Optimum Parameters for Production of Chitin and Chitosan from Squilla (*S. empusa*)

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ABSTRACT: Chitin and chitosan of high quality were produced from squilla, a by-catch of Indian Ocean fisheries, by demineralization, deproteination, and deacetylation. Optimum conditions for the production of chitin and chitosan were determined. The quality of chitin was assessed from its ash and protein content. Ash content was below 1% after treatment with 4% HCl for 12 h at 50°C. A protein content of less than 1% could be achieved by treatment with 4% NaOH in 12 h but only at a temperature of 70°C or higher. Production of chitin was also tested by a three-stage treatment with altering sequence of sodium hydroxide and hydrochloric acid (HCl–NaOH–HCl or NaOH–HCl–NaOH). This three-step treatment appeared to be successful to achieve a mineral content and protein content below 1% within 30 h and at a temperature not exceeding 50°C. The chitin obtained under optimum conditions was tested for deacetylation using NaOH concentrations of 40 and 50% for 12–44 h at 30, 50 and 70°C. The chitosan obtained had a degree of deacetylation of 77–86%, a viscosity of 8.2–16.2 $\times$ 10^2 cps, solubility of 98%, and molecular weight of $\sim 1 \times 10^6$ dalton. The data show that processing of squilla waste can lead to a high quality chitosan, useful for a broad range of applications. J Appl Polym Sci 103: 3694–3700, 2007

Key words: chitin; chitosan; squilla

INTRODUCTION

Chitin is the most abundant natural polymer after cellulose and is found in almost all crustaceans.[1] Chitin and its deacetylated derivative chitosan have numerous applications in biotechnology, pharmacy, medicine, food processing, textile, and waste water treatment.[2] The use of these environmental friendly biomaterials is constantly growing.[3]

The quality of chitin and chitosan depends mainly on the source and production conditions. Shrimp and crab shell are the most popular sources of chitin due to their availability in large quantity.[4] In addition, cuttlefish and squid are used. In the family of crustaceans, squilla is another candidate that has not been explored as a source of chitin and chitosan. It is a by-catch with an extremely low economic value and is available in large quantities in the seas of countries in the tropical region.[5] Squilla, which is also referred to as mantis shrimp, has a relatively thin skin, similar to that in shrimp.[1] This makes it a good candidate for production of chitin. For shrimp, isolation of chitin through chemical treatment is the most common method, where NaOH and HCl are used for deproteination and demineralization, respectively. The quality of chitin and chitosan obtained varies considerably with the change in concentration of acid and alkali, the processing temperature and incubation period.[6] In general, the process conditions should be as mild as possible in order not to damage the structure of these biopolymers.[7]

In this study, the effect of reaction conditions i.e., time, temperature and concentration of HCl and NaOH, on the removal of minerals and protein from squilla waste were investigated. In addition, the sequence of NaOH and HCl treatment was altered. The chitin produced was deacetylated under various combinations of NaOH concentration, incubation period and temperature. The quality of resulting chitosan was analyzed for viscosity, solubility, degree of deacetylation, and molecular weight and compared with that of chitosan from shrimp and crab shells.

EXPERIMENTAL

Determining the optimum conditions for chitin production

Squilla waste was obtained from the harbor in plastic containers loaded with ice. It was stored at −10°C and crushed using a blender before experiments. In all experiments, 10 g samples of squilla waste were used with 100 mL HCl or NaOH solution (1 : 10 ratio) in 250 mL conical flasks. Initially the effect of HCl concentration (3, 4, and 5%) during

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3–24 h was investigated. This was followed by experiments with 4% HCl to study the effect of the demineralization temperature (30–90°C) on the removal of minerals from squilla waste. Deproteination was tested with different concentrations of NaOH (3, 4, and 5%) for 12 and 24 h. Subsequently the effect of the deproteination temperature was studied at 30–90°C for 12 and 24 h using 4% NaOH.

Effect of altering and combination treatment on chitin production

The quality of chitin and chitosan depends substantially on the production route chosen.[7] The squilla shells were subjected to two production routes A and B (Table II). In Route A, decalcification by 3% HCl was done twice for 3 h each and 4% NaOH treatment during 24 h was given between the two HCl treatments. In Route B, the squilla shell was deproteinated with 4% NaOH twice for 12-h period each and HCl treatment (4.5%, 6 h) was given in between.

Determining optimum conditions for chitosan production

For the experiments to determine the effect of various process parameters on the quality of chitosan, chitin was prepared using optimum conditions obtained in this study. Chitin samples (10 g each) were put in a series of 250 mL conical flasks containing 100 mL of different concentrations of NaOH (40 and 50%) incubated at temperatures between 50°C and 70°C during 12 to 44 h. Chitosan obtained from each treatment was analyzed.

General analytical procedures

Moisture content was measured by drying the samples in an oven at 105°C for 24 h. Ash content was determined by burning the samples in a crucible at 600°C in a muffle furnace (Sanyo Gallenkamp, UK). Protein content was measured by using standard Biuret protein assay with Bovine Serum Albumin (Sigma, St. Louis, MO) as standard.[8] The micro-Biuret method was used in case when the protein content was low. In the micro-Biuret method, 0.5 g sample was mixed with 10 mL 3% NaOH and incubated at 80°C for 8 h and subsequently centrifuged. Two milliliters of the supernatant obtained was diluted with 4 mL 3% NaOH, mixed with 200 μL micro-Biuret reagent and left for 15 min. The OD value at 330 nm was determined against a blank consisting of 4 mL of 3% NaOH and 200 μL of micro-Biuret reagent.[8] Chitin recovery (% CR) was computed as chitin obtained (g) in reference to the original amount of chitin present in squilla waste. Chitin yield (% CY) was calculated as chitin (db, g) obtained in reference to the original wet sample quantity of squilla shells.

Chitin and chitosan analysis

Solubility

To determine the solubility of chitin, the sample was first dried for 24 h at 50°C in a vacuum oven. One gram of dried chitin was dissolved in 100 mL dimethyl acetamide–lithium chloride (DMA–LiCl) solution for 12 h and subsequently centrifuged to determine the percentage of insoluble chitin. The DMA–LiCl was prepared by dissolving 8 g of anhydrous lithium chloride overnight in 100 mL DMA. Solubility of chitosan was determined by dissolving 1% (w/v) chitosan in a solution of 1% glacial acetic acid with continuous stirring for 24 h. The solution was centrifuged to determine the % insoluble chitosan.

Degree of deacetylation (%DD)

Degree of deacetylation was determined by the acid hydrolysis HPLC method.[9] The %DD is obtained by quantifying the acetic acid released upon hydrolysis of chitin by sulfuric acid in the presence of oxalic acid. Complete hydrolysis is obtained within 1 h. A known amount (10–50 mg) of vacuum oven dried chitin or chitosan sample was placed in a 5 mL ampoule into which 1.5 mL 12*M* sulfuric acid and 1 mL of 63 mg/L oxalic acid were added. The ampoule was sealed gas tight, incubated in an oven at 110°C for 2 h and cooled in ice water for 2 h. The sample was diluted 10-fold and filtered through a 0.45 μm membrane filter before injection into a HPLC (Waters, Milford, MA) 300 mm × 7.8 mm column packed with cation exchange resin (ORH-801). A flow rate of 0.8 mL/min of 5 m*M* sulfuric acid under 1600 psi pressure was maintained. The oven and compartment temperatures were set to 45°C and 25°C, respectively. Injection volume was kept at 30 μL. A tunable absorbance detector (Waters™ 486) was used at 210 nm. Acetic acid standard solutions were prepared with Merck GR grade acetic acid (purity $\gg$ 99.8%). At the conditions stated above, the acetic acid peak eluted at 9.00 min and was usually separated from other peaks ($R_s \gg 1.84$).

Molecular weight and polydispersity determination

The weight average molecular weight and polydispersity were determined by gel permeation chromatography (GPC). Waters Ultra-Hydrogel 500, 1000, 2000 GPC columns were set up in series in a HPLC system consisting of Waters™ 600 controller and Waters GPC software. A solvent system with 0.2*M*

TABLE I
Ash, Protein, and Chitin Contents of Squilla

Sample type	Moisture (%)	Ash (%) db	Protein[a] (%) db	Chitin[b] (%) db
Unprocessed squilla	78.1 ± 1.3	26.4 ± 0.5	18.1 ± 1.1	55.5
Demeated squilla	71.3 ± 1.8	42.8 ± 0.6	21.2 ± 0.9	36.0
Dried squilla	34.4 ± 1.2	39.7 ± 0.8	22.4 ± 1.4	37.9

[a] Determined by Biuret method.
[b] [100 – %(ash+protein)].
db = dry basis.

acetic acid–0.1*M* sodium acetate buffer as a carrier medium flowing at a rate of 0.8 mL/min and column temperature of 35°C, was applied. Refractive Index (RI) detector maintained at 30°C was used. Dextran standard solutions (0.2% v/v) of known molecular weights were prepared and calibration curve was obtained. 150 μL of chitosan solution was injected into the column. The number average molecular weight ($M_n = [\Sigma(N_iM_i)/\Sigma(N_i)]$) and the weight average molecular weight ($M_w = [\Sigma(N_iM_i^2)/\Sigma(N_i/M_i)]$) of the samples were obtained by comparing the elution pattern of the sample and the standard dextran solutions. N_i is the number of moles of species present and M_i is the molecular weight of the species *i*. Polydispersity, which is the measure of the width of the molecular weight distribution, was calculated from the ratio of M_w/M_n. Hypothetically polydispersity should be 1 for a monodispersed polymer.

Apparent viscosity

Chitosan solution was prepared by dissolving 1% (w/v) chitosan in 1% (v/v) glacial acetic acid for 24 h. The solution was then filtered through a nylon cloth to remove nonsoluble particles. The viscosity of chitosan solution was measured using a Brookfield DV II+ Viscometer with a spindle no. 63.

RESULTS AND DISCUSSION

Analysis of squilla products

The ash and protein content of fresh, dried, and demeated squilla were measured (Table I). The dried squilla waste with residual moisture content of 34% was obtained after subjecting the demeated squilla to sun drying for a period of 5 days on a cemented floor. The ash content in squilla waste (~ 40%) was found to be much higher than that in shrimp shells (~ 20%), however comparable to that in crab.[10] The protein content in the three sample types was in the range of 18–22%, similar to that in shrimp.[11] The shell of squilla appeared to be harder than that of shrimp. This is attributed to the high content of minerals in the squilla shells. Assuming that the shells contain only chitin, proteins and minerals, the chitin content in dried squilla was derived to be 38%.

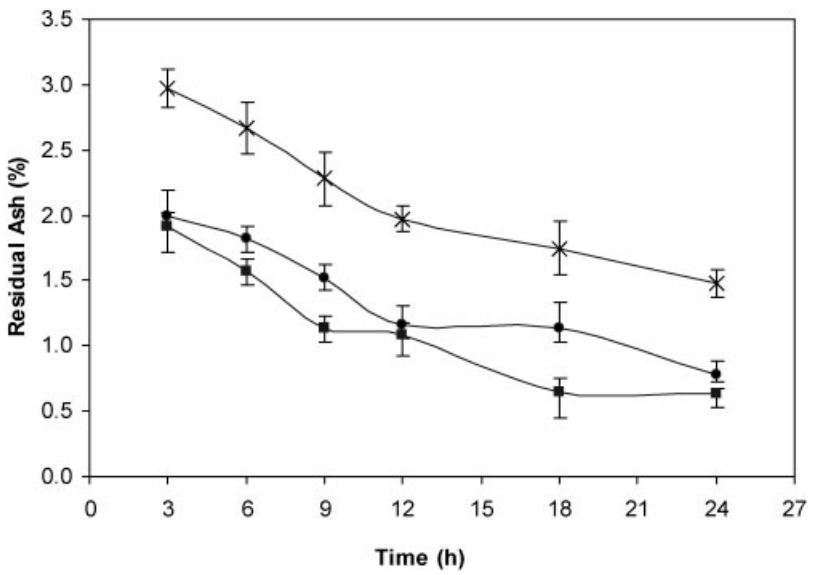

Figure 1 Effect of treatment time on residual ash content of squilla waste treated at room temperature with 3% (×), 4% (●) and 5% (■) HCl.

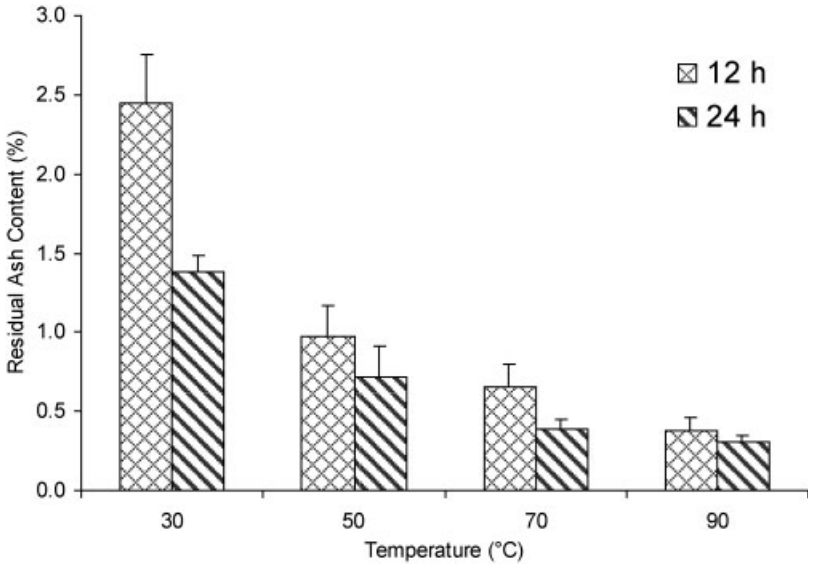

Figure 2 Effect of temperature on residual ash content (%) of squilla waste treated for 12 h and 24 h with 4% HCl.

Optimum parameters for production of chitin

Effect of HCl concentration and incubation time on demineralization efficiency

Experiments with varying concentration of HCl were conducted at room temperature during 3–24 h. Treatment of squilla waste with 4% HCl during 12 h resulted in ash content between 1 and 1.2% (Fig. 1). Even with 5% HCl, the residual ash was more than the desired value of 1%. The data indicated that with 4% HCl and an incubation period of 24 h, the resulting ash content was 0.78%, whereas with 5% HCl, in 18 h the ash content came down to 0.65%. Since HCl affects the molecular weight of chitin and chitosan, the choice between a higher concentration and a longer incubation time will depend on the desired objective of the processor. With 3% HCl concentration, even after 24 h the ash content was around 1.5%.

Effect of temperature on demineralization

To determine the effect of temperature on demineralization of squilla, 4% HCl was applied for 12 h and 24 h at 30, 50, 70, and 90°C. The residual ash content in squilla shells decreased with increasing temperature (Fig. 2). At 50°C, the ash content after 12 h was lower than 1%, which is an acceptable value in industry.

Demineralization at higher temperature is not attractive because of higher costs of energy, corrosion of equipment, and damage to the structure of chitin.

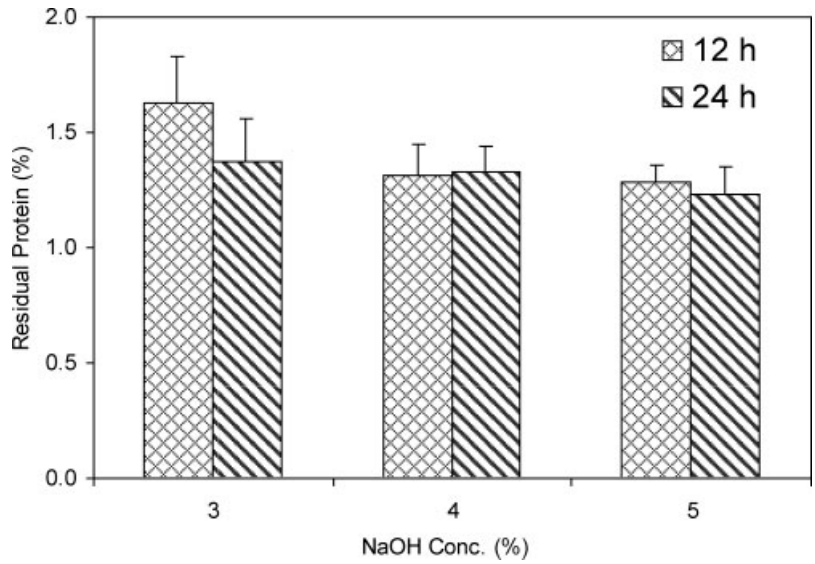

Figure 3 Effect of NaOH concentration on residual protein content (%) of squilla waste treated for 12 h and 24 h at room temperature.

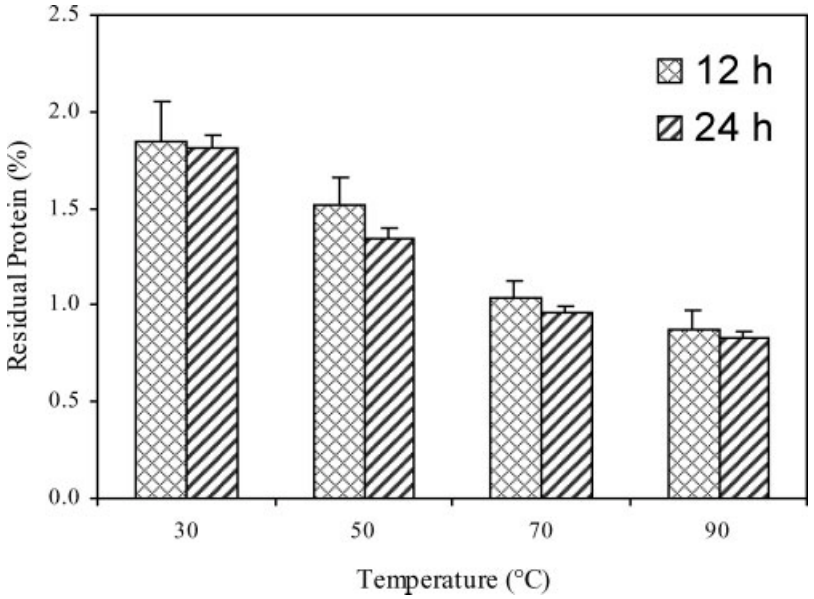

Figure 4 Effect of temperature on residual protein content (%) in squilla waste treated for 12 h and 24 h with 4% NaOH.

Effect of different concentrations of NaOH and temperature on deproteination

The effect of NaOH concentration on protein removal was determined at room temperature (30°C). The amount of residual protein on demineralized squilla shells did not differ significantly after deproteination with 4 and 5% NaOH. In all cases, the increase in incubation time did not lower the residual protein content (Fig. 3). Even with 5% NaOH and an incubation period of 24 h, the protein content was between 1.2–1.4%, a value higher than acceptable (1%) for most potential applications. There was no significant difference between the value of residual protein content after treatments with 4 and 5%.

Temperature plays a significant role in deproteination. To determine an optimum value, experiments were conducted at 30, 50, 70, and 90°C with 4% NaOH for 12 and 24 h. Only at elevated temperatures of 70°C and 90°C, the residual protein decreased to values less than 1% (Fig. 4). In shrimp chitin, the protein content is below 1% after 12 h when treated with 4% NaOH and a temperature as low as 30°C.[11] This indicates that protein packaging within the squilla shell is more compact. Since protein is bound to chitin (shell) and the minerals, it might be possible that deproteination could be more efficient, if the shells are decalcified first.

Variation in chitin treatment

Deproteination treatment before demineralization affects the ash removal considerably. Once the protein layer is removed, the acid is able to attack more efficiently. It is expected that a lower concentration of HCl and a shorter treatment time would suffice. On the other hand in the case when demineralization is done before deproteination, HCl is less effective because the mineral is protected by the protein layer.[7] The effect of sequence has been tested in two 3-step procedures (Table II). The chitin recovery and chitin yield in both 3-step procedures was almost equal but resulted in chitin preparations with a protein and ash content below 1% (Table III). The chitin recovery was $\sim$ 50% in both production routes. In case of shrimp the chitin recovery is between 35 and 45%.[11]

Determining optimum parameters for chitosan production

Deacetylation of chitin was conducted in a series of experiments with combination of temperatures at 50 and 70°C, NaOH concentration of 40 and 50% and incubation period between 12 and 44 h

TABLE II
Production of Chitin Through Different Routes

Path A	Path B
3% HCl for 3 h (1 : 8)	4% NaOH for 12 h (1 : 8)
↓	↓
Wash	Wash
↓	↓
4% NaOH for 24 h (1 : 8)	4.5% HCl for 6 h (1 : 8)
↓	↓
Wash	Wash
↓	↓
3% HCl for 3 h, 1 : 4	4% NaOH for 12 h, 1 : 4
↓	↓
Wash and dry	Wash and dry
↓	↓
Chitin	Chitin

All treatments at 50°C.

TABLE III
Analysis of Chitin Residues Obtained after Chemical Treatment

Sample type	Sample wt (g)	MC (%)	DW (g)	Protein (%) db	Protein (g) db	Ash (%) db	Ash (g) db	Chitin (g) db	CR (%)	CY (%)
Original	1000	65	350	21.0	73.5	41.4	144.9	131.6		
Path A										
R 1	70	10.8	62.4	0.85	0.53	0.15	0.10	61.8		
R 2	72	10.5	64.4	0.90	0.58	0.12	0.08	63.7		
Average	71	10.7	63.4	0.88	0.56	0.14	0.09	62.7	47.6 ± 0.8	6.3 ± 0.09
Path B										
R 1	74	10.4	66.3	0.93	0.62	0.20	0.13	65.5		
R 2	76	10.4	68.1	0.95	0.65	0.25	0.17	67.3		
Average	75	10.4	67.2	0.94	0.63	0.23	0.15	66.4	50.5 ± 0.6	6.6 ± 0.09

For details on Path A and B, refer to Table II.
R1 and R2 (replicates) are residues after treatment.
MC = moisture content, DW = dry weight of residue, CR = chitin recovery, CY = chitin yield, and db = dry basis.

(Table IV). Preliminary experiments conducted at 30°C resulted in samples with a very low degree of deacetylation and only after 3–5 days of treatment. Therefore deacetylation at 30°C is not reported. In general, it was observed that a higher initial temperature was required to treat the chitin from squilla.

At 40% NaOH and temperatures of 50–70°C, chitosan with low degree of deacetylation and higher turbidity was obtained. Unlike the trend in %DD that was sensitive to concentration of NaOH, the final viscosity was more dependent on the changes in temperature and time, than on NaOH concentration. At lower temperatures, the viscosity was higher. However with increase in incubation time, except for samples treated at 70°C and 50% NaOH, an increase in viscosity was observed. Similar to %DD trend, the solubility increased with NaOH concentration, though solubility was high in all samples (≫97%). Considering the optimum values of %DD and viscosity, it is recommended to use 50% NaOH concentration at 50°C between 12 and 44 h for converting squilla chitin to chitosan (Table IV). Polydispersity was low in all treatments indicating a rather homogeneous distribution of the molecular weight of the chitosan polymer produced.

The protein and chitin linkage in squilla is much stronger than what is observed in shrimp. This makes the removal of protein more difficult. The samples were not bleached and as a result the color of chitosan was pink. If the chitosan is treated with bleach or is dried under sun, the whiteness appears, but viscosity and molecular are reduced.[7]

CONCLUSIONS

It is concluded that squilla is a cheap and useful raw material to produce chitin and chitosan. In comparison to shrimp, it requires more treatment to reduce

TABLE IV
Properties of Chitosan Derived from Squilla Using Different Conditions

Treatments Temp (Time) Con (°C h %)	MC (%)	Viscosity (cp)	Turbidity (NTU)	Solubility (%)	DD (%)	Peak M_p (MDa)	M_w (MDa)	M_n (MDa)	PD
50(20)40	9.7	1205 ± 233	38 ± 6	98.2 ± 0.3	79.5 ± 2.2	1.95	1.58	0.92	1.72
50(30)40	9.8	1272 ± 231	38 ± 1	98.4 ± 0.2	80.6 ± 0.3	1.94	1.53	0.86	1.78
50(44)40	9.7	1624 ± 235	49 ± 9	98.4 ± 0.9	81.9 ± 1.6	1.95	1.69	1.36	1.23
70(12)40	9.3	995 ± 134	35 ± 3	97.6 ± 0.4	77.1 ± 1.7	1.94	1.50	0.83	1.80
70(18)40	9.5	1042 ± 82	43 ± 9	97.7 ± 0.1	78.8 ± 0.1	1.94	1.58	1.04	1.52
70(24)40	6.2	1045 ± 120	35 ± 1	97.3 ± 0.4	79.2 ± 0.7	1.95	1.54	0.76	2.02
50(20)50	9.9	1144 ± 67	31 ± 1	98.2 ± 0.3	81.7 ± 0.5	1.93	1.53	1.01	1.51
50(30)50	9.5	1124 ± 71	32 ± 6	98.6 ± 0.0	84.8 ± 0.3	1.95	1.58	0.96	1.65
50(44)50	9.7	1202 ± 235	32 ± 3	98.5 ± 0.2	86.6 ± 0.4	1.93	1.50	0.83	1.80
70(12)50	9.3	963 ± 29	32 ± 3	97.5 ± 0.9	85.6 ± 0.8	1.91	1.49	1.05	1.42
70(18)50	6.9	821 ± 228	34 ± 1	98.5 ± 0.3	86.8 ± 0.0	1.95	1.59	1.03	1.54
70(24)50	7.4	815 ± 71	36 ± 3	98.4 ± 0.1	86.9 ± 0.2	1.89	1.31	0.54	2.42

The treatments specified are denoted as ''Temperature (time) concentration,'' represented in ''°C (h) %'' respectively. Treatment at 50°C for 20 h with 50% NaOH is represented by 50(20)40.
DD(%) = degree of deacetylation, M_p = peak molecular weight (MDa, million daltons), M_w = weight average molecular weight (MDa, million daltons), M_n = number average molecular weight, and PD = polydispersity (M_w/M_n).

protein and mineral content, but squilla chitosan has a high degree of deacetylation, a high molecular weight, and an attractive solubility and viscosity.

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