Iran. J. Biotechnol. April 2023;21(2): e3253 DOI: 10.30498/ijb.2023.323958.3253

Research Article

Preparation and Characterization of Crayfish (*Astacus leptodactylus*) Chitosan with Different Deacetylation Degrees

Ali Eslem Kadak[1*], Aygül Küçükgülmez[2], Mehmet Çelik[3]

[1]Kastamonu University, Fisheries Faculty, 37150 Kastamonu, Türkiye
[2]Çukurova University, Faculty of Fisheries, 01330 Adana, Türkiye
[3]Çukurova University, Faculty of Ceyhan Veterinary Medicine, 01330, Adana, Türkiye

[*]*Corresponding author*: Ali Eslem Kadak, Kastamonu University, Fisheries Faculty, 37150 Kastamonu, Türkiye. Tel: +90-3662802307, Fax: +90-3662802313, E-mail: akadak@kastamonu.edu.tr

Received: 2022/01/10; **Accepted:** 2022/08/29

Background: In this study, chitosan with various deacetylation degrees was extracted from crayfish (*Astacus leptodactylus*) shells with the purpose of examining the effect of deacetylation on the characterization of chitosan.

Objectives: Recycling of wastes has become an important issue with the advancement of shellfish processing technology. Therefore, this study examined the most important and conventional characterization parameters of chitosan extracted from crayfish shells and investigated whether crayfish chitosan can be an alternative to commercial products.

Material and Methods: In order to determine the characterization of the chitosan; degree of deacetylation, yield, molecular weight, apparent viscosity, water binding capacity, fat binding capacity, moisture content, ash content, color properties, Fourier transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM), and X-ray diffraction analyses (XRD) were applied.

Results: The low (LDD) and high (HDD) deacetylated crayfish chitosan characterization results in terms of yield, molecular weight, apparent viscosity, water binding capacity, fat binding capacity, moisture content, ash content were 17.50%, 424.03-334.66 kDa, 16.82-9.63 cP, 481.29-428.04%, 419.30-355.75%, 3.32-1.03%, 0.98-1.01%, respectively. As detected by two different methods, potentiometric titration and elemental analysis, the deacetylation degrees of low and high crayfish chitosan were found close to each other, which were 76.98-94.98% and 73.79-92.06%, respectively. As the deacetylation period extended, acetyl groups were removed, and the degree of the deacetylation of crayfish chitosan increased while the apparent viscosity, molecular weight, water and fat binding capacity decreased.

Conclusions: The findings of the present study are important to obtain the chitosan having various physicochemical characteristics from unevaluated crayfish wastes and to use it in many different sectors, especially biotechnology, medicine, pharmaceutical, food, and agriculture.

Keywords: Biopolymer characterization, Crayfish chitosan, Fisheries waste, Physicochemical parameters

1. Background

Crayfish *(Astacus leptodactylus),* which is originally from Western Asia and Eastern Europe, is extensively introduced to many countries. *A. leptodactylus,* most commonly known as ‘‘Turkish crayfish”, is a native crayfish species widely distributed in Turkey, and can be found in lakes, ponds, and rivers throughout the country (1). Approximately 80% of crayfish consists of wastes (2). The shell in the wastes consists of 40% calcium carbonate, 30% protein, and 30% chitin (3-5). Chitin ranks as third in the shell composition and are remarkably hydrophobic and insoluble in most of the organic solvents and water. Roughly 10^{11} tons of chitin/ per year are produced from waste crustacean carapaces.

Chitosan, a copolymer of D-glucosamine and N-acetyl-D-glucosamine, is a nontoxic biopolymer obtained after the deacetylation of chitin in an alkaline condition (6-9). Traditional isolation of chitin and chitosan from crustacean shell waste is applied in four stages, e.g., demineralization, deproteinization, decolorization, and deacetylation (10). The first three steps form the standard procedure for chitin production. In the deacetylation process, which is the final stage, chitosan is obtained by the removal of acetyl groups (CH_3-CO) from the chitin molecular chains and the formation of amine (NH_2) groups (6, 11).

2. Objectives

Chitosan has successfully increased commercial interest as being suitable resource materials thanks to its prominent characteristics and its widely used in various sectors, particularly in food packaging, paper making, environmental protection, cosmetics, dentistry, pharmaceutical, medicine, textile, veterinary, agriculture, chemistry, and biotechnology (12-16). For these sectors, physicochemical characteristics can range from deacetylation degree (DD), viscosity, molecular weight, solubility, and color (17). The most important of chitosan characteristics is "the deacetylation degree", which represents the ratio of the number of the deacetylated N-acetyl-D-glucosamine units to the total number of the units (18-20). In many different industries, a high degree of deacetylation chitosan is generally preferred thanks to being easier to dissolve and other superior physicochemical characteristics. The present study examined the effect of deacetylation on the characterization of chitosan extracted from crayfish shells. In the characterization of the chitosan, deacetylation degree, molecular weight, moisture, ash content, yield, water-fat binding capacity, and apparent viscosity were measured using Fourier transform infrared spectroscopy, X-ray diffraction, and scanning electron microscopy.

3. Material and Methods

3.1. Materials

Shell materials were obtained from the crayfish (*Astacus leptodactylus*) shell wastes. Crayfish samples were caught in Germeçtepe Dam Lake, Kastamonu, Türkiye. After catching, the samples were put in ice to store during transportation to the laboratory. Thereafter, shells were separated completely from the crayfish samples, washed in pure water, and dried at 60 °C. Chemicals and compounds, all of the high analytical purity, such as NaOH (≥98%, anhydrous pellets), HCl (37%, EMPROVE® ESSENTIAL), H_2O_2 (30%, stabilized, EMPROVE® ESSENTIAL), and acetic acid (≥99%, glacial, ReagentPlus®) were purchased from Sigma-Aldrich.

3.2. Methods

3.2.1. Chitosan Preparation

Chitosan from the crayfish shells was extracted by the method of Fernandez-Kim (5) with some modifications. Deproteinization, demineralization, decolorization, and deacetylation process were made for chitosan extraction, respectively. Deproteinization and demineralization stages were performed with 3.5% NaOH ($w.v^{-1}$) at 65 °C and 1 N HCl ($v.v^{-1}$) at room temperature, respectively. For decolorization, the chitin residue was treated with 10:1 H_2O_2/HCl ($v.v^{-1}$) and dried. Two different deacetylated chitosan (low and high) were prepared by alkali treatment of chitin using 50% NaOH ($w.v^{-1}$) at 120 °C at different times, which were 30 min and 8 h. (**LDD:** Low deacetylation degree, **HDD:** High deacetylation degree)

3.2.2. Characterization of Chitosan

3.2.2.1. Moisture, Ash Contents and Yield

The moisture was determined after drying samples at 103 °C for 4 h. Ash content was determined by heating at 550 °C for 4 h. Weight measurements of the raw material and chitosan acquired after treatment were compared in order to calculate the chitosan yield.

3.2.2.2. Determination of the Deacetylation Degree

Potentiometric titration: A potentiometric titration method was used to determine the deacetylation degree of chitosan. 10 mL from 0.30 M HCl ($v.v^{-1}$) were used to dissolve 250.0 mg of chitosan. After being diluted to 50.0 mL by using ultrapure water, the solution was titrated with 0.10 M NaOH ($w.v^{-1}$). Consumed NaOH ($w.v^{-1}$) solution volume that corresponds to the number of amine groups in chitosan was calculated according to the difference between two inflection points of acid-base titration (21). Elemental analysis: The amount of C, H, and N in chitosan was determined by using Eurovector (EA-3000 Single) elemental analysis apparatus. Briefly, samples were heated to 1000 °C, then 2 mg of chitosan

were placed inside a silver capsule and dropped into the CHNS-932 furnace where it is combusted completely.

3.2.2.3. Determination of Molecular Weight

The molecular weight of chitosan was measured with the Size Exclusion Chromatography (SEC, Agilent Technologies Inc.), which standardized between 100-2000000 Da molecular weight standard. Results were given in kDa.

3.2.2.4. Apparent Viscosity

Moisture-free chitosan sample was prepared in 1% (v/v) acetic acid at 1% (w/v) chitosan concentration on a basis. Then the chitosan solution was filtered for the removal of all insoluble materials. Apparent chitosan viscosity was determined with a Rheometer (Anton-Paar, Physica MCR 301) at 23 °C. Values were determined in centipoises units (cP).

3.2.2.5. Water and Fat Binding Capacity

In order to measure water (WBC) and fat binding capacities (FBC) of chitosan, the method of Wang & Kinsella (22) was modified. Water absorption was performed by weighing a centrifuge tube that contains 0.5 g sample, adding 10 mL water, and mixing by vortex for 1 min. The solution was incubated at room temperature for 30 min by shaking for 5 s every 10 min. After the tube was centrifuged at 3500 rpm for 25 min, the supernatant was discarded, and the tube was weighed again. The same process was applied for fat binding capacity. WBC and FBC parameters were calculated according to the formulas below:

$$WBC\ (\%) = \frac{Water\ bound}{Sample\ weight} \times 100 \quad (1)$$

$$FBC\ (\%) = \frac{Fat\ bound}{Sample\ weight} \times 100 \quad (2)$$

3.2.2.6. Color Measurement

Chitosan sample was placed into a transparent petri dish and the color measurement was carried out by using a Hunter lab (Hunter Associates Laboratory, Inc., Reston, VA, USA) which was calibrated with white and black plates. The results were recorded as *L**, *a**, *b**.

3.2.2.7. Fourier Transform Infrared Spectroscopy (FT-IR)

Chitosan was previously dried in an oven at 90 °C, and the infrared spectrum (IR) characterization was performed in a Bruker Spectrometer (Alpha Platinum, ATR), in the region 3-4000 cm^{-1}.

3.2.2.8. Scanning Electron Microscopy (SEM)

The dried chitosan was initially coated with Gold-Palladium (Au/Pd) with an automatic coating machine (Cressington Sputter Quater 108 Auto). SEM characterization was carried out using an FEI (Quanta FEG 250 model) type instrument in a vacuum environment. Images of the samples surfaces were recorded at different areas and magnifications.

3.2.2.9. X-Ray Diffraction (XRD)

X-ray diffraction data were collected on a Bruker diffractometer with 2θ and a scan angle from 5 to 50. Chitosan was prepared by compressing it in the cassette sample holder without any adhesive substances.

4. Results

4.1. Moisture, Ash Contents and Yield

The yield values of the chitosan from the crayfish shell are given in **Table 1**. The yield values were determined by the calculation of the data obtained at the end of all the steps performed until the chitosan was obtained from the cleaned shell. The production stages of chitosan are deproteinization, demineralization, decolorization, and deacetylation, respectively. The yield of deproteinization was detected as 63.80%, while the yield of chitin was 23.72%, and finally, the yield of chitosan was 17.50%.

Moisture and crude ash contents of the high and low deacetylated chitosan were found to be 1.03-3.32% and 1.01-0.98%, respectively.

The results of the physicochemical parameters (deacetylation degree, moisture, crude ash, apparent viscosity, molecular weight, water binding capacities, fat binding capacities, and color measurement) of chitosan extracted from crayfish shells are presented in **Table 2**.

4.2. Deacetylation Degree

The deacetylation degree indicates the ratio of the amount of deacetylate N-Acetyl-D-glucosamine units to the total unit number. The deacetylation degree is the most important parameter among the physicochemical characteristics of chitosan. It is effective on all other features of chitosan and is generally divided into low and high deacetylated chitosan. In the present study, chitosan was produced with two different deacetylation

Table 1. The yield of chitosan extracted from crayfish during the extraction process (%)

Processing steps	Weight
Deproteinization	63.80±3.31
Demineralization	27.39±1.33
Decolorization	23.72±1.71
Chitin	**23.72±1.71**
Deacetylation	17.50±1.36
Chitosan	**17.50±1.36**

Table 2. Physicochemical characterization parameters of chitosan with different deacetylation degrees

		Deacetylation Degrees	
Parameters		**Low**	**High**
Deacetylation Degree(%)*	Titration**	76.98±3.76	94.98±3.99
	Elemental**	73.79±4.20	92.06±4.66
Moisture(%)*		3.32±0.11	1.03±0.07
Crude ash(%)*		0.98±0.06	1.01±0.14
Molecular weight(kDa)*		424.03±20.07	334.66±25.66
Apparent viscosity(cP)*		16.82±0.31	9.63±0.54
Water binding capacity(%)*		481.29±16.97	428.04±25.44
Fat binding capacity(%)*		419.30±8.06	355.75±7.51
Colour Measurement	L*	57.24±1.08	55.86±0.37
	a*	3.82±0.52	4.38±0.70
	b*	19.28±1.04	18.89±1.06

* Indicates a significant difference between the two groups ($p<0.05$).

**Deacetylation degrees were examined in different analysis methods on the same sample.

degrees based on the duration of deacetylation. In addition, the deacetylation degree was determined by two different methods (titration and elemental), and no statistically significant difference was observed between the methods (**Table 2**). In the highly deacetylated group, the deacetylation degree was found between 92.06-94.98%, while in the lowly deacetylated group, it was found to be between 73.79-76.98%.

4.3. Molecular Weight and Apparent Viscosity

The molecular weight of chitosan was determined in SEC in the study. SEC is a chromatographic method that separates molecules by particle size. This method is used for the fast and precise determination of the molecular weight and molecular weight distribution of polymers. In the present study, molecular weights decreased significantly with increasing deacetylation levels of chitosan ($p<0.05$). The highly deacetylated group had a lower molecular weight (334.66 kDa), while the lower deacetylated group had a higher molecular weight (424.03 kDa).

The deacetylation degree is affects the entire physicochemical characteristics of chitosan, primarily molecular weight and viscosity. Apparent chitosan viscosity was determined with a Rheometer and result was observed 16.82 and 9.63 cP for low and high deacetylated chitosan, respectively.

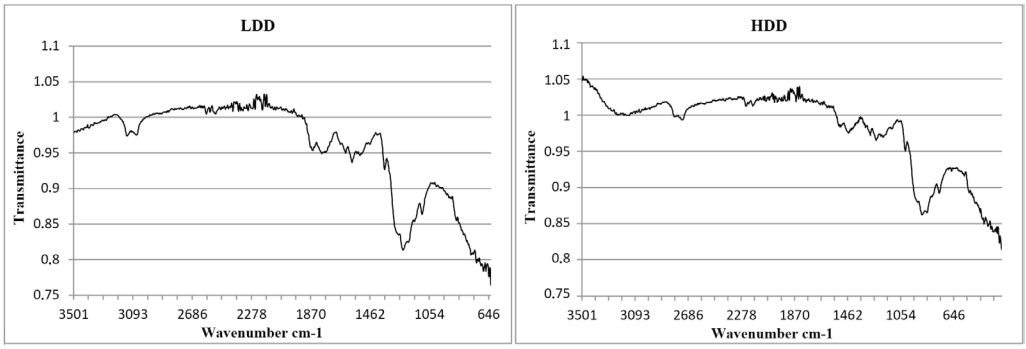

Figure 1. FTIR spectra of crayfish chitosan (LDD: Low deacetylation degree, HDD: High deacetylation degree)

4.4. Water and Fat Binding Capacity

Water and fat binding capacities of the chitosan group with the high deacetylation degree (428.04 and 355.75 %) were found to be lower compared to the group with a low deacetylation degree (481.29 and 419.30 %).

4.5. Color Measurement

Color values of chitosan. *L* (lightness), *a* (redness), and *b* (yellowness) values were measured in Hunter Lab for color measurements. Accordingly, significant differences were obtained between the *L* (55.86-57.24), *a* (3.82-4.38), and *b* (18.89-19.28) values. In the present study, the color of crayfish chitosan was light brown-white.

4.6. Fourier Transform Infrared Spectroscopy (FT-IR)

Figure 1 presents the FT-IR spectrums of the crayfish chitosan with various deacetylation degrees. To detect the chitosan structure, Fourier transform infrared spectroscopy was applied. According to FTIR analysis results, the peaks of the chitosan with low and high deacetylation were found to be similar to each other. Among the main groups of chitosan compounds, are glucosamine (C=O) peak was found as 1023 cm^{-1}; ring structure C-O-C peak as 1149 cm^{-1}; $CH\text{-}CH_3$ peak as 1373 cm^{-1}; the stretching vibration peak in the CH_2 group as 1417 cm^{-1} and the C = O-C-N peak as 1581 cm^{-1} wavelength. In addition, peaks belonging to OH bonds of the hydroxyl groups were detected between 3102 cm^{-1} and 3400 cm^{-1}.

4.7. Scanning Electron Microscopy (SEM)

The morphology of chitosan was investigated by SEM, and the SEM images with various magnifications (2.000x, 10.000x, 20.000x, and 40.000x) of crayfish chitosan with different deacetylation degrees are presented in **Figure 2**. Changes in the deacetylation degree of chitosan as well as changes in morphological

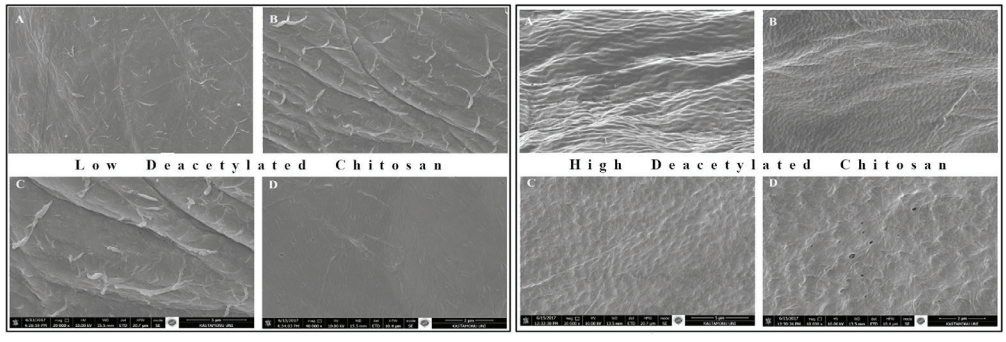

Figure 2. Scanning Electron Microscopy (SEM) of Low and High Deacetylated Chitosan. **A**) 2.000x, **B**) 10.000x, **C**) 20.000x, **D**) 40.000x.

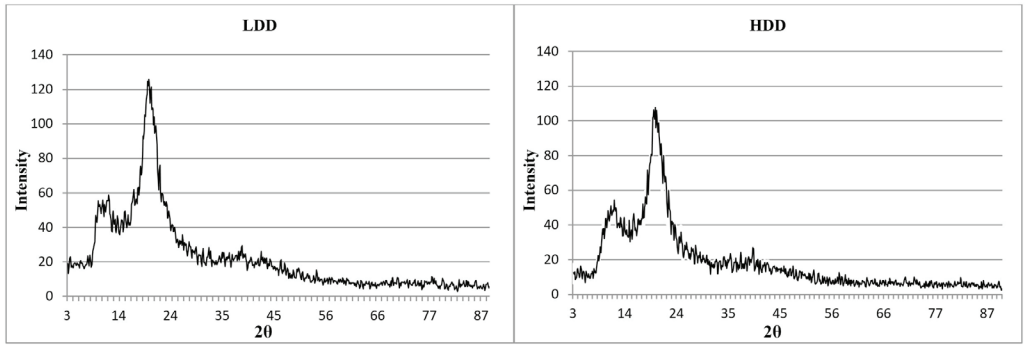

Figure 3. X-Ray Diffraction Patterns of Chitosan (LDD: Low deacetylation degree, HDD: High deacetylation degree)

characteristics, can be seen in forms. In morphological terms, it can be seen that as the deacetylation levels of chitosan increase, the number of filamentous structures decreases.

4.8. X-Ray Diffraction (XRD)

X-Ray Diffraction (XRD) results of crayfish chitosan are shown in **Figure 3**. XRD is based on the principle of the breakage of each crystal phase.

5. Discussion

The yield values are in parallel with the findings of the present study, the chitosan yield obtained from chemically-treated crayfish shells was reported as 25.70% (3); between 16.7-18.8 % in another crayfish study (6). The chitosan yield of crustacean seafood caught by the South East Asian coasts varied between 15-30% (23) while it was 15.25% for those obtained from northern Iranian region (24).

The deacetylation degree results are in a similar study, it was determined that as the duration of 4h and 6h alkaline treatment extended, the formation of amine groups increased, and the degree of deacetylation degree increased accordingly (18). The studies conducted on chitosan obtained from many different crustacean species so far have reported different deacetylation degrees from low too high for the chitosan (6, 10, 25-29). As a matter of fact, the findings of both the present study and the literature are at these intervals, where the most important parameters are the deacetylation method for the chitosan yield from chitin, temperature, NaOH concentration, and reaction time. The deacetylation degree should be adjusted according to the application area as it affects the entire physicochemical characteristics of chitosan, primarily molecular weight, and viscosity.

In a similar study, the moisture values of chitosan obtained from shrimp and crayfish were found to be 1.7 % and 0.8 %, respectively (3). Fernandez-Kim (6) reported ash values in the range of 0.3-0.7 % and moisture values in the range of 0.3-1.6 % in crayfish chitosan obtained through different methods.

The molecular weight of chitosan was determined in SEC in the study and parallel to the present findings, it was reported that high alkaline concentration in the deacetylation process might help depolymerization of chitosan, resulting in a decrease in molecular weight (18). Similarly, in parallel with the findings related to molecular weight, low (9.63 cP) viscosity values were detected in the highly deacetylated group, and higher (16.82 cP) values were found in the lowly deacetylated group. Viscosity value is closely related to molecular weight; hence viscosity increases (30) as molecular weight increases. As reported by Jeon *et al*. (31), the viscosity of the chitosan was found closely related to the deacetylation duration; and chitosan had the highest viscosity in the shortest deacetylation duration, which is in parallel with the findings of the present study. Both molecular weight and viscosity values of chitosan vary by the production method of chitosan, especially deacetylation conditions (temperature, time and NaOH concentration).

For the water and fat binding capacities of the chitosan different results have been reported for obtained from different sources (3, 31). The water and fat binding capacity of chitosan vary by the source of chitosan, the methods of obtaining chitosan, the chemicals used and the temperature.

Color measurement of similar chitosan has been obtained in different chitosan studies (25, 32), and the most important parameters in the formation of chitosan color are thought to be the chitosan source and decolorization method. In addition, the color of the chitosan found in commercial powder form can vary from light yellow to white.

For the FT-IR analysis in similar research, researchers found the C2 position of glucosamine (C=O) peak on 1033cm^{-1}, CH-CH$_3$ peak on 1380cm^{-1}, and C=O-C-N peak on 1558cm^{-1}, the peaks were similar, and no significant difference was verified (4, 33).

Morphological results in similar research, it was reported that the surface morphologies of chitosan obtained from other sources are very different from the electron microscopy images; accordingly, the surface of the chitosan obtained from shrimp shells was smoother, and that of the chitosan obtained from crayfish shells was a three-dimensional surface (3).

X-rays in a characteristic order, depending on their specific atomic sequences, X-ray diffraction is often used to measure polymorphic differences and crystal planes and also to measure crystalline content (34). The first crystallization peaks of low and high deacetylated chitosan were measured at the range of 9.24° 2θ and 11.3° 2θ, respectively. The second crystallization peaks of low and high deacetylated chitosan were measured at the range of 19.8° 2θ and 19.6° 2θ, respectively. Various XRD patterns of chitosan have two characteristic peaks that are generally around 2θ = 10° and 2θ = 20° (35).

6. Conclusion

This study's primary focus was on process modifications of the crayfish chitosan during the production stage (deacetylation process) that can affect the physicochemical and functional characteristics of chitosan. However, it will be very uncertain to conclude that only one deacetylation process is optimum for the production of chitosan because the chitosan obtained in different physicochemical characteristics can be presented to different sectors according to their usage areas. Therefore, the relationship between the process and the resulting specific characteristics of chitosan must be monitored properly and continuously, and the results of the present characterization will be the basis for all the industrial areas where chitosan is used. The deacetylation degree of chitosan was calculated in two different ways, which were the elemental analysis and potentiometric titration method. The analyses of other physical parameters such as moisture, ash, WBC, FBC, molecular weight, and viscosity changed depending on the deacetylation degree properties. The chemical parameters such as FT-IR, XRD, and SEM also changed depending on the different deacetylation degrees. In general, these results showed that different deacetylation degrees could affect both physical and chemical properties. At the same time, these results were found in accordance with the literature and showed that it could be an alternative product to the widely used commercial chitosan.

Acknowledgments

This work was part of PhD Thesis of Ali Eslem KADAK and was supported by the Çukurova University, Unit of Research Projects with the grant number: FDK-2014-2290.

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