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Characterization of Chitosan Extracted from Exoskeletons of Pomacea spp.

Unnis P. Aguilor*, Renjith Raju, Jitty Raju, Julius T. Capili

Cagayan State University, Carig Campus, Tuguegarao City, Cagayan Valley, Northern Philippines

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Abstract

This study focused mainly on the characterization of the chitosan sample extracted from exoskeletons of *Pomacea spp*., a freshwater mollusk. FTIR was used to characterize the chitosan sample and was compared to the commercial chitosan, which has a DD value of 95%. The FTIR spectra formed characteristic bands in the frequency range between 4000 and 400 cm^{-1}. The FTIR of chitosan samples isolated from shells of *Pomacea spp*. and the commercial chitosan yielded spectra with functional groups where the hydroxyl (OH) group is at 3650 to 3400 cm^{-1}; carbonyl (C=O) group vibration is at 1730 cm^{-1} and amide I group is at 1650 to 1550 cm^{-1}. Moreover, the locally extracted chitosan samples from *Pomacea spp*. shells showed FTIR spectra that are nearly comparable with the commercial chitosan and those locally extracted chitosan samples used in the previous studies. Analysis by IR estimated the DD as 60.47% for this chitosan. This DD value was 56% to 99% when a sample can be considered chitosan.

* **Corresponding Author:** Unnis P. Aguilor ✉ jchua@csu.edu.ph

Introduction

Chitin is the second most abundant natural polymer, next to cellulose. And like cellulose, it functions as a structural polysaccharide, and annually, it is estimated to be produced almost as much as cellulose. It is insoluble in most solvents because of its compact structure. Therefore, chemical modifications of chitin are performed to obtain more soluble analogs, among which are the so-called chitosan that can be derived by deacetylation of chitin. Hence, this chitosan is the most common form of chitin derivative.

Chitin and its derivative, chitosan, are of commercial interest due to their excellent biocompatibility, biodegradability, non-toxicity, chelating- and adsorption power (Chabbra, 2001). Both can be used as components and ingredients in the development and production of various medical and veterinary products that serve as agents for anti-infectious, antiviral, anti-tumor (Synowiecki and Al-Khateeb, 2003; Younes and Rinaudo, 2015;), bacteriostatic and fungistatic (Chabbra, 2001; Synowiecki and Al-Khateeb, 2003; Younes and Rinaudo, 2015; Loutfy *et al.*, 2016; Murugan *et al.,* 2017; Bariuan *et al.*, 2020;), and antioxidants (Murugan *et al.,* 2017).

According to Kaewboonruang (2016), chitin is insoluble under many physiological conditions, but it can be chemically modified into its soluble derivative –Chitosan. Therefore, chitosan is the most common derivative of chitin. Chitosan is given lots of attention in the biomedical field because of its valuable biochemical and physiological properties like antimicrobial, biodegradability, biocompatibility, non-immunogenicity, reactivity, solubility, and non-toxicity.

Typically, the primary sources of chitin are obtained from shells and are considered waste products. However, in terms of chitin and chitosan content of their exoskeletons, the rich diversity of freshwater mollusks in the Cagayan Province, particularly the Pomacea spp., in terms of chitin and chitosan content of them is not yet studied and are just waiting to be explored. Therefore, it is fascinating to extract and characterize the chitosan from the chitin of exoskeletons of *Pomacea spp*. Hence, this study was conducted to characterize the chitosan sample extracted from exoskeletons of *Pomacea spp*.

They are using FTIR (Fourier Transform-Infrared Spectroscopy) by determining the functional groups of the FTIR spectrum and computing the degree of deacetylation (%DD).

Materials and methods

Locale and duration of the study

The *Pomacea spp*. were collected from the rice paddies and ponds of Gattaran, one of the municipalities of Cagayan Province, Cagayan Valley, Philippines. First, the taxonomical identification and verification of *Pomacea spp*. was certified by the Bureau of Fisheries and Animal Resources.

Then, the chitosan extraction was conducted in the laboratory of Cagayan State University – Carig Campus, ROCO Building. After extraction, exoskeletons were submitted to the Philippine Institute of Pure and Applied Chemistry (PIPAC) for FITR analysis. The laboratory tests were performed from April to July 2021.

Chitin-Chitosan Extraction Procedure

The procedure of Murugan *et al.,* 2017 was utilized for chitin-chitosan extraction. The chitosan was extracted from shells of *Pomacea spp*. by following the process of chitosan recovery, demineralization, and deacetylation.

The step-by-step sequences of chitosan recovery or deproteinization involved first washing crushed shell samples using distilled water. Then, soak the pieces in a boiling sodium hydroxide (NaOH) (4% w/v) for 1 hour. This process is used to dissolve proteins and sugars, thus, isolating the crude chitin. Moreover, 4% NaOH is the concentration used by the scientists at the Sonat Corporation in chitin preparation. After boiling, the beakers containing the shell samples are removed from the hot plate and allowed to cool for 30 min at room temperature.

Demineralization involved soaking the shell samples into 1% HCl (v/v) with four times the quantity of the samples for 24 hours. This particular process is to remove the minerals (mainly calcium carbonate). Next, the demineralized shell sample powders were treated with 50 mL 2% NaOH solution for 1 hour.

This is to decompose the albumen into water-soluble amino acids. Then, the remaining chitin was washed with deionized water and drained. Finally, the chitin was further converted into chitosan by deacetylation. The deacetylation process was carried out by adding 50% NaOH to the samples, boiled at 100 °C for 2 hours on a hot plate. Then, the samples were placed under the hood for 30 minutes at room temperature during the cooling process. Then again, samples were washed with 50% NaOH and filtered to retain the solid matter, which is the chitosan already. Finally, the samples were left uncovered and oven-dried at 110 °C for 6 hours.

Fourier Transform Infrared Spectroscopy (FTIR)

Ten (10) grams of chitosan samples were submitted to the Philippine Institute of Pure and Applied Chemistry (PIPAC) for FTIR analysis. The spectrum of chitosan samples was obtained using Shimadzu FT-IR Spectrometer with a frequency range of 4000-400 cm^{-1}. The methodology for this particular analysis was per the protocol (Dimson and Knepper, 2015) used by the PIPAC. Locally isolated chitosan from the shells of freshwater mollusks, *Pomacea spp*., used in this present study were compared to the standard commercial chitosan, with a degree of deacetylation of 95%, provided by PIPAC. In the study of Ssekatawa *et al*., 2021, this was the procedure for FTIR analysis. Three milligrams (3 mg) of each sample (Chitosan) and 5 g of Potassium bromide (KBr) were dried at 60 °C and 120 °C, respectively, under reduced pressure for 12 h. Each dried chitosan sample was homogenized with 100 mg of KBr and then compressed to form fragile discs of approximately 0.2 mm thickness. Finally, the chitosan samples were examined at 4000–400 cm^{-1} range using a Spectrometer.

Results and discussion

This present study used FTIR to characterize the chitosan samples isolated from shells of *Pomacea spp*. The FTIR spectra formed characteristic bands in the frequency range between 4000 and 400 cm^{-1}.

Table 1. FTIR spectra of chitosan samples isolated from shells of *Pomacea spp*. and commercial chitosan with their corresponding functional groups.

Wave number cm $^{-1}$	*Pomacea spp*	Commercial Chitosan (Sigma Aldrich)	Present Functional Group/ Molecule
4000–3700	------		O-H
3650-3400	3634.53 3400.50	3427.1	OH hydroxyl group
3360 NH	------		Group-stretching vibration
2919–2868	2916.37	2918.8 2875.7	Stretching band C–H and –C=O of the amide group CONH-R of the polymers
2920, 2880	2916.37		Symmetric or asymmetric CH_2 stretching vibration
2349	2520.96	------	Carbon dioxide O=C=O
2140-1990	------	------	Isothiocyanate
1730	1788.01	------	Carbonyl group vibration
1650-1550	------	1651.7	C=O in amide group (amide I band)
1590	------	1599.2	NH_2 in amino group
1560	------	------	NH-bending vibration in amide group
1415, 1320 1430, 1320	1479.4	1422.1	Vibrations of OH, CH in the ring Symmetric or asymmetric CH_2 stretching vibration

1390-1370	------	1383.2	CH_3 in amide group Amide III
1310-1250	------	1321.1 1261.4	Aromatic band C-O
1275, 1245	------	------	Attributed to pyranose ring
1150-1040	------	1153.1	–C–O–C– in glycoside linkage
1124–1087	1082.07	1077.5 1029	Stretching band C–O–C
900-890	------	895.9	–C–O–C– bridge and glycosidic linkage of amides
850, 838	862.18	------	CH_3COH group
800-700	711.73	------	Amide VI
600	------	661.8	Amide VI
400	------	------	Amide VI

The FTIR of chitosan samples isolated from shells of Pomacea spp. and the commercial chitosan yielded spectra with functional groups are shown in Table 1 and Figs 1 and 2. According to Ssekatawa *et al.* (2021), Nandiyanto and Risti Ragadhita (2019), Thillai *et al.* (2017), and Kaewboonruang (2016), the hydroxyl (OH) group is at 3650 to 3400 cm^{-1}; carbonyl (C=O) group vibration is at 1730 $cm^{-1,}$ and amide I group is at 1650 to 1550 cm^{-1}. Fig. 1 shows the FTIR spectrum of the chitosan sample extracted from the *Pomacea* spp. shells. The spectrum displayed absorption bands at 3643.53, 3400.50, 2916.37, 2520.96, 1788.01, 1479.4, 1082.07, 862.18 and 711.73 cm^{-1}.This chitosan FTIR spectrum showed sharp peaks at 711.73 cm^{-1} (out-of-plane bending NH), 1082.07 cm^{-1} (C– O–C stretching), 2916.37 cm^{-1} (CH2 stretching), and 3400.50 and 3643.53 cm^{-1} (–OH stretching). The vibrational mode of amide C=O stretching was not observed at 1650 to 1550 cm^{-1}, but there was an observable vibrational mode of the carbonyl group at 1730 cm–1, which was 1788.01 cm^{-1}. This result was similar to Varma and Vasuden's (2020) study. This spectrum was also compared with the standard Chitosan (Fig. 2).

Table 2. Comparison of bands representing the amide I ($NHCOCH_3$) and the OH molecules and the computed DA (%) and DD (%) among the chitosan samples extracted from *Pomacea* spp. shells and commercial chitosan.

	AMIDE I ($NHCOCH_3$) cm^{-1}	OH molecule cm^{-1}	DA (%)	DD (%)
Commercial Chitosan	1651.7	3427.1		95
	Carbonyl Group cm^{-1}	OH molecule cm^{-1}	DA (%)	DD (%)
Pomacea spp.	1788.01	3400.50	39.53	60.47

In this chitosan sample extracted from the shells of *Pomacea spp.*, the absorption bands at 1540 cm^{-1} were not observed; therefore, steps of deproteinization were executed correctly. However, there were observable bands at 1798, 1420-1430, and 876 cm^{-1}; these were at 1788.01, 1479.4, and 862.18 cm^{-1}. This means that there was inefficiency during the demineralization process.

The FTIR spectrum of the chitosan samples extracted from *Pomacea spp*. shells did not show the vibrational mode of amide I C=O stretching at 1650 to 1550 cm^{-1}, unlike in the studies of Domszy and Roberts (1985), Khan *et al.*, (2002), Biskup *et al.,* (2012), Paul *et al.*, (2014), Vilar Junior *et al.*, (2016), Anwar *et al.,* (2017), Oyekunle and Omoleye (2019), Boukhlifi (2020), and Ssekatawa *et al.* (2021). But instead, there were bands in the carbonyl group at 1730 cm^{-1}. Likewise, the study by Varma and Vasuden (2020) considered the band with a wave number of 1795, a peak that implies the presence of the carbonyl group.

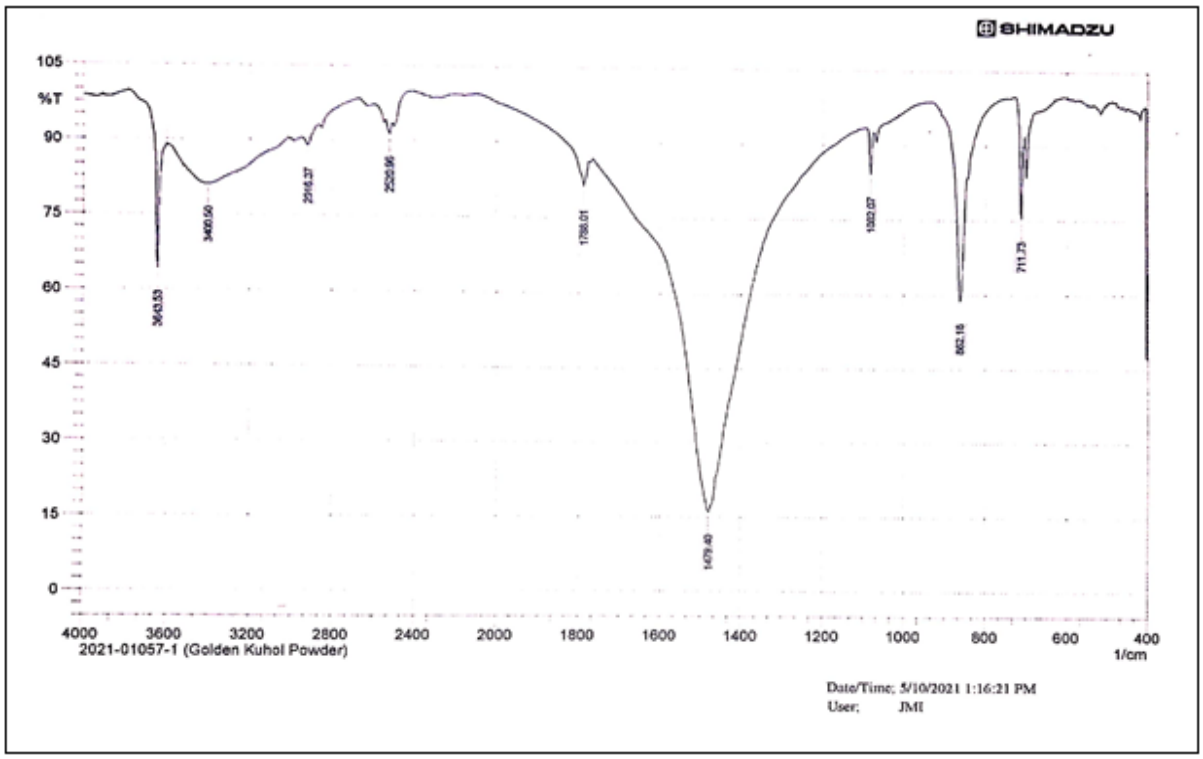

Fig. 1. FTIR spectrum for the chitosan sample extracted from the shells of *Pomacea spp*.

As for the OH group, the FTIR spectrum of the chitosan samples extracted from the shells of *Pomacea spp*., and the commercial chitosan and the chitosan samples from the studies of Khan *et al*., (2002), Biskup *et al*., (2012), Paul *et al*., (2014), Vilar Junior *et al*., (2016), Anwar *et al.,* (2017), Oyekunle and Omoleye (2019), Varma and Vasuden (2020), Boukhlifi (2020), and Ssekatawa *et al.* (2021) were comparable with each other. It was within the given range of 3650-3400 cm^{-1}. This means the FTIR spectrum of the chitosan samples extracted from *Pomacea spp*. shells were nearly comparable with the commercial chitosan and with those chitosan samples used in the previous studies.

To further validate this claim, the researcher of this present study determined the degree of acetylation (DA) and the degree of deacetylation (DD) of chitosan samples used in this study. The DA and DD of Chitosan extracted from different shell samples were determined using FTIR spectra. These were done by correlating some absorbance bands linked to some of the amide, methyl, and hydroxyl bands registered by the FTIR spectra. This study used the ratio of the absorbance of the carbonyl group to that of the hydroxyl group and used formulas 1 and 2 in the study of Ssekatawa *et al.* (2021). The computation of the DD (%) of the chitosan sample extracted from shells of *Pomacea spp*. in this present study, bands 1788.01 cm–1 corresponding to the carbonyl group, as alternative values for acetylated residues of amide I ($NHCOCH_3$) and 3400.50 cm^{-1} associating to the vibration of the OH molecule were used. Analysis by Infrared spectroscopy estimated the percentage DD (%) as 60.47% for *Pomacea spp,* as seen in Table 2.

Varma and Vasuden (2020) stated that the degree of acetylation (DA), on average, ranges from 40 to 13%; when the fraction of acetylated amine groups is reduced to 40-35%, the resultant copolymer is then called chitosan as cited by Goy *et al*., (2009). Kaewboonruang (2016) said that 40% is an important parameter to consider that it is chitosan (%DD), which means 60% eliminated of the acetyl group was eliminated. It was stated in the study of Kalut (2008) that the degree of deacetylation of chitosan ranges from 56% to 99%, with an average of 80%, depending on the crustacean species and the preparation methods; also, a degree of deacetylation of 75% or above is known as chitosan. The analysis by Infrared

spectroscopy estimated the percentage DD (%) as 60.47% and 95% for *Pomacea spp*. and commercial chitosan, respectively, and were comparable. Furthermore, this present study's computed DD (%) was similar to Varma and Vasuden's (2020) study. The deacetylation rate for *M. edulis* (69.60 ± 0.12%); *L. attenuatum* (37.30 ± 0.31) (Majekodunmi *et al*., 2017); 54.65% deacetylation in shrimp shell (Al-Hassan, 2016); however, this study computed a lower DD as compared to the deacetylation rate of *Aspergillus niger* mycelium which is 73.6% (Muñoz *et al.,* 2015); the deacetylation rate of 77.8%, 78.1%, 79.1% for Banana weevils, Mushroom and Nile perch scales Chitosan (Ssekatawa *et al*., 2021).

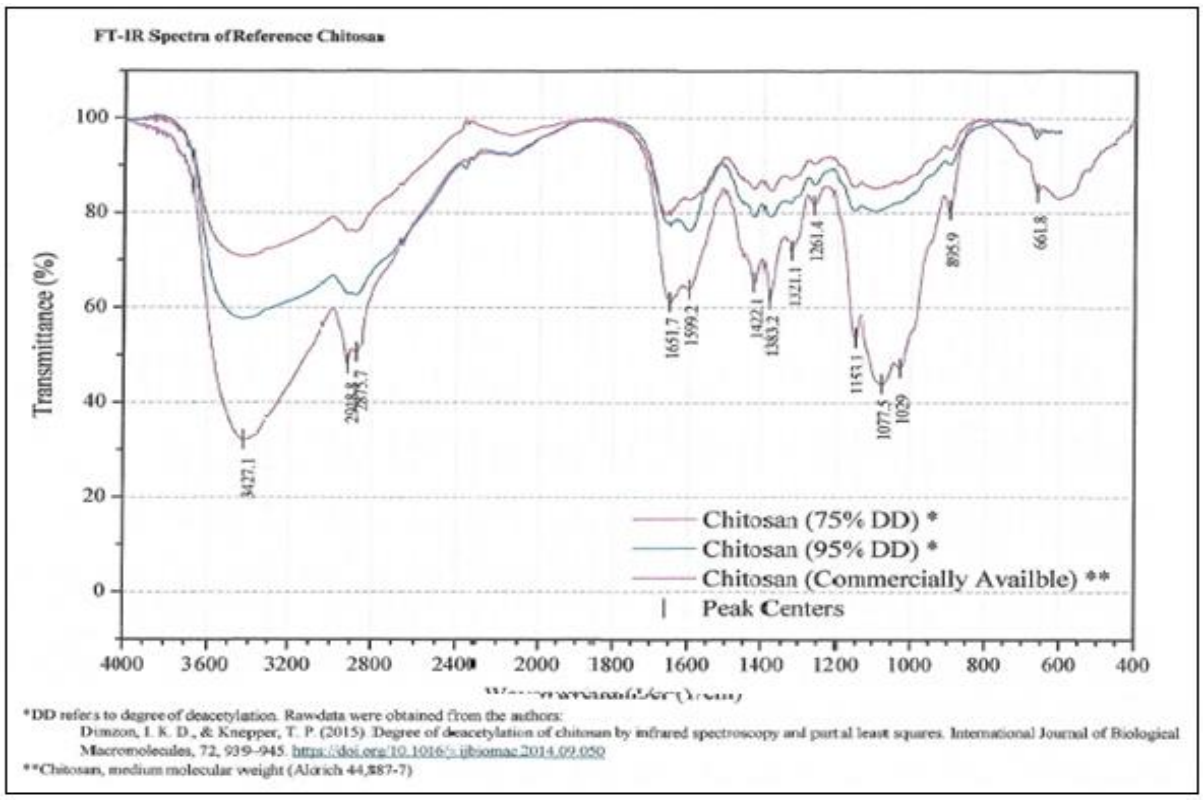

Fig. 2. FTIR spectrum of the commercial chitosan.

The degree of deacetylation varies depending on the various factors. First, the raw materials being used – type species or the type of organism (Oyekunle and Omoleye, 2019; Kaewboonruang, 2016). Second, the methods/steps/preparation processes used during deacetylation include soaking, boiling, and stirring the sample with the reagent (Oyekunle and Omoleye, 2019; Kaewboonruang, 2016). Third, this particular step's time/reaction time or duration had been carried out (Oyekunle and Omoleye, 2019; Kaewboonruang, 2016). Fourth is the temperature used during this process. Fifth, the types and ratios/concentrations of the reagents used. Sixth is the quality of chitin or the purification of raw materials (Oyekunle and Omoleye, 2019). Lastly, the analytical methods and the types of instruments used in determining or estimating the degree of deacetylation of chitosan (ninhydrin test, linear potentiometric titration, near-infrared spectroscopy, nuclear magnetic resonance spectroscopy, hydrogen bromide titrimetry, infrared spectroscopy, and first derivative UV-spectrophotometry) (Khan *et al*., 2002; Kaewboonruang, 2016;Oyekunle and Omoleye, 2019).

Conclusion

The FTIR spectra exhibited typical banding in the frequency range of 4000 to 400 cm-1. The hydroxyl (OH) group is at 3650 to 3400 cm^{-1}, the carbonyl (C=O) group is at 1730 cm^{-1}, and the amide I group is at 1650 to 1550 cm^{-1} in the FTIR spectra of chitosan samples isolated from the shells of *Pomacea spp*. and commercial chitosan. In addition, the FTIR spectra of

the locally extracted chitosan samples from *Pomacea spp.* shells are virtually identical to commercial chitosan and the locally extracted chitosan samples utilized in earlier investigations. IR analysis indicated the DD for this chitosan to be 60.47%. This DD result was within the range of 56 to 99%, within which a sample is deemed chitosan.

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