Extraction and Physicochemical Characterization of Chitosan from *Litopenaeus vannamei* Shells

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ABSTRACT: *The chitosan was extracted from a whiteleg shrimp shell (Litopenaeus vannamei) by the deacetylation of chitin, which is carried out using 45% NaOH at 110 ºC for 6 h. The prepared chitosan was characterized by using the Fourier Transform InfraRed (FT-IR) spectroscopy, X-Ray Diffraction (XRD), Scanning Electron Microscope (SEM), Energy Dispersive X-ray Spectroscopy (EDXS), and ThermoGravimetric Analysis (TGA). The physicochemical property of this extracted polysaccharide, including the degree of deacetylation, apparent viscosity, molecular weight, Water Binding Capacity (WBC), and Fat Binding Capacity (FBC) from whiteleg shrimp shell was evaluated. The current study contrasted the characteristics of chitosan with the commercial type. Accordingly, to obtain the degree of deacetylation, the titration method and elemental analysis were considered, while the viscometric methods were used to achieve the molecular weight. The SPSS software was used for the analysis of the obtained data. Based on the comparison between the studied chitosan and commercial one, some behaviors were observed, including increased deacetylation and viscosity, decreased molecular weight, and higher water and fat binding capacities. The degree of deacetylation was determined as 86% for the titration method and 83% for elemental analysis. WBC and FBC of chitosan from shrimp were reported as 673.58 and 491.32, showing that all chitosan properties experienced a good improvement compared with the commercial type.*

KEYWORDS: *Chitin; Chitosan; Shrimp waste; Deacetylation; Demineralization; Deproteinization.*

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INTRODUCTION

The vast quantities of waste processed shrimps, fish, and lobsters are due mainly to their shell has created a huge environmental problem, as many researchers have tried to optimize the use of these lesions [1]. According to *Nithya* [2], about 30 to 40% protein, 30 to 50% calcium carbonate, and 20 to 30% chitin on a dry basis are included in shrimp waste. Besides, approximately half of the processed seafood contains shrimp in that the exoskeleton and cephalothoraxes are the major components of the shrimp waste [3, 4].

Chitin (b-1,4-poly-N-acetyl-D-glucosamine) is a polymer that exists to a large extent in nature of the polymer, known as cellulose. It can be found in the shells, crustaceans, and exoskeleton of marine zooplankton, the cuticle of insects, and the cells of fungicide. The extraction of the chitin and chitosan from the waste is one of the most influential and valuable methods. For a long, Chitin was not in the center of attention; however, chitosan and chitooligosaccharides have been widely studied in recent years [1, 5]. Many industries consider chitosan for their productions: food, chemistry, biotechnology, agriculture, cosmetics, medicine, environment, and textile [6, 7, 8-12]. In order to obtain the Chitosan (poly-b-1,4-2-amino-2-deoksi-b-D-glukopiranoz), chitin needs to be under deacetylation [8, 13-16]. One characteristic of chitin is that this polysaccharide cannot be solved in water; however, the chitosan (obtained after deacetylation) can be solved if the condition is acidic. Three properties of biodegradability, biocompatibility, antifungal activity, and antibacterial activity of chitosan made it an essential factor in many studies in recent years, also considering its usage to prepare hydrogels, films, and fibers [14, 15].

In recent decades, many researchers have tried extracting chitosan from aquatic animals, the Mediterranean and Aegean shrimp shells. They investigated some properties of the extracted chitosan such as efficiency, moisture, ash, WBC, FBC, viscosity, deacetylation degree, molecular weight and compared its physicochemical properties with commercial chitosan. Their results showed that the shrimp shell was a rich source of chitosan (17.45 % of the dry weight of the shell), and the extracted chitosan had low molecular weight, high deacetylation extent, high viscosity, and high WBC and FBC compared with the commercial type [8, 17-19]. *Al Sagheer et al.* [20] extracted chitin and chitosan from several shrimp using conventional and microwave heating methods. They observed that microwave heating had reduced deacetylation time from 6-10 h to 10-15 min to yield the same degree of deacetylation and higher molecular weight chitosan. Palpandi et al. [21] extracted chitosan from the shell and operculum of N. crepidularia through de-mineralizing and de-proteinizing the powder. Chitosan was extracted from the chitin using a deacetylation process with a degree of deacetylation of about 50%. They found that the yield of chitin and chitosan was to be 23-35% and 31-44%. *Heidari et al.* [22] produced the natural chitosan with different deacetylation degrees from shrimp shells. The different deacetylation degree of chitosan was obtained using the boiling of chitin in NaOH at 110 ℃ for 4 h. The deacetylation degrees of chitin became in ranges of 50-82.5%.

One of the world's trending industries decades ago was agriculture, and growing shrimp has been one of the agricultural sectors produced at an average rate of 16.8% per year [23, 24]. Litopenaeus vannamei are shrimp with some properties such as the ability to defend themselves against disease, fast growth at salinity and a high temperature of the Persian Gulf water, cost-effectiveness, and high demands in the market [25, 26]. The objective of the present study is to extract the valuable polymers chitin from the waste byproducts of the shrimp obtained in the Persian Gulf water. The extracted chitin will be characterized and deacetylated to the more functional chitosan. The produced chitosan was characterized using FTIR, XRD, SEM, and TGA techniques. Finally, the physicochemical property of this extracted polysaccharide from whiteleg shrimp shell was evaluated and compared with the commercial type.

EXPERIMENTAL SECTION

Materials and apparatus

Shrimp and vannamei source chitosan were purchased from a local market in Ahvaz. Commercial chitosan was purchased from Merck (Darmstadt, Germany). The morphology was investigated by scanning electron microscopy (TESCAN VEGA TS5136MM, Japan), CHNOS elemental analysis was used to measure the extracted chitosan using Perkin Elmer 2400 Series II. Gel strength analysis was measured with RV2 viscometer (Spain), Hydrochloric acid (95-97% HCl, GR > 95.0% for analysis), and NaOH (Sharlo, Spain)

Chitin extraction

In this research, chitosan was prepared from Litopenaeus vannamei shrimp shell according to the procedures described in the literature [17]. Afterward, the shells of these species were modified to remove the loose tissue and then became wet and dried at 60 ˚C for 2 hours. Moreover, the shells were grounded so that they could move through a 250 µm sieve. The final step was the demineralization and deproteinization of the shells.

Demineralization

The demineralization was conducted in dilute HCl solution. Since the minerals inside the exoskeleton of shrimp are different per species, the extracted chitin does not require a similar treatment procedure. Putting the cuttlefish aside, it was processed in 0.25 M HCl solution at surrounding temperature by a solution to solid ratio of 40 mL/g, while cuttlefish pens received 1.0 M HCl for demineralization. During the last step, the distilled water was utilized to achieve the solid in natural form.

Deproteinization

To deproteinize the chitin, 1.0 M NaOH (20 mL/g) at 70 ºC was used, and this procedure was carried out several times. During the first 24 h of deproteinization process, the medium had been become dirty and dark, thus the medium was replace with fresh solution every 2 h. After one extra day, the solution became to be finally clear, showing the complete deproteinization. Then, to achieve neutrality, the procedure of washing the solution was conducted with hot ethanol. During the purification stage, the sample was boiled in acetone, and then it was dried. The differences in the value of measured weights for the raw materials and the chitin were calculated to determine the chitin content following the acid and alkaline treatments. Then the sample was burnt at 600 ºC in a muffle furnace to obtain the Ash content of the dried chitin. The deproteinization process lasted three days. The colour of the medium had been become dark during the first 24 h, thus the medium was changed every 2 h and fresh sodium hydroxide was added. After two days, the colour gradually changed. The dark colour was not observed after the end of the third and the solution became to be finally clear, which was supposed as an index of full deproteinization.

Chitosan preparation

The Chitin taken from various species was processed with 45% NaOH at 110 ºC for 6 h, with a solution to solid ratio of 15 mL/g. Following the reaction, the washing procedure was done for the resulting material with distilled water so that the pH value was around 7. Then it was dried at 60 ºC in an oven to reach a steady weight. The suspended materials in the sodium hydroxide solution were smoothened, and the remaining chitosan on the filter was washed using distilled water until a pH value of 7 was achieved. Finally, the same drying temperature was used for the resulting chitosan for about one hour.

Chitin and chitosan specifications

Fourier Transform InfraRed (FT-IR) spectroscopy

Infrared spectra was used to measure the chitosan sample over the frequency range 4000-400 1/cm at a resolution of 4 1/cm using a model 800 Nicolet spectrometer. Before the test, the sample was in the desiccators with silica gel for about 48 h [17, 18, 20].

ThermoGravimetric Analysis (TGA)

Thermogravimetric analysis TGA was conducted *via* METTLER TOLEDO TGA/DSC instrument at a heating rate of 10 ºC/min under hydrogen atmosphere in the range of 20-700 ºC.

Differential Scanning Calorimetric (DSC)

DSC was recorded on a DSC 3100 (MAC SCIENCE) thermal analyzer under N_2 atmosphere at a heating rate of 108 ºC/min and a cooling rate of 58 ºC/min for a sample weighing 5 mg.

Scanning electron microscopy and Energy Dispersive X-ray Spectroscopy (SEM/EDS)

The characteristic of SEM was defined using a tool called TESCAN VEGA in an environment devoid of matter space. Before doing the analysis, the chitosan was put onto adhesive Al, and it was attached to discs that had Au on the surface. This treatment provided the visualization for surface and cross-section. For cross-section observations, films were cryo-fractured by immersion of the samples in liquid nitrogen. The accelerating voltage of 10 kV was used to test all samples [18, 27]. Energy dispersive X-ray spectroscope (Oxford EDS detector) was used to identify the elemental composition of the samples.

X-Ray Diffraction (XRD)

X-ray diffraction analysis (XRD) was used to evaluate the crystallinity of the extracted chitin and chitosan. The X-Ray Diffraction (XRD) patterns were recorded with Cu Kα-radiation in the 2θ range of 10 to 80° on a Rigaku D max 2000 machine at 40 kV, 30 mA. The relative crystallinity of samples was computed by dividing the area of the crystalline peaks into the total area under the curve.

Determination of the deacetylation degree

Potentiometric titration

The Degree of Deacetylation (DD) of the sample chitosan, as one of the crucial factors, was calculated by using the potentiometric titration method as an excellent way to characterize the acid. 150 mg portion of chitosan was dissolved in 25 mL of 0.20 M HCl. In the next step, the sample was diluted to 50 mL of water with high purity, and it was titrated with 0.10 M NaOH. The used volume of NaOH solution was obtained concerning the difference between acid-base titration inflation points [28]. The following formula can be used to calculate the DD of chitosan:

$$\mathrm{DD\%} = (0.0016\Delta \mathrm{V} \cdot \mathrm{C_{NaOH}})/(0.0994\mathrm{M_{Chitosan}}) \quad (1)$$

Where ΔV was the volume of NaOH and $M_{Chitosan}$ was the weight of chitosan.

Elemental analysis

Since it was essential to obtain the extents of C and N in chitosan, a useful machine for elemental analysis, called A CHNOS. For this purpose, the sample was heated until it reached the temperature of 1000 ºC. Next, it was time to insert about 2 mg of chitosan inside a silver capsule to be thrown into the CHNOS-932 furnace. The carbon weight percentage was calculated based on the infrared detection feature on this instrument. Moreover, that value of nitrogen was obtained using thermal conductivity detection. The DD of chitosan was determined by the formula below [29]:

$$\mathrm{DD\%} = 100\left(\frac{\mathrm{C}}{\mathrm{N}} - 5.145\right)/1.716 \quad (2)$$

Viscosity and Mark–Houwink parameters determination

The study adopted *Lai* and *Yang* [30] to evaluate the parameters, namely the intrinsic viscosity and Mark–Houwink related to the polysaccharides. Next, to determine the time allocated for the passage time of the fraction, an Ubbelohde glass capillary viscometer was utilized. This tool was used for each mixed fraction dissolved in a PBS (phosphate buffer solution) in acetic acid equal to a pH of 4.3 (0.2 M acetic acid and 0.3 M sodium acetate) that passed through capillary in the constant temperature at 25 ± 0.5 °C. The process of centrifugation (3000 rpm, 10 min) was performed on the whole studied samples, and they were filtered through a 0.45 µm filter membrane before starting the measurement process. Moreover, the difference of the passage was controlled under ± 0.1 s. The resulting data was calculated by the equation of viscosity [31, 32]. Relative viscosity (η_r) was calculated according to the following equation:

$$\eta_{\mathrm{rel}} = \eta/\eta_0 \quad (3)$$

Where η_0 represents the viscosity of solvent and η represents the viscosity of the solution at the same temperature. The viscosities obtained from the above formula were modified to a specific equation dedicated to viscosity (η_{sp}).

$$\eta_{sp} = (\eta - \eta_0)/\eta_0 = \eta_{\mathrm{rel}} - 1 \quad (4)$$

Intrinsic viscosity was defined as equation below (equation 5):

$$[\eta] = \left(2\eta_{\mathrm{sp}} - 2\ln(\eta_{\mathrm{rel}})\right)^{\frac{1}{2}}/\mathrm{C} \quad (5)$$

According to Lai and *Yang* [33], the high molecular polymers follow the Mark–Houwink model:

$$|\eta| = \mathrm{KM_V^{\alpha}} \quad (6)$$

Where M_w represents the average molecular weight, k is the Mark–Houwink constant (k = 0.0074 cm^3/g), and α (dimensionless) is the constant in each solvent polymer system (α = 0.95).

Water and fat binding capacity

The current study adopted the method provided by *Wang* and *Kinsella* [34] to measure Water Binding Capacity (WBC) and Fat Binding Capacities (FBC) of the chitosan. Since the procedure requires the absorption of water and fat, the following steps were taken. First, a centrifuge tube containing 0.5 g of chitosan was weighted. Second, about 10 mL of water or soybean oil was added to the sample and mixed for a minute to spread the sample. The ambient temperature was ideal to leave the content

Table 1: Description of IR band (1/cm) of Shrimp chitosan extracted in this study compared with Eddya et al. [36].

This study	*Eddya et al.* [36]	Description
3300	3392	Intermolecular –H bands
1555	1680	($-NH_2$) amide II
1415	1413	Angular deformation of CH_2
1075	1026	C-C and C-O stretching
-	873	Asymmetric stretching of the CO_3^{2-} ion
670	-	Out plane bending -OH vibrations
-	564	Vibration of cis C-H

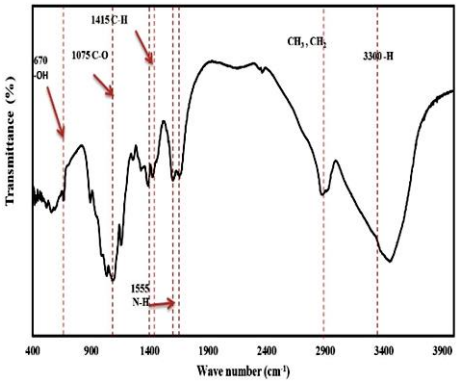

Fig. 1: FT-IR spectra of the chitosan extracted from shrimp shells.

for half an hour while shaking it for 5 seconds every 10 minutes. Then it took about 25 minutes to centrifuge it at 3500 rpm. After the supernatant was poured into another container, the seconded weighting of the tube was done. WBC and FBC were obtained based on these formulas:

WBC (%) = Water bound / sample weight * 100;
FBC (%) = Fat bound / sample weight * 100;

RESULTS AND DISCUSSION

Fourier Transform InfraRed (FT-IR) spectroscopy of chitosan

Based on the shrimp shell, the structure was achieved via a technique called Fourier Transform Infrared Spectroscopy (FTIR). The infrared spectrum of the extracted chitosan was shown in Fig. 1 and their bands' descriptions are shown in Table 1. The spectrum indicated that the peak was approximately 1555 1/cm. This value is primarily due to N–H bending of the secondary amide II bands related to –CONH– group. However, the bands existing in the region of 1380–1460 1/cm are because of the methyl groups' symmetric and asymmetric bending vibrations. According to the recent study [35], the peak at 1415 1/cm is related to the C–H bending vibrations of $–CH_2$.

Moreover, the previous studies observed that the C–O stretching of the structure was 1075 1/cm. In addition, there were some wider bands with approximately 3300 cm^{-1}, and it was related to intermolecular –H bands. The out plane bending –OH vibrations are observed at 670 1/cm. It should be noted that the strong amide II bands were not found because of the high deacetylation degree of the produced chitosan.

ThermoGravimetric Analysis (TGA)

Fig. 2 indicates the TGA curves of chitin, in that two stages are involved in the weight loss. During the first stage, the weight loss is weak in that the temperature increment is from 30 ºC to 120 ºC since the chitosan as polysaccharides has a stronger connection with the water making it prone to hydration. During the second stage, the temperature of about 300 corresponds to the thermal decomposition of chitin, the CH-Cr-M (a-Chitin). It can be decomposed at a higher temperature than the CH-Cut (b-Chitin). Therefore, it can be concluded that one a-chitin has a stable structure to the thermal decomposition compared with the b-chitin.

Scanning Electronic Microscopy (SEM)

The SEM was used to investigate the morphology of chitosan that had various enlarging features and different parts of chitosan (Fig. 3). The SEM images of the extracted chitosan show that the layers of flakes and porous were apparent in some areas while fibril structures are not hard to find. When the enlargement turned to be more prominent in some areas of chitosan, the fibril body started to break into small fragments, as in the study of *Yen et al.* [37].

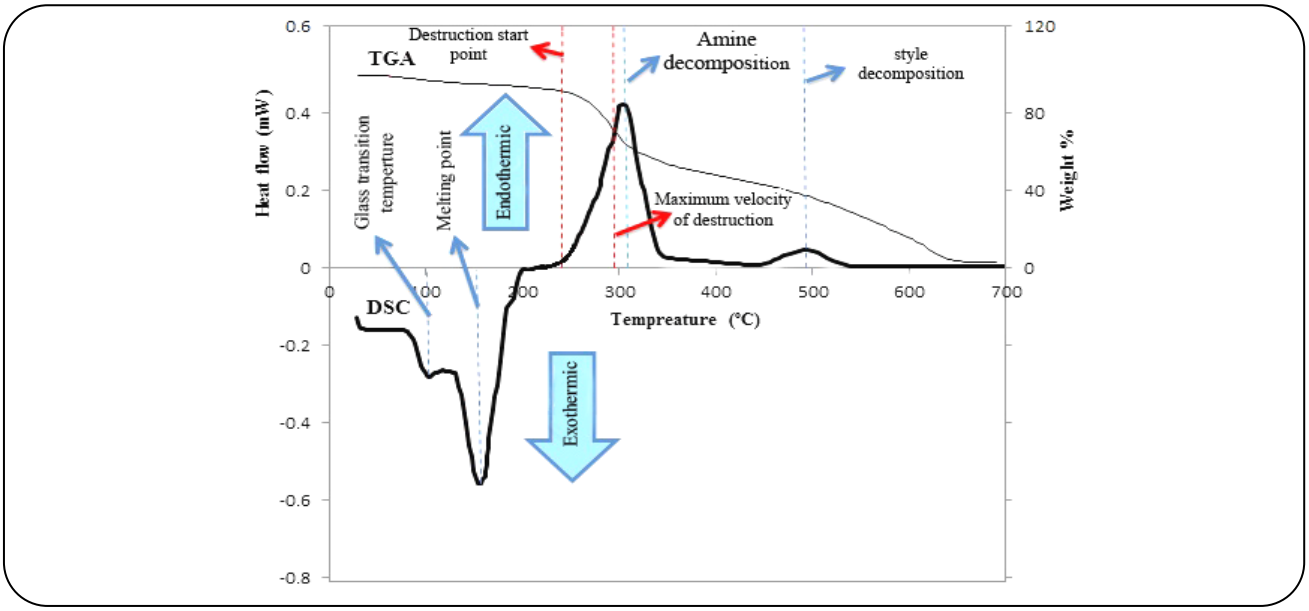

Fig. 2: TGA and DSC analysis of extracted chitosan.

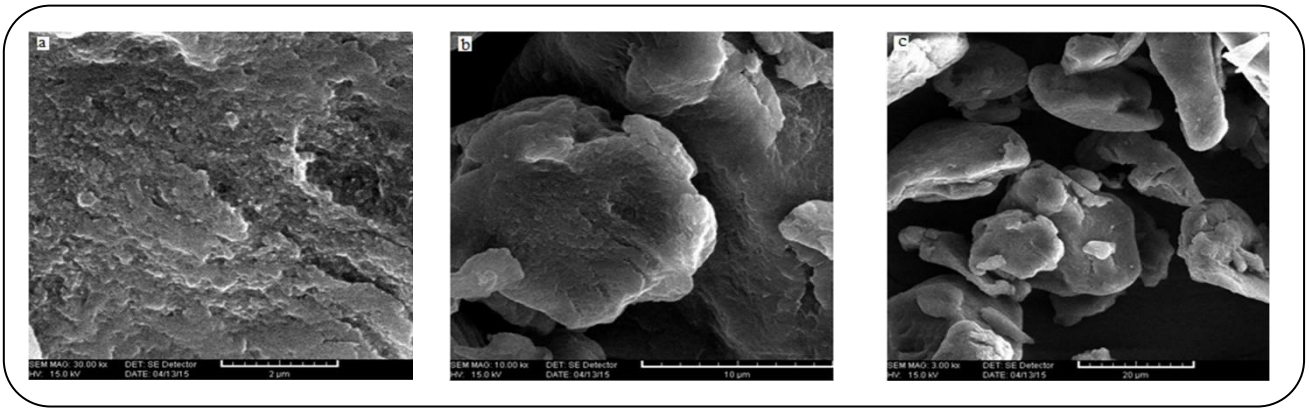

Fig. 3. SEM images of chitosan (a) 10000×, (b) 5000×, (c) 1000×

Energy Dispersive X-ray (EDX) spectroscopy

The EDS spectrum analysis for prepared Chitosan is shown in Fig. 4, and the results of each sample elemental weight and atomic composition are reported in Table 2.

It can be seen that the presence of the peaks for following element: the carbon C, the nitrogen N and the oxygen O with a different intensities related to their concentration in each chitosan samples. However, the small peaks of the phosphorus P, and the calcium Ca are appeared and it proves the ability of this method to remove all minerals from the shrimp shells.

X-Ray Diffraction (XRD)

The XRD patterns of shrimp chitin and extracted chitosan are plotted in Fig. 5. The strong reflection for both chitin and chitosan are at 2θ value of around 19.5°. The XRD analysis of chitosan from shrimp chitin displayed one more characteristic peak at 2θ values of 32° that is the characteristic region of calcium and phosphate groups. The results show that concentrated sodium hydroxide is the favorite way to extract chitosan from shrimp chitin, and it can remove all minerals.

Deacetylation degree of extracted chitosan

The result of element analysis for the extracted chitosan from shrimp shell and vannamei showed that the chitosan consists of 44.64 and 6.78 w% carbon and nitrogen, respectively, and the values are comparable with *Sarwar et al.* [38]. According to these results and using Eq. (2), the degree of deacetylation to chitosan was83%.

Table 2: Elementary EDS analysis for extracted Chitosan.

Element	Weight%	Atomic%
C	41.6	50.8
N	5.13	5.37
O	43.4	39.7
P	4.82	2.28
Ca	5.05	1.85

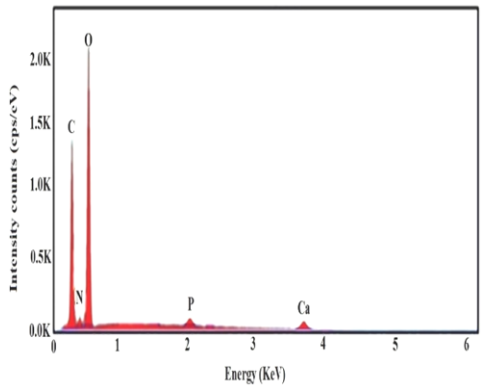

Fig. 4: EDS spectrum analysis for extracted chitosan.

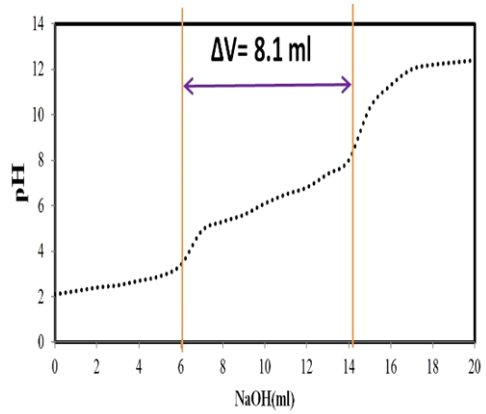

Fig. 6. Result of element analysis for extracted chitosan

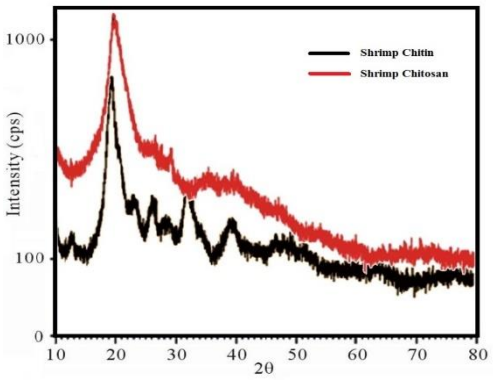

Fig. 5: X-ray diffraction patterns of Shrimp chitin and extracted chitosan.

To determine the degree of deacetylation by Potentiometric, titration method and chitosan solution was titrated with sodium hydroxide solution, and the pH was measured.

As shown in Fig. 6, the chart has two equivalence bending points. The first and second bending points are the equivalence points from the additional titration of hydrochloric acid in the solution and the protonated chitosan titration. The amount of the required acid for the chitosan amine groups was determined by the differences in volume (ΔV) of sodium hydroxide used between the two bending points. The deacetylation of chitosan was obtained as 86 percent considering the amount of ΔV in the Fig. (8.1 mL) and Eq. (1).

Viscosity and molecular weight of chitosan

In this research due to an intrinsic viscosity of three solution (0.5, 1, 5 g/L) chitosan in the solvent sodium acetate, 3.0 M, and acetic acid, 2.0 M, the numerical value of 253.71 kDa was reported as average molecular weight of the extracted chitosan. Viscosity and molecular weight of chitosan are shown in Table 3.

Chitosan water and fat binding capacity

Water Binding Capacity (WBC) and Fat Binding Capacity (FBC) extracted from shrimp shell were measured, and they were about 673.58 and 491.32, respectively. The physicochemical property of extracted chitosan was compared to commercial chitosan, as shown in Table 4.

According to the resulting data, the extracted chitosan (2.20 kDa) had a lower molecular weight than the commercial chitosan. The difference in the molecular weight is due to the difference in the deacetylation degree

Table 3: Viscosity of chitosan solution and molecular weight of chitosan.

Solution concentration C (g/L)	Solution viscosity Ƞ (cP)	Buffer viscosity $Ƞ_0$ (cP)	Relative viscosity $Ƞ_{rel}$	Special viscosity $Ƞ_{sp}$	intrinsic viscosity [ɳ] (mL/g)	Molecular weight M_W (kDa)
0.5	1.657	1.042	1.5902	0.5902	1005.34	253.71

Table 4: Water and fat binding capacity for chitosan.

Property	Extracted chitosan	Commercial chitosan	Test/sig.
Deacetylation degree (%)	86 ± 0.02	82 ±0.01	0.036
Molecular weight (kDa)	253.071 ± 1.62	480 ± 5.00	0.00
WBC(%)	673.58 ± 1.89	492.67 ± 1.90	0.00
FBC(%)	491.32 ± 3.49	383.04 ±1.42	0.00

and the various sources of the chitosan. Furthermore, some effective factors impact the molecular weight of chitosan, namely, the high temperature, concentration of alkali, reaction time, previous treatment of the chitin, particle size, chitin concentration, dissolved oxygen concentration, and shear stress level [22, 39-41].

CONCLUSIONS

The current study aimed to investigate the physicochemical properties of chitosan extracted from whiteleg shrimp shell (Litopenaeus vannamei) by the deacetylation of chitin, which is carried out using 45% NaOH at 110 °C for 6 h. It can be concluded that the extracted chitosan is pure with a significant Degree of Deacetylation (DD) 86%. Also, it provides a different degree of crystallinity towards biodegradable sutures. It offers more heat resistance until 317 °C for starting thermal degradation, than commercial chitosan, which starts degradation at 295 °C. Moreover, the higher water binding capacity and fat binding capacity of chitosan from shrimp, 673.58 and 491.32, are shown a good improvement compared with the commercial type. Finally, the results indicated that more chitosan could be found in shrimp shells compared to commercial chitosan.

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