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Comparison of Physicochemical, Binding, and Antibacterial Properties of Chitosans Prepared without and with Deproteinization Process

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Physicochemical, binding, and antibacterial properties of chitosans prepared without and with deproteinization (DP) process (5, 10, 15, and 30 min at 15 psi/121 °C) were compared. Chitosan from DP 0 min had comparable nitrogen content, lower degree of deacetylation and solubility, but higher molecular weight and viscosity than chitosans from DP 5−30 min. The latter four chitosans showed differences only in molecular weight. Deproteinization treatment resulted in slightly decreased *L** values and increased *a** and *b** values compared with those of DP 0 min. Chitosan from DP 0 min had comparable water and fat-binding capacity (FBC) except for chitosan from DP 15 min, which had a higher FBC but lower dye-binding capacity than those of the four chitosans from DP 5−30 min. The antibacterial activities of chitosans against seven different bacteria showed that the inhibitory effects varied with the deproteinization time and the particular bacterium.

KEYWORDS: Chitosan; deproteinization; physicochemical characteristics; binding capacity; antibacterial activity

INTRODUCTION

Chitosan is a natural biopolymer derived by deacetylation of chitin, a major component of the shells of crustacea such as crab, shrimp, and crawfish. During the past few decades, chitosan has been receiving increased attention for its commercial applications in biomedical, food, and various chemical industries (*1*, *2*). In studies on the functional properties of chitinous polymers, chitosan has been documented to possess several distinctive properties for use in water and fat uptake, emulsification (*3*), dye binding (*4*), and gelation (*5*). Recently, chitosan has attracted notable interest due to its biological activities, that is, antimicrobial (*6*, *7*), antitumor (*8*), and hypocholesterolemic functions (*9*).

Isolation of chitin from crustacean shell generally consists of three basic steps: demineralization, deproteinization, and decoloration. Among them, deproteinization is accomplished by extraction with dilute sodium hydroxide solution (1−10%) at elevated temperature (65−100 °C) for 1−6 h. Conversion of chitin to chitosan is achieved by treatment with concentrated sodium hydroxide solution (40−50%), usually at 100 °C or higher, to remove some or all of the acetyl groups from the polymer (*10*). In the isolation of chitin, elimination of the deproteinization step, or less reaction time than for optimum deproteinization, would yield a chitin containing mostly, or partly, the proteins present in the original crustacean shell. However, if these residual proteins can be removed during the deacetylation step that involves harsh alkali treatment, considerable reduction in chitosan production cost is anticipated due to reduction in NaOH usage, process time, and voluminous wastewater discharge.

The physicochemical characteristics of chitosan affect its functional properties, which differ with preparation methods. More recent studies have revealed notable variability in the water-, far-, and dye-binding capacities (*11*−*13*) as well as in the antibacterial activities (*14*, *15*) of various chitosans. The aforementioned elimination of deproteinization, or reduction of alkali treatment time, may affect the physicochemical characteristics of the final chitosan product. Therefore, it is mandatory that the physicochemical characteristics and functional properties of chitosan products be examined to effectively utilize these products for particular usages.

The objective of the present research was to compare physicochemical, binding, and antibacterial properties of chitosans prepared without and with deproteinization process (5, 10, 15, and 30 min at 15 psi/121 °C) for development of an ultimate economic chitosan production process for particular applications.

MATERIALS AND METHODS

Materials. Dried crab (*Chionoecetes opilio*) leg shell was obtained from Keumho Chemical (Seoul, Korea). The shell was ground through

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a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ) with a 2 mm mesh screen and sifted with 20 mesh (0.841 mm) and 40 mesh (0.425 mm) sieves using a portable sieve shaker (JISICO, Seoul, Korea). Ground shell of 0.841−0.425 mm particle size was used throughout this research to obtain reproducible and consistent results.

The dye used for evaluation of binding capacity was FD&C Red No. 40 [disodium salt of 6-hydroxy-5-[(2-methoxy-5-methyl-4-sulfophenyl)azo]-2-naphthalenesulfonic acid]. Commercially available refined soybean oil was used for the fat-binding study.

Production of Chitosan. Production of chitosan involved demineralization, deproteinization, decoloration, and deacetylation. The shell was demineralized with 1 N HCl for 30 min at ambient temperature with a solids/solvent ratio of 1:15 (w/v) (*16*). Following demineralization, the decalcified shell was collected on a 100 mesh sieve, washed to neutrality in running tap water, rinsed with deionized water, and filtered to remove excess moisture.

Deproteinization was accomplished under standard autoclaving conditions (15 psi/121 °C), a relatively simple, rapid, and effective process for deproteinization compared with conventional methods (*17*). The demineralized shell was treated with 3% NaOH for 0, 5, 10, 15, and 30 min at 15 psi/121 °C and a solids/solvent ratio of 1:10 (w/v). The residue was then washed and filtered as above.

The chitin residue was bleached with 0.32% sodium hypochlorite solution for 3 min with a solids/solvent ratio of 1:10 (w/v) (*16*). The decolored chitin was collected, washed as above, and dried at 60 °C for 4 h in a forced-air oven.

Deacetylation was achieved by treatment of chitin under conditions of 15 psi/121 °C with 45% NaOH for 30 min and a solids/solvent ratio of 1:15 (w/v) (*18*). The resulting chitosan was collected, washed as above, and dried at 60 °C for 4 h in a forced-air oven.

Water- and Fat-Binding Capacities. Water-binding capacity (WBC) and fat-binding capacity (FBC) of chitosan were measured using a modified method of Wang and Kinsella (*19*). Water or fat absorption was initially carried out by weighing a centrifuge tube containing 0.5 g of sample, adding 10 mL of water or soybean oil, and mixing on a vortex mixer for 1 min to disperse the sample. The contents were left at ambient temperature for 30 min with shaking for 5 s every 10 min and centrifuged at 3200 rpm for 25 min. After the supernatant was decanted, the tube was weighed again. WBC and FBC were calculated as follows: WBC (%) = [water bound (g)/sample weight (g)] × 100; FBC (%) = [fat bound (g)/sample weight (g)] × 100.

Dye-Binding Capacity. Dyeing of chitosan was achieved by shaking 0.2 g of chitosan and 10 mL of aqueous dye solution (containing 2.5 mg of dye) in horizontally positioned screw-capped test tubes at 23 °C for 1 h using a shaking water bath (80 rpm), followed by procedures described by Cho et al. (*11*). The amount of dye bound to chitosan was determined by calculating differences in concentrations between the initial dye solution and the combined filtrate. Dye-binding capacity (DBC) was expressed as percent adsorption.

Assays for Antibacterial Activity. Seven bacteria were tested for antibacterial activity of chitosans. These include three Gram-negative bacteria (*Escherichia coli* ATCC 11775, *Salmonella typhimurium* ATCC 14028, and *Vibrio parahaemolyticus* ATCC 17802) and four Gram-positive bacteria (*Bacillus megaterium* KCTC 3007, *Bacillus cereus* ATCC 21366, *Listeria monocytogenes* Scott A, and *Staphylococcus aureus* ATCC 29737).

The antibacterial activity of chitosans was assayed as follows: Chitosan solutions were prepared in 1% (v/v) acetic acid at concentrations of 0.0 (control) and 1.0% (w/v) before being applied to broth, and each solution was added to Mueller Hinton broth (MHB, Merck, Darmstadt, Germany) to give final chitosan concentrations of 0.0 and 0.1% (w/v). Then 0.05 mL of each bacterium [10^9 colony-forming units (CFU)/mL] subcultured in tryptic soy broth (Difco Laboratories, Detroit, MI) at 37 °C for 24 h was inoculated into 10 mL of MHB (adjusted to pH 5.9) and incubated at 37 °C for 24 h with shaking at 100 rpm. Viable cells (log CFU/mL) were enumerated on tryptic soy agar (Difco) by pour plating 1 mL of serial dilutions of MHB followed by incubation at 37 °C for 48 h.

Proximate and Astaxanthin Analyses. Crude protein was calculated by multiplying the nitrogen content of the sample by 6.25. Nitrogen was determined using an elemental analyzer (EA 1110, CE Instrument, Rodano-Milan, Italy). Moisture content was determined using a halogen moisture analyzer (HG53, Mettler Toledo, Greifensee, Switzerland). Ash was determined according to a standard method (*20*). Chitin was determined according to the method of Black and Schwartz (*21*). The extractable astaxanthin was determined following the solvent extraction procedure described by Lee (*22*).

Table 1. Chemical Composition of Crab Leg Shell

composition	content[a]
crude protein, %	24.3 ± 0.3
ash, %	43.8 ± 0.3
chitin, %	24.4 ± 0.5
astaxanthin, ppm	trace

[a] Mean ± standard deviation of triplicate determinations, on a dry basis.

Degree of Deacetylation (DD) and Molecular Weight (MW). The DD was determined according to a colloid titration method (*23*) using N/400 potassium polyvinyl sulfate ($f = 1.006$, Wako Pure Chemicals, Osaka, Japan). For the determination of viscosity-average MW of chitosan, chitosan was dissolved in 0.1 M acetic acid/0.2 M NaCl and an automated solution viscometer (relative viscometer model Y501, Viscotek Corp., Houston, TX) was used to measure the intrinsic viscosity [η]. The Mark−Houwink equation, $[\eta] = K(\mathrm{MW})^a$, was used to calculate the molecular weight. Values of K and a of 1.81×10^{-3} cm^3/g and 0.93, respectively, were used.

Viscosity and Solubility. Viscosity was determined with a Brookfield viscometer, model LVDV-II+ (Brookfield Engineering Laboratories, Stoughton, MA). Chitosan solution was prepared in 1% acetic acid at a 1% concentration on a moisture-free basis. Measurements were made using a small sample adapter on solution (8 mL) at 23 ± 0.3 °C with values reported in centipoise (cP) units. Percentage of solubility of chitosan was determined at a 0.5% chitosan concentration in 1% acetic acid.

Color. Color was measured using a portable Minolta chroma meter CR-200 (Minolta Camera Co. Ltd., Osaka, Japan). Results were recorded as L^*, a^*, and b^* values, where L^* describes lightness, a^* redness, and b^* yellowness.

Statistical Analysis. All experiments were carried out in triplicate, and average values or means ± standard deviations are reported. Means of the main effects were separated by Duncan's multiple-range test using the SAS software package.

RESULTS AND DISCUSSION

Chemical Composition of Crab Shell. Chemical analyses (**Table 1**) of crab leg shell indicate that the shell is an excellent source of chitin, 24.4% by dry weight. The average crude protein and ash contents of the shell were 24.3 and 43.8%, respectively. The level of the carotenoid astaxanthin extractable with organic solvents was negligible.

Crustacean shell mainly consists of 30−40% protein, 30−50% calcium carbonate, and 20−30% chitin (*24*). These portions vary with species and season (*25*). The chemical compositions of crab shell used in our study were comparable to those previously reported (*16*) with crab shell. Pigments were not extractable with organic solvents, indicating that decoloration is needed when a bleached chitinous product is desired.

Characteristics of Chitosans. The physicochemical characteristics of various chitosans prepared under different deproteinization (DP) times were determined. Results are shown in **Table 2**.

No significant differences ($p > 0.05$) in nitrogen content were observed among chitosans, irrespective of deproteinization times. According to Cho and No (*17*), optimal conditions for deproteinization of crab shell for chitin preparation under autoclaving conditions (15 psi/121 °C) were 3% NaOH for 15 min with a solids/solvent ratio of 1:10 (w/v). This indicates that elimination of the deproteinization step (0 min) or DP 5 and 10 min in our

Table 2. Physicochemical Characteristics[a] of Chitosan Products Prepared under Various Deproteinization (DP) Times at 15 psi/121 °C

DP time (min)	N (%)	DD[b] (%)	MW[c] (kDa)	viscosity (cP)	solubility (%)
0	7.53 ± 0.14a	78.4 ± 1.5a	813 ± 14d	248 ± 19b	72.8 ± 5.9a
5	7.65 ± 0.04a	88.1 ± 2.9b	730 ± 1c	40 ± 3a	88.8 ± 3.2b
10	7.58 ± 0.04a	89.0 ± 2.4b	723 ± 1c	40 ± 1a	88.6 ± 0.4b
15	7.57 ± 0.02a	87.9 ± 1.3b	660 ± 1b	38 ± 1a	86.9 ± 4.3b
30	7.54 ± 0.07a	88.6 ± 1.5b	549 ± 1a	32 ± 1a	90.4 ± 1.1b

[a] Mean ± standard deviation of triplicate determinations, on a dry basis. Means with different letters within a column indicate significant differences ($p < 0.05$). [b] DD = degree of deacetylation. [c] MW = molecular weight.

Table 3. Color Values of Chitosan Products Prepared under Various Deproteinization (DP) Times at 15 psi/121 °C

	color value[a]		
DP time (min)	*L**	*a**	*b**
0	60.67 ± 0.34c	10.89 ± 0.11a	13.62 ± 0.58a
5	57.93 ± 0.25a	11.29 ± 0.10b	16.91 ± 0.68b
10	58.40 ± 0.42ab	11.14 ± 0.09b	16.38 ± 0.40b
15	58.65 ± 0.19b	11.25 ± 0.11b	16.91 ± 0.40b
30	58.30 ± 0.12ab	10.88 ± 0.11a	15.93 ± 0.75b

[a] Mean ± standard deviation of triplicate determinations. Means with different letters within a column indicate significant differences ($p < 0.05$).

Table 4. WBC, FBC, and DBC of Chitosan Products Prepared under Various Deproteinization (DP) Times at 15 psi/121 °C

DP time (min)	WBC[a] (%)	FBC[a] (%)	DBC[a,b] (%)
0	626 ± 7a	374 ± 12a	60.0 ± 1.3a
5	628 ± 12a	392 ± 6ab	62.7 ± 0.5b
10	636 ± 13a	372 ± 3a	64.9 ± 3.0b
15	619 ± 17a	401 ± 13b	63.3 ± 2.2b
30	622 ± 5a	379 ± 18ab	63.7 ± 1.8b

[a] Mean ± standard deviation of triplicate determinations, on a dry basis. Means with different letters within a column indicate significant differences ($p < 0.05$). [b] At 2.5 mg of dye concentration/0.2 g of sample.

study would yield chitins which contain mostly, or partly, the proteins present in the crab shell. Nevertheless, comparable nitrogen contents of five chitosans reveal that the residual proteins in chitin must be removed in the deacetylation step involving harsh alkali treatment. These results support earlier observations that chitins from crustaceans contain significant amounts of amino acids; however, in the course of deacetylation to chitosan, these amino acids are solubilized, and resultant chitosans are generally free of amino acids (*26*).

Chitosan from DP 0 min had lower DD and solubility but higher MW and viscosity than chitosans from DP 5−30 min. Four chitosans from DP 5−30 min showed no significant differences in DD, viscosity, and solubility except for MW. Earlier, Karuppaswamy (*27*) noted that more complete removal of protein in chitin results in higher chitosan viscosity values. In the present study, however, higher MW and viscosity of chitosan from DP 0 min compared with other chitosans were probably due mainly to decreased depolymerization by elimination of the deproteinization step. The presence of some swollen particles in the chitosan solution from DP 0 min may have partly affected its increase in viscosity.

Table 3 shows color values of chitosans prepared under various deprotenization times. Deproteinization treatment resulted in slightly decreased *L** values and increased *a** and *b** values compared with those of DP 0 min. There was no notable difference in color values among DP 5−30 min except for *L** at DP 5 min and *a** at DP 30 min.

Binding Capacities of Chitosans. WBC, FBC, and DBC of chitosans were measured, with results given in **Table 4**. No significant differences ($p > 0.05$) in WBC were observed among the five chitosans tested. FBC of chitosan (374%) from DP 0 min was comparable to that of other chitosans except for chitosan (401%) from DP 15 min. However, DBC of chitosan from DP 0 min was lower than that of the four chitosans from DP 5−30 min, which had comparable DBC.

Cho et al. (*11*) and No et al. (*13*) reported that WBC, FBC, and DBC of commercial chitosans differed with individual products. According to No et al. (*13*), WBC, FBC, and DBC of six commercial chitosan products were in the range of 355−611, 217−403, and 21.3−100%, respectively. The binding capacities of chitosans observed in this study were comparable to those of commercial chitosan products reported by Cho et al. (*11*) and No et al. (*13*).

Antibacterial Activities of Chitosans. The antibacterial activity of chitosans was examined at concentrations of 0.0 (control) and 0.1% for seven different bacteria incubated for 24 h at 37 °C. As seen in **Table 5**, chitosans markedly inhibited the growth of most bacteria tested; however, the inhibitory effects differed with the deproteinization time of the chitosan and the type of bacterium. In general, chitosan showed relatively stronger bactericidal effects for Gram-positive bacteria than for Gram-negative bacteria in the presence of 0.1% chitosan, as observed by Jeon et al. (*14*) and No et al. (*15*).

Chitosan from DP 0 min showed similar or greater antibacterial activity against *E. coli* (Gram-negative), *B. megaterium*, *B. cereus*, and *S. aureus* (Gram-positive) compared with other DP times; however, the former possessed weak antibacterial activity against *S. typhimurium*. Growth of *B. megaterium* and *S. aureus* was completely, except for DP 5 min, or effectively suppressed by all chitosans independent of deprotenization time.

Uchida et al. (*28*) found that chitosan hydrolysate, slightly hydrolyzed with chitosanase, was more active as an antibacterial

Table 5. Antibacterial Activities of Chitosan Products Prepared under Various Deproteinization (DP) Times at 15 psi/121 °C

			DP time[a]				
	bacteria	control[a]	0 min	5 min	10 min	15 min	30 min
Gram-negative	*Escherichia coli*	9.01e	4.44a	5.18c	4.75b	4.50a	5.63d
	Salmonella typhimurium	8.64d	8.10c	5.74a	5.95a	7.52b	7.59b
	Vibrio parahaemolyticus	8.07e	0.48c	NDa	2.50d	0.30b	0.48c
Gram-positive	*Bacillus megaterium*	6.95	ND[b]	0.60	ND	ND	ND
	Bacillus cereus	7.42e	0.60a	1.04d	0.95c	0.70b	0.60a
	Listeria monocytogenes	8.70e	2.42d	1.11b	1.91c	NDa	2.59d
	Staphylococcus aureus	8.20b	2.65a	2.10a	1.97a	2.28a	2.54a

[a] Viable cells (log CFU/mL) after incubation without (control) and with 0.1% chitosan for 24 h at 37 °C. Values are average of triplicate determinations. Means with different letters within a row indicate significant difference ($p < 0.05$). [b] ND = not detected.

agent than was native chitosan. Cho et al. (*29*) reported that the antibacterial activity of chitosan for *E. coli* and *Bacillus* spp. increased with decreasing viscosity from 1000 to 10 cP. In the present study, molecular weight and viscosity of chitosan decreased from 813 to 549 kDa and from 248 to 32 cP, respectively, with increasing deproteinization time from 0 to 30 min (**Table 2**). However, no comparable trend was observed in this study.

The present study revealed that chitosans (DP 0, 15, and 30 min) possessed weak antibacterial activity against *S. typhimurium* at 0.1% concentration. Similarly, Wang (*30*) found that 0.5% chitosan was ineffective in the inhibition of *S. typhimurium*. Elsewhere, Allan et al. (*31*) reported that *S. aureus* was negligibly inhibited at a chitosan concentration of 0.1% and that *E. coli* was only slightly affected at a level as high as 1.0%; however, in the present work, *S. aureus* was effectively inhibited and *E. coli* was inhibited by 4−5 log cycles by 0.1% chitosan treatment.

Conclusion. This study has demonstrated that elimination of the deproteinization step (DP 0 min) in chitosan preparation warrants consideration for the particular usage of the final chitosan product. Elimination of the deproteinization step yields a chitosan with comparable nitrogen content, lower degree of deacetylation (DD) and solubility, but higher molecular weight (MW) and viscosity than those of chitosans prepared from DP 5−30 min. In binding studies, chitosan from DP 0 min had comparable water- and fat-binding capacities except for chitosan from DP 15 min, which had a higher FBC but lower DBC than those of four chitosans from DP 5−30 min. Chitosan from DP 0 min showed similar or greater antibacterial activity against *E. coli*, *B. megaterium*, *B. cereus*, and *S. aureus* compared with chitosans from DP 5−30 min. Elimination of the deproteinization step ultimately would result in considerable reduction in chitosan production cost due to reduction in NaOH usage, process time, and voluminous wastewater discharge. Further extensive studies are needed with chitosan from DP 0 min for its wider promising commercial applications. Preparation of chitosan from DP 5 min appears to be feasible if a chitosan product with a higher degree of deacetylation and solubility is needed.

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