, 45, and 31 kDa; 90% DDA of α-chitosan with M_w of 111, 74, and 27 kDa; 60% DDA of β-chitosan with M_w of 73 kDa; 75% DDA of β-chitosan with M_w of 72, 31, and 20 kDa; and 90% DDA of β-chitosan with M_w of 76, 40, and 17 kDa, whereas acetic acid was applied as a control. M_w, 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 M_w. Figures 1 and 2 present partial FT-IR spectra (2200–3700 cm^{-1}) that illustrate the intra- or intermolecular hydrogen bonds and CH stretching in α- and β-chitosan at a wide range of DDA and M_w, 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} was attributed to the vibration of CH stretching, assuming the free hydroxymethyl (CH$_2$OH) 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 2).

In 75% DDA of α-chitosan, the peak intensities of OH and NH$_2$ strengthened as M_w decreased, indicating that OH and NH$_2$ would more readily form hydrogen bonds at lower M_w (≤31 kDa). The peak intensity of CH stretching was the highest in 45 kDa chitosan and decreased in other M_w samples (Figure 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 M_w decreased (≤31 kDa) along with increasing CI (Table 2). In 90% DDA of α-chitosan,

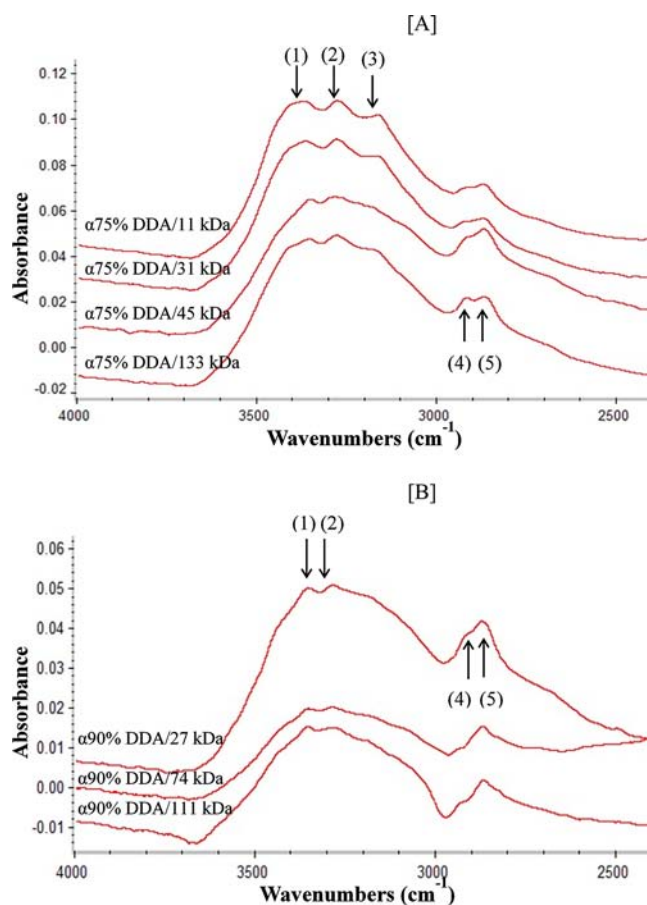**Figure 1.** Partial FT-IR spectra (2400–3700 cm^{-1}) of 75% DDA of α-chitosan (A) and 90% DDA of α-chitosan (B) at different molecular weights (M_w); (1) and (2) are attributed to the vibration of OH; (3) is attributed to the vibration of NH; (4) and (5) are attributed to the vibration of CH stretching.

no NH bands were distinguished, and the OH bands had no significant difference among 27–111 kDa chitosan (Figure 1B). However, the peak intensities of CH stretching in higher M_w (74 and 111 kDa) were relatively more intense than those in lower M_w (27 kDa) (Figure 1B). Crystallites of (120) planes were observed in lower M_w (≤27 kDa) and CI increased as M_w decreased (Table 2). Hence, the hydrogen bonds in 90% DDA/74 and 111 kDa α-chitosan were weaker than those in the 27 kDa sample.

For 60% and 75% DDA of β-chitosan, the peak of OH bands was 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 M_w (Figure 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 M_w samples (Table 2). Hence, the hydrogen bonds associated with OH could be weaker in 75% DDA/20 and 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 (Figure 2B). However, the bands of CH stretching were strengthened in lower M_w sample (≤40 kDa) (Figure 2B), which had no significant difference from 76 kDa sample in CI and crystal formation (Table 2). Hence, the hydrogen bonds
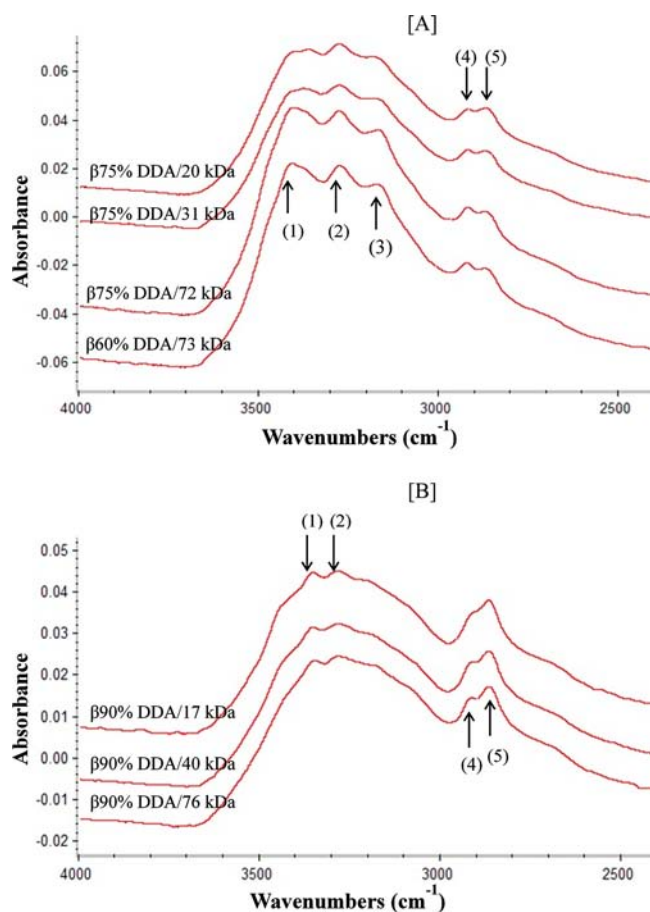
Figure 2. Partial FT-IR spectra (2400−3700 cm^{-1}) of 60% DDA and 75% DDA of β-chitosan (A) and 90% DDA of β-chitosan (B) at different molecular weights (M_w); (1) and (2) are attributed to the vibration of OH; (3) is attributed to the vibration of NH; (4) and (5) are attributed to the vibration of CH stretching.

associated with OH or NH$_2$ could be weaker in 90% DDA/17 and 40 kDa β-chitosan.

In summary, α- and β-chitosan had different structural properties in association with DDA and M_w. The intra- and intermolecular 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. Therefore, 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 1 shows the depolymerizing reaction of β-chitosan during 1−4 h in comparison with α-chitosan at 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 those of the 60% DDA of β-chitosan (Table 2). In contrast, the depolymerization 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 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.[30] M_w of 75% DDA/72 kDa and 90% DDA/76 kDa β-chitosan were significantly lower than those of 75% DDA/ 133 kDa and 90% DDA/111 kDa of α-chitosan at the first 1 h,

Table 1. M_w of Different Degrees of Deacetylation (DDA, %) of α- and β-Chitosan by Cellulase Hydrolysis at Different Reaction Times (h)^a

reaction time (h)	α-chitosan			β-chitosan	
	~75% DDA	~90% DDA	~60% DDA	~75% DDA	~90% DDA
0	2175	1773	ND^b	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}	d	ND^c	^{B} 17 ^{c}

^{a}Means preceded by the same capital letter in the same row were not significantly different ($P > 0.05$). Means proceeded by the same small letter in the same column were not significantly different ($P > 0.05$). ^{b}ND: nondetected since prepared chitosan was unable to be solubilized in the solution for measuring viscosity-average M_w. ^{c}ND: nondetected as M_w was significantly lower than 10 kDa. ^{d}No further experiment was carried out due to nonsignificant degradation.

indicating β-chitosan was more reactive to cellulase than α-chitosan. On the basis of the structural properties of the native 75% DDA and 90% DDA of α- and β-chitosan prior to the depolymerization process (Table 2), the crystallite of (020) plane appeared in 75% DDA of α-chitosan, and CI of the 75% DDA and 90% DDA of β-chitosan was slightly lower than that of 90% DDA of α-chitosan even though M_w of β-chitosan was significantly higher than that of α-chitosan (Table 2). Hence, polymorphic β-chitosan with lower CI and less crystallites was more susceptible to cellulase than α-chitosan regardless of the initial M_w.

With respect to the influence of DDA on the depolymerizing reaction in α- and β-chitosan, M_w of 75% DDA α- and β-chitosan at 4 h was significantly lower than that of 90% DDA samples (Table 1). Zhang et al. (2001) indicated that the degradation rate decreases as DDA increases since the higher DDA chitosan has a lower affinity for the enzyme.[31] Although it has been known that cellulase randomly cleaves the β-1,4-glycosidic bond along chitosan polymeric chains,[30] 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 $M_w < 10$ kDa at 4 h and was 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 M_w. Table 3 presents the antibacterial activity (IR or MIC) of α- and β-chitosan against *L. innocua* and *E. coli* at different DDA and M_w values. 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 (Figure 3). Similarly, Chung et al. (2004) reported that the cellular adsorptive amount of chitosan is higher for the Gram-negative strain with higher electrostatic interaction in comparison with the Gram-positive strain.[27] Hence, the protonated amino groups in chitosan solubilized in acidic solution were better able to bind with stronger negatively charged *E. coli*, allowing the cytoplasm to flow out of the bacterial cells. Although the hydrophilicity of *L. innocua* at a

Table 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 (M_w)

chitosan samples form (DDA, M_w)	CI (%)[a]	(020) d (Å)[b]	(020) RI (%)	(110) d (Å)	(110) RI (%)	(120) d (Å)	(120) RI (%)	(101) d (Å)	(101) RI (%)	(130) d (Å)	(130) RI (%)
α (60%, ND[c])	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%, 27 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%, 73 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						

[a]Crystallinity was determined by X-ray diffraction (XRD) using the equation: $(I_{110} - I_{am})/I_{110} \times 100$, 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 $2\theta = 12.6°$. [b]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. [c]ND: M_w was undetectable since prepared chitosan was unable to be solubilized in the solution for measuring viscosity-average M_w.

Table 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 (M_w, kDa)[a]

	L. innocua						*E. coli*					
	degree of deacetylation (DDA, %)											
	60		75		90		60		75		90	
form (M_w)	IR	MIC	IR	MIC	IR	MIC	IR	MIC	IR	MIC	IR	MIC
α (27−31 kDa)	b		98.5[a]	0.03	86.6[a]	0.03				0.03		0.02
β (17−20 kDa)			c	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 (M_w, kDa)											
	high[d]		med[+2]		low[+++]		high[d]		med[+2]		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, M_w	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%, 17−27 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		

[a]Means preceded by the same letter in the same raw within each bacterium were not significantly different ($P > 0.05$). ++M_w 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. +++M_w 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. [b]The blank cell meant that 60% DDA of α- and β-chitosan were unable to be hydrolyzed to lower M_w. [c]Since MIC was significantly different among the samples, no IR needed to be reported. [d]M_w 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.

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 a hexane/water ratio of 0:1 or 1:1 (Figure 3). Since the actual antibacterial
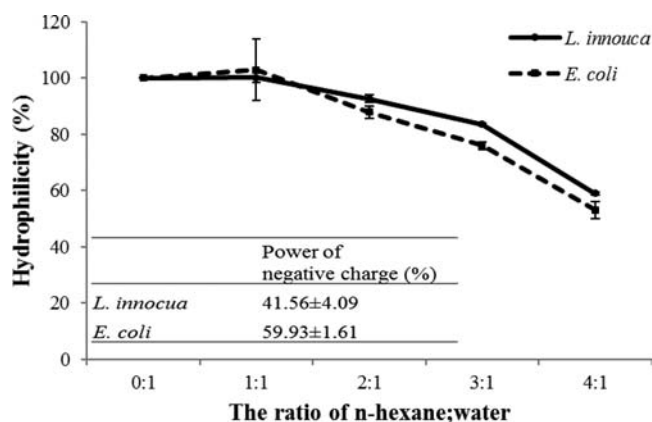
Figure 3. The hydrophilicity of *L. innocua* and *E. coli* at different ratios of *n*-hexane and water and the power of negative charges of *L. innocua* and *E. coli*; power of negative charge (%) = 100-relative cell density (RCD), RCD = O.D. of cell suspension treated with Dowex 1X8/O.D. of cell suspension without Dowex 1X8 × 100.

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 negatively 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 M_w of 17−20 kDa (Table 3). This result was contradicted by the previous report, in which the antimicrobial activity was enhanced in higher DDA chitosan.[32] This difference was probably associated with the tested range of M_w in different studies as well as the distribution of acetyl groups. In 75% DDA of *β*-chitosan, the IR values of Low M_w (95.1%) and Med M_w (96.2%) samples were significantly higher than that of the High M_w one (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 M_w 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 M_w of 72−76 kDa (Table 3). Med M_w of chitosan showed enhanced inhibition against *E. coli* than Low and High M_w of 75% and 90% DDA of *α*-chitosan and 90% DDA of *β*-chitosan, respectively. According to Eaton et al. (2008), higher M_w chitosan could exert a higher inhibitory effect as it formed an impermeable layer around the cell wall to block the transportation of essential nutrients into the cells.[33] However, the electrostatic interaction between the cationic amino groups of chitosan and the anionic bacterial cell membrane can be decreased with increased M_w due to the electrostatic repulsion between extensively charged amino groups in high M_w 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.[34] Hence, the Med M_w 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 out a comparative study of the antibacterial activity between *α*- and *β*-chitosan, DDA and M_w of the two forms of chitosan were retained as similar as possible since these two parameters significantly affect the antibacterial activity (Table 3). As stated in the previous studies and confirmed in this study, chitosan extracted from *α*- and *β*-chitin have different structures and interactions between the polymeric chains because the different

forms of chitin responded differently to the deacetylation and depoymerization processes.[8,10] The different intra- or intermolecular 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.[35,36]

For *L. innocua*, no significant difference in the antibacterial activity between *α*- and *β*-chitosan was observed (Table 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 result in different antibacterial activities between the two forms of chitosan. However, the electrostatic interaction between *α*- and *β*-chitosan was difficult to differentiate against *L. innocua* since the power of negative charges of *L. innocua* was significantly weaker than that of *E. coli* (Figure 3). In contrast, the different antibacterial activity against *E. coli* between the two forms of chitosan might be due to the stronger negative charge of *E. coli* that showed more association with the structural properties of *α*- and *β*-chitosan (Figure 3).

MIC of 75% DDA/31 kDa *β*-chitosan (0.02) against *E. coli* was lower than that of *α*-chitosan (0.03) (Table 3). At 75% DDA/31 kDa, *α*-chitosan had a higher CI with the presence of the crystal formation in (120), (101), and (130) planes in comparison with *β*-chitosan (Table 2). In addition, the band of CH stretching in *β*-chitosan was slightly more intense than that in *α*-chitosan (Figures 1 and 2). Hence, *α*-chitosan conformation in the solution could be more rigid than *β*-chitosan conformation due to strong intra- or intermolecular hydrogen bonds, thus decreasing the contacts with the bacterial cells in the suspension. Reversely, at 90% DDA/74−76 kDa *α*-chitosan exerted a significantly higher IR (58.4%) compared to *β*-chitosan (43.3%) against *E. coli* (Table 3). Although CI of *α*-chitosan was relatively higher than that of *β*-chitosan (Table 2), the band of CH stretching in *α*-chitosan was intense in comparison with that in *β*-chitosan, and 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 M_w. For fully understanding the differences in the antibacterial activity between *β*- and *α*-chitosan, it is necessary to investigate the distribution of acetyl groups and the physicochemical properties of *β*- and *α*-chitosan solutions by using H NMR spectroscopy and/or mass spectroscopy and coordinating with their structural properties in the solid form.

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Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Campana-Fillho, S. P.; De Britto, D.; Curti, E.; Abreu, F. R.; Cardoso, M. B.; Battisti, M. V.; Sim, P. C.; Lvall, R. L. Extraction, structures, and properties of alpha and beta-chitin. *Quim. Nova* **2007**, *30*, 644−650.

(2) Kumirska, J.; Czerwicka, M.; Kaczynski, Z.; Bychowska, A.; Brzozowski, K.; Thoming, J.; Stepnowski, P. Application of spectroscopic methods for structural analysis of chitin and chitosan. *Mar. Drugs* **2010**, *8* (5), 1567−1636.

(3) Jang, M. K.; Kong, B. G.; Jeong, Y. I.; Lee, C. H.; Nah, J. W. Physicochemical characterization of α-chitin, β-chitin, and γ-chitin separated from natural resources. *J. Polym. Sci., Part A: Polym. Chem.* **2004**, *42* (14), 3423−3432.

(4) Chandumpai, A.; Singhpibulporn, N.; Faroongsarng, D.; Sornprasit, P. Preparation and physico-chemical characterization of chitin and chitosan from the pens of the squid species, *Loligo lessoniana* and *Loligo formosana*. *Carbohydr. Polym.* **2004**, *58* (4), 467−474.

(5) Lavall, R. L.; Assis, O. B. G.; Campana-Filho, S. P. β-chitin from the pens of *Loligo sp.*: Extraction and characterization. *Bioresour. Technol.* **2007**, *98* (13), 2465−2472.

(6) Abdou, E. S.; Nagy, K. S. A.; Elsabee, M. Z. Extraction and characterization of chitin and chitosan from local sources. *Bioresour. Technol.* **2008**, *99* (5), 1359−1367.

(7) Tsai, G. J.; Su, W.-H. Antibacterial activity of shrimp chitosan against *Escherichia coli*. *J. Food Prot.* **1999**, *62* (3), 239−243.

(8) Lamarque, G.; Viton, C.; Domard, A. Comparative study of the first heterogeneous deacetylation of α- and β-chitins in a multistep process. *Biomacromolecules* **2004**, *5* (3), 992−1001.

(9) Kurita, K.; Tomita, K.; Tada, T.; Ishii, S.; Nishimura, S. I.; Shimoda, K. Squid chitin as a potential alternative chitin source: Deacetylation behavior and characteristic properties. *J. Polym. Sci., Part A: Polym. Chem.* **1993**, *31* (2), 485−491.

(10) Kurita, K.; Ishii, S.; Tomita, K.; Nishimura, S. I.; Shimoda, K. Reactivity characteristics of squid β-chitin as compared with those of shrimp chitin: High potentials of squid chitin as a starting material for facile chemical modifications. *J. Polym. Sci., Part A: Polym. Chem.* **1994**, *32* (6), 1027−1032.

(11) Kumar, A. B. V.; Gowda, L. R.; Tharanathan, R. N. Non-specific depolymerization of chitosan by Pronase and characterization of the resultant products. *Eur. J. Biochem.* **2004**, *271* (4), 713−723.

(12) Ogawa, K. Effect of heating an aqueous suspension of chitosan on the crystallinity and polymorphs. *Agric. Biol. Chem.* **1991**, *55* (9), 237502379.

(13) Liu, N.; Chen, X. G.; Park, H. J.; Liu, C. G.; Liu, C. S.; Meng, X. H.; Yu, L. J. Effect of Mw and concentration of chitosan on antibacterial activity of *Escherichia coli*. *Carbohydr. Polym.* **2006**, *64* (1), 60−65.

(14) Gerasimenko, D. V.; Avdienko, I. D.; Bannikova, G. E.; Zueva, O. Y.; Varlamov, V. P. Antibacterial effects of water-soluble low-molecular-weight chitosans on different microorganisms. *Appl. Biochem. Microbiol.* **2004**, *40* (3), 253−257.

(15) Tikhonov, V. E.; Stepnova, E. A.; Babak, V. G.; Yamskov, I. A.; Palma-Guerrero, J.; Jansson, H.-B.; Lopez-Llorca, L. V.; Salinas, J.; Gerasimenko, D. V.; Avdienko, I. D.; Varlamov, V. P. Bactericidal and antifungal activities of a low molecular weight chitosan and its *N*-/2(3)-(dodec-2-enyl)succinoyl/-derivatives. *Carbohydr. Polym.* **2006**, *64* (1), 66−72.

(16) Helander, I. M.; Nurmiaho-Lassila, E. L.; Ahvenainen, R.; Rhoades, J.; Roller, S. Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. *Int. J. Food Microbiol.* **2001**, *71* (2−3), 235−244.

(17) Feng, F.; Liu, Y.; Hu, K. Influence of alkali-freezing treatment on the solid state structure of chitin. *Carbohydr. Res.* **2004**, *339* (13), 2321−2324.

(18) Huang, J.; Mao, J. W.; Hu, S.; Zhao, D. K.; Mei, L. H.; Liu, S. W.; Wu, Y. F.; Fang, S.; Shao, Q. Ultrasonic-assisted preparation, characterization and antibacterial activity of β-chitosan from squid pens. *Adv. Mater. Res.* **2011**, *236−238*, 282−287.

(19) Shimojoh, M.; Masai, K.; Kurita, K. Bactericidal effects of chitosan from squid pens on oral *Streptococci*. *Nippon Nogei Kagaku Kaishi* **1996**, *70* (7), 787−792.

(20) Jung, J.; Zhao, Y. Characteristics of deacetylation and depolymerization of β-chitin from jumbo squid (*Dosidicus gigas*) pens. *Carbohydr. Res.* **2011**, *346* (13), 1876−1884.

(21) Jung, J.; Zhao, Y. Comparison in antioxidant action between α-chitosan and β-chitosan at a wide range of molecular weight and chitosan concentration. *Bioorg. Med. Chem.* **2012**, *20* (9), 2905−2911.

(22) Tolaimate, A.; Desbrieres, J.; Rhazi, M.; Alagui, A. Contribution to the preparation of chitins and chitosans with controlled physico-chemical properties. *Polymer* **2003**, *44* (26), 7939−7952.

(23) Mao, S.; Shuai, X.; Unger, F.; Simon, M.; Bi, D.; Kissel, T. The depolymerization of chitosan: Effects on physicochemical and biological properties. *Int. J. Pharm.* **2004**, *281* (1−2), 45−54.

(24) No, H. K.; Lee, S. H.; Park, N. Y.; Meyers, S. P. Comparison of physicochemical, binding, and antibacterial properties of chitosans prepared without and with deproteinization process. *J. Agric. Food Chem.* **2003**, *51* (26), 7659−7663.

(25) Chang, K. L. B.; Tsai, G.; Lee, J.; Fu, W.-R. Heterogeneous *N*-deacetylation of chitin in alkaline solution. *Carbohydr. Res.* **1997**, *303* (3), 327−332.

(26) Huang, R.; Du, Y.; Zheng, L.; Liu, H.; Fan, L. A new approach to chemically modified chitosan sulfates and study of their influences on the inhibition of *Escherichia coli* and *Staphylococcus aureus* growth. *React. Funct. Polym.* **2004**, *59* (1), 41−51.

(27) Chung, Y. C.; Su, Y. P.; Chen, C. C.; Jia, G.; Wang, H. L.; Wu, J. C.; Lin, J. G. Relationship between antibacterial activity of chitosan and surface characteristics of cell wall. *Acta Pharmacol. Sin.* **2004**, *25* (7), 932−936.

(28) Focher, B.; Beltrame, P. L.; Naggi, A.; Torri, G. Alkaline *N*-deacetylation of chitin enhanced by flash treatments. Reaction kinetics and structure modifications. *Carbohydr. Polym.* **1990**, *12* (4), 405−418.

(29) Focher, B.; Naggi, A.; Torri, G.; Cosani, A.; Terbojevich, M. Structural differences between chitin polymorphs and their precipitates from solutions-evidence from CP-MAS 13C-NMR, FT-IR and FT-Raman spectroscopy. *Carbohydr. Polym.* **1992**, *17* (2), 97−102.

(30) Lin, S. B.; Lin, Y. C.; Chen, H. H. Low molecular weight chitosan prepared with the aid of cellulase, lysozyme and Chitinase: Characterisation and antibacterial activity. *Food Chem.* **2009**, *116* (1), 47−53.

(31) Zhang, H.; Neau, S. H. In vitro degradation of chitosan by a commercial enzyme preparation: effect of molecular weight and degree of deacetylation. *Biomaterials* **2001**, *22* (12), 1653−1658.

(32) Andres, Y.; Giraud, L.; Gerente, C.; Le Cloirec, P. Antibacterial effects of chitosan powder: Mechanisms of action. *Environ. Technol.* **2007**, *28* (12), 1357−1363.

(33) Eaton, P.; Fernandes, J. C.; Pereira, E.; Pintado, M. E.; Xavier Malcata, F. Atomic force microscopy study of the antibacterial effects of chitosans on *Escherichia coli* and *Staphylococcus aureus*. *Ultra-microscopy* **2008**, *108* (10), 1128−1134.

(34) Uchida, Y.; Izume, M.; Ohtakara, A., *Preparation of Chitosan Oligomers with Purified Chitosanase and Its Application*; Elsevier: London, 1989.

(35) Domard, A. A perspective on 30 years research on chitin and chitosan. *Carbohydr. Polym.* **2012**, *84* (2), 696−703.

(36) Du, J.; Hsieh, Y. L. PEGylation of chitosan for improved solubility and fiber formation via electrospinning. *Cellulose* **2007**, *14* (6), 543−552.