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Research paper

Isolation and Identification of Chitin and Chitosan Horseshoe Crab Shrimp Shell Using Infrared Spectroscopy

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Abstract

Isolation and identification of chitin and chitosan Horseshoe Crab Shrimp Shell has been carried out. There were several reports about chitin isolation on crabs and shrimp shells with different reagent concentrations due to the shell hardness difference. Mechanically, the Horseshoe Crab Shrimp Shell was harder than the two. The characterization of chitin and chitosan is implemented through Infra Red Spectroscopy. Isolation method consists of three steps. First, it was deproteination with various NaOH concentrations of 3.5%, 4.5%, 5.5%, 6.5% and 7.5%. The second was demineralization with various HCl concentrations of 1 M, 1.5 M, 2 M, 2.5 M, and 3 M. The two steps would produce chitin. The third step was deacetylation with a NaOH concentration of 50% to produce chitosan. The results showed that the optimum concentration of NaOH in deproteination step is 4.5% with the optimum concentration of released protein by 601 ppm. In addition, the optimum concentration of HCl in the demineralization step was 2.5 M with remaining ash in the chitin of 0,972%. Moreover, deacetylation degree (D%) of chitin and chitosan were 45.4% and 50.5% respectively. Infrared spectroscopy showed that secondary NH amide stretching of chitin was vanished on chitosan. Meanwhile, secondary C=O amide stretching shifted in chitin and disappeared on chitosan due to deasetylation.

Keywords: *Horseshoe Crab, Chitin and chitosan.*

1. Introduction

Mimi (local name at Madura island, Indonesia) as known as horseshoe crab (Tachypleus gigas) is an animal that has legs on the abdomen extending like a foot on the crab. Mimi taxonomy, as described in World Conservation Monitoring Centre (1996), is Arthropoda phylum, Merostomata class, Xiphosura order, Limulidae family. Most types in this order is already extinct.

Mimi is kosher seafood but it is not suitable for food consumption because it contains toxic. According to local people, there were several toxication occurrences. An epidemic of poisoning caused by toxic horseshoe's egg in Thailand in 1995 was reported (Kanchanapongkul and Krittayapoositpot, 1995). There was also a study concerning toxicity degree of horseshoe crab in Cambodia (Ngy, et al., 2007). This does not mean mimi has no benefit because all of God's creation that has specific purpose and benefits as it is written in the Qur'an, surah Ali Imran (191) which means:

"Who remember Allah while standing or sitting or [lying] on their sides and give thought to the creation of the heavens and the earth, [saying], "Our Lord, You did not create this aimlessly; exalted are You [above such a thing]; then protect us from the punishment of the Fire".

Many usages of horseshoe crab have been developed extensively, such as its antibacterial activity (Powers, et al., 2005 and Zhang, et al., 2000) including chitin and chitosan production from the shells.

1.1. Isolation of chitin and chitosan

Isolation of chitin was prepared in two steps. The initial step began with the separation of proteins with an alkaline solution called deproteination step. Deproteination aims to separate proteins in the shell material. The next step was demineralization to remove minerals from shell material.

The method of chitin/chitosan preparation can vary with sources to meet compositional differences. The physical and chemical characteristics of chitin and chitosan differ with species and preparation methods. Some studies have clearly demonstrated that specific properties of these products, i.e., molecular weight and degree of deacetylation, vary with different process conditions such as type and strength of acid and alkali solutions, reaction temperature and atmospheric conditions (No and Meyers, 1995).

Isolation of chitin from crab shells use 2M HCl as demineralization reagent and 1M NaOH as deproteination reagent (Marganof, 2003). There was also an isolation of chitin from shrimp shells using 1M HCl and NaOH 3.5% (Amaria and Sari, 2005). The physical differ-

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ences viewed in terms of hardness shell between Mimi shells, crab shells and shrimp shells are the crab shells is harder than the shrimp shells, while shells of mimi is harder than the crab shells. Isolating chitin by varying the concentration of reagent will provide information on the optimum concentration of the reagent usage.

1.2. Qualitative identification of infrared spectrophotometer

Identification of chitin and chitosan can be performed using Infrared spectrophotometry. Infrared spectrophotometry is used to determine the structure, particularly the functional groups of organic compounds. Infrared spectrophotometry provides maximum peaks as clear as the minimum peak. Spectrum absorption is a graph relating wave number on X axis and percentage of transmittance (T) on Y axis.

1.3. Quantitative methods of infrared spectrophotometer

Quantitative methods using infrared spectrophotometry was performed by calculating % transmittance or absorbance. The degree of deacetylation (D%) of infrared spectra on chitin and chitosan is calculated by comparing the absorbance of wave number for amide group-NH (1650-1500) cm-1 (A1655) and absorbance of wave number for a primary amine group (3500-3200) cm-1 (A3450), with absorbance value of 1.33 on a perfect deacetylation process (Basttaman, 1989).

$$D\ \% = 1 - \left[\frac{A_{1655}}{A_{3450}} X \frac{1}{1{,}33} \right]$$

2. Material and Method

2.1. Chitin production

2.1.1. Deproteination.

Shells of mimi were washed with water and dried under the sun, then they were washed in hot water twice, and boiled for 10 minutes, then those were drained and dried under the sun again. The dried materials were milled to be a powder size of 60 mesh. The powder was mixed with NaOH 3.5% (w/v) (in ratio of 6:1 NaOH to shell powder by weight), followed by stirring with a magnetic stirrer for 1 hour, left briefly, and then heated at 90°C for 1 hour. The mixture was filtered, and the residues were cooled to obtain some solid residues. The solid residues were washed with distilled water until reaching neutral pH condition and dried at 80°C for 24 hours or dried under the sun until they were dried thoroughly, then those were weighed for further analysis. The filtrate was saved for N-Total test. The same procedure was carried out for various concentrations of NaOH i.e. 4.5%; 5.5%; 6.5% and 7.5%. N-Total test employed Nessler Method using Spectronic 20 Milton Roy.

2.1.2. Demineralization.

20 grams of mimi shell powder resulted from optimum deproteination condition were mixed with 1 M hydrochloric acid solution in a ratio of 10: 1 (HCl: mimi shell powder by weight). The mixture was stirred using a magnetic stirrer about 1 hour, left briefly, and then heated at 90°C for 1 hour and filtered. The solid residues were washed with distilled water until reaching neutral pH condition, then dried at 80°C for 24 hours or dried under the sun until they were dried thoroughly, and then weighed for further analysis of ash. The same procedure was performed for the hydrochloric acid concentration of 1.5 M; 2 M; 2.5 M; and 3 M.

2.2. Formation of chitosan by chitin deacetylation

Chitosan was made by adding NaOH 50% (w/v) in a ratio of 20: 1 (NaOH: chitin by weight). The mixture was stirred with a magnetic stirrer for 1 hour and left to stand for 30 minutes, then heated for about 90 minutes at 140oC. The mixture was then filtered to obtain some solid residues. The solid residues were washed with distilled water until reaching neutral pH condition, and then dried in an oven at 70°C for 24 hours or sun dried. The final form of chitosan can be either a powder or flakes.

2.3. Functional group identification through infrared spectrofotometry

Functional group identification of Mimi shells, Chitin and Chitosan had been done using Infrared spectrophotometry Shimadzu IR. Solid samples were prepared as 1% powder in dry KBr. These samples were placed on agate mortar to obtain a particle size of about less than 2 µm. Samples were placed onto the pellet press evenly. The pellet press was connected to a hydraulic compression pump with the power of 100 tons (kg newton) as well as a vacuum pump for 15 minutes. The good pellets formed are about 0.3 mm in thick or in transparent visual. Infrared spectrophotometry recording devices were set up with the speed of a position paper on "normal" and the expansion of "100 X" transmission. The instrument spectrum was standardized by polystyrene film first.

3. Results and Discussion

The carapace or shells of such animal is a biological multiphase nano-composite consisting of an organic matrix (crystalline chitin and non-crystalline proteins) and biominerals (calcite, phosphate). Horseshoe crab generally does not seem to contain notable amounts of crystalline minerals (Raabe, et al., 2005).

3.1. Optimum concentration of naoh in deproteination process

Isolation of chitin from the shells of mimi needs two shells filler removal steps. The first step was deproteination to remove protein from mimi shell structure using alkaline reagent. In this study, NaOH was used as alkaline reagent. Molecular form of the protein is a fiber to the type of keratin, which is the form of a secondary structure. In the form of secondary structure protein, the major interaction constituting the structure is intramolecular hydrogen bond (Poedjiadi, A., 2006). Thus, the bond that might occur between proteins and chitin is the intermolecular hydrogen bonding. In accordance with the hardness of mimi shells, at this deproteination step, some concentration variation of NaOH i.e. 3.5; 4.5%; 5.5%; 6.5% and 7.5% (w/v) were used. To identify the protein removal capacity, Nessler method was employed which is a method for determining N total content as ammonia. Amino acids are amphoteric which may be acidic or alkaline. In alkaline environment (pH> 7), amino acids will be acidic which acts as H+ donor. On the contrary, in acidic conditions (pH <7), amino acids will be alkaline which act as H+ acceptor. In the Nessler method, the deproteination filtrate was treated with concentrated H2SO4 and heated. The reaction aimed to change the nitrogen contained in the filtrate into ammonia, then reacted with Nessler reagent under alkaline conditions to produce a brownish filtrate was then analyzed by spectronic 20 at a wavelength of 410 nm. The results of treatment of each concentration of NaOH are showed at Table 1.

According to Table 1, the optimum N total content released from mimi shells was at 4.5% NaOH concentration in the value of the N content of 601 ppm. In accordance to the graph, it appears that the value of N content dropped after treatment with NaOH above 4.5%. Judging from the type of bonds between proteins and chitin that is intermolecular bond that has a binding energy lower than energy of covalent bond found in the inner structure of chitin, the decreasing of N content at concentration of NaOH above 4.5% treatment apparently was caused by the no longer break of intermolecular hydrogen bond between chitin and protein but breaking of acetyl group (No and Meyers, 1995). The following is deproteination mechanism proposed in this study as described in Figure 1.

Table 1: Protein degree as nitrogen content of shells deproteination process

NaOH concentration (%)	Absorbance	N content (ppm)
3,5	0,389	572
4,5	0,409	601
5,5	0,342	503
6,5	0,201	295
7,5	0,119	175

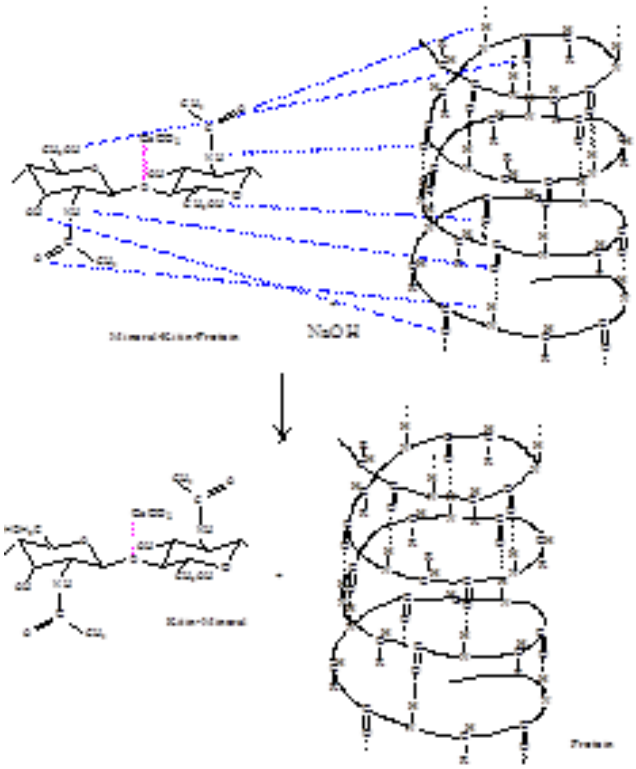


Fig 1: Deproteination mechanism of chitin.
----- = Intramolecular hydrogen bond
----- = Intermolecular hydrogen bond
----- = Intramolecular bond

This was confirmed by the shift of NH secondary amide (-CO-NH-) and C=O secondary amide infrared spectra absorption peak between mimi shells and chitin produced. NH secondary amide (-CO-NH-) peak of mimi shells located at 3277 cm-1 was shifted to 3268 cm-1 at corresponding chitin, while C=O secondary amide peak of mimi shells at 1638 cm-1 was shifted to 1661 cm-1 at corresponding chitin. This shifting is the result of deproteination process that caused the loss of intermolecular hydrogen bonds between chitin and proteins. The breaking of those bonds changed the bond length of the two N-H and C=O bonds, causing a wave number shifting of infrared peaks correspondingly.

3.2. Optimum concentration of hcl in the demineralization process.

Demineralization had used HCl in several concentration variations. The variety was used to determine the optimum concentrations of HCl for demineralization. The method used to determine the amount of mineral released in demineralization process was ash content test. The demineralized sample with lowest value of ash content has an optimum HCl concentration treatment, while the data obtained is shown at Table 2.

Table 2: Ash levels of demineralized mimi shells in HCl variation concentration

HCl Concentration (M)	Ash level (%)
1	4,407
1,5	2,968
2	1,243
2,5	0,821
3	1,616

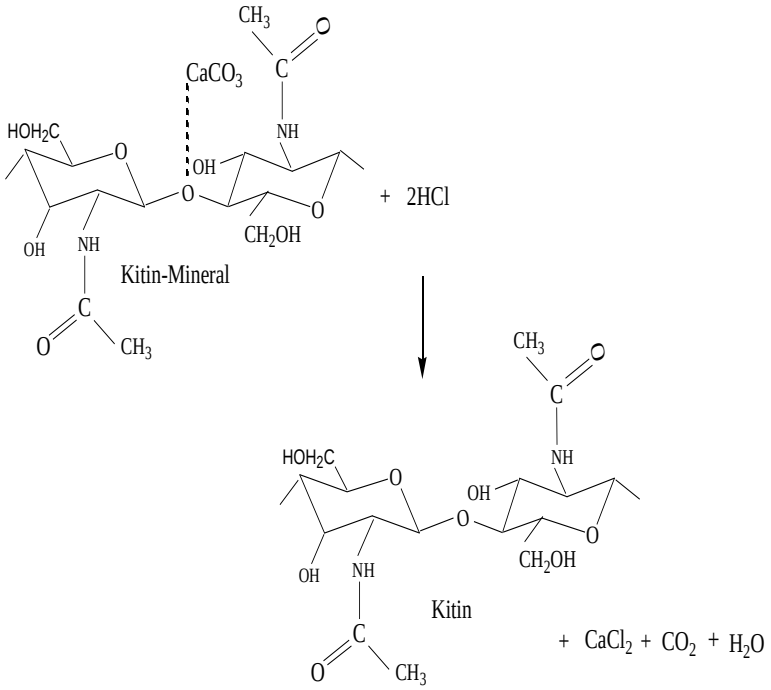


Fig 2: Demineralization mechanism
----- = Intermolecular interaction

Table 2 provides information that the optimum HCl concentration for demineralization process was 2.5 M with ash content of 0.821%. Sea animal's shells like fish, shrimp and crab naturally bind minerals such as Si, Ca, I, and Br [12], while the main mineral is CaCO3 (No and Meyers, 1995). Mineral shells are diluted by HCl treatment in deproteination process. As confirmed by infrared spectra, there were differences in the infrared spectra peak between mimi shells and corresponding chitin to be suspected as result of mineral released. There was shifting peak of asymmetric ether aliphatic C-O stretching from 1155.28 cm-1 at mimi shells into 1157.21 cm-1 at corresponding chitin spectra. In addition, there was symmetric ether aliphatic C-O stretching shifting peak from 1033 cm-1 at mimi shells into 1026 cm-1 at corresponding chitin. The demineralization mechanism is described as Figure 2.

3.3. Infrared characterization of functional groups of mimi shells, chitin and chitosan.

Infrared spectra of functional group area of mimi shells (Fig. 3(a)) showed that medium less-broad absorption band in the area 3442.70 cm-1 is due to N-H vibration with intermolecular O-H vibration. Moderate absorption band at 3277.80 cm-1 is overtone peak due to vibration of secondary amide N-H stretching. Moderate absorption band at 2958.60 cm-1 is vibration spectra of asymmetric C-H of CH3 group, while the sharp absorption at 2920.03 cm-1 and 2851.56 cm-1 is CH stretch vibration of alkanes.

Further inspection at finger print area showed that there was sharp absorption band at 1636.49 cm-1 due to vibration of secondary amide C=O stretching. Weak absorption band at 1540.05 cm-1 is vibration from secondary amine N-H stretching, and an absorption band at 1458.08 cm-1 is due to CO3 group vibration of CaCO3. C-H vibration of secondary alcohol gives a weak absorption at 1401.19 cm-1. Moderate absorption band at 1155.28 cm-1 is an asymmetric ether aliphatic C-O stretching vibration. Absorption band at 1110.92 cm-1 is due to vibration of secondary alcohol C-O, while the absorption band at 1073.31 cm-1 was derived from the vibrations of primary alcohol C-O. Moderate absorption band at 700.11 cm-1 and 669.25 cm-1 were the result of deformation vibration of primary amide NH2, while the medium absorption bands at 574.75 cm-1 and 471.56 cm-1 were the result of the bending vibration of aliphatic primary amide NCO.

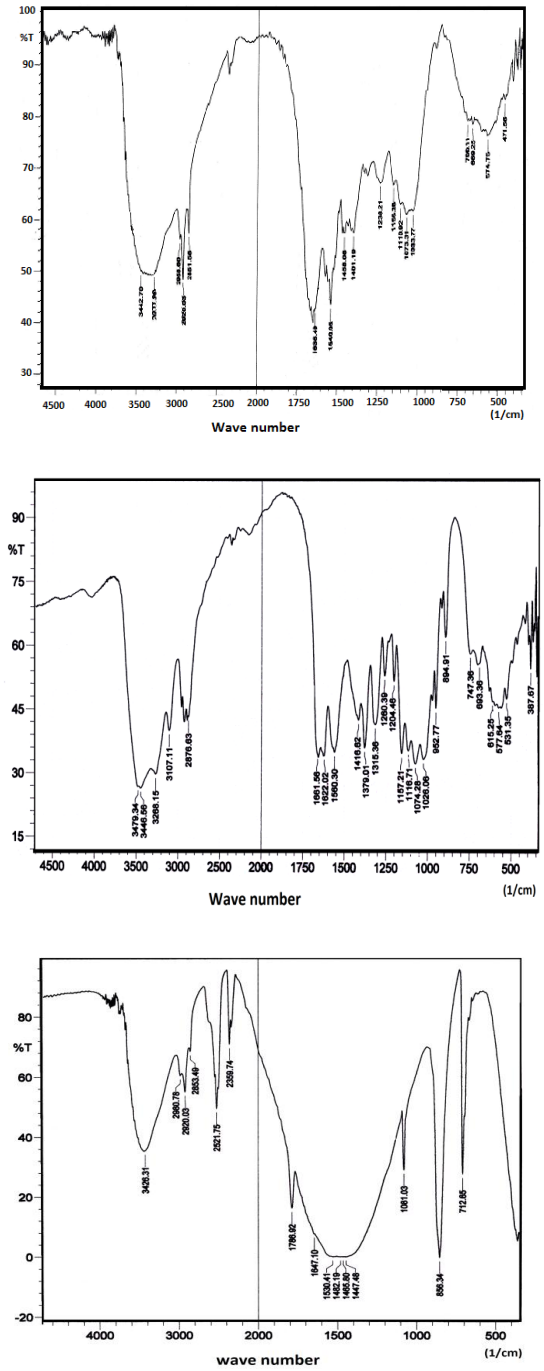


Fig 3: Infrared spectra of: (a) Mimi shells, (b) chitin and (c) chitosan.

Infrared absorption difference between chitin (Fig. 3(b)) and mimi shells are as follows: The narrower less-broad peak at 3442.70 cm-1 in chitin compared to mimi shells peak means a decrease in the number of intermolecular O-H bind to N-H. This is because of O-H intermolecular vibration number was getting down due to the breaking of intermolecular O-H bond between protein and chitin structure in deproteination process. There was shifting vibration absorption range of secondary amide N-H at wave number 3277.80 cm-1 in mimi shells spectra into 3368.15 cm-1 in chitin due to the breaking of intermolecular hydrogen bonds between protein and chitin molecules caused stiffer secondary amide N-H bond resulting the wave number shifted to the left. Shifting peak of secondary amide C=O at 1636.49 cm-1 in mimi shells into 1661.56 cm-1 in chitin was due to the breaking of intermolecular hydrogen bonding, consequently C=O bond was getting stiffed so that the wave number shifted to the left. The emergence of the primary amide of N-H vibrational wave number at 1622.02 cm-1 led to the formation of primary amide groups due to partially deasetylation of cithin. The shift of the absorption range of the asymmetric ether aliphatic C-O stretching at 1155.28 cm-1 into 1157.21 cm-1 was a result of the demineralization with HCl solution.

Chitosan is a chitin with further deasetylation. The difference between the infrared spectra of chitin and chitosan were as follows: very narrow peaks of N-H absorption at wave number 3426.31 cm-1 was caused by OH intermolecular binds to N-H which was mostly vanished leaving primary N-H absorption. Disappearing vibration absorption range of secondary amide N-H at wave number 3277.80 cm-1 in mimi shells spectra which was shifted into 3368.15 cm-1 in chitin showed the secondary amide deasetylation to produce primary amine. The emergence of a primary amine N-H stretch at wave number 2521.75 cm-1, 2359.74 cm-1 and 1786.92 cm-1, the loss of sec-

ondary amide N-H absorption at wavenumber 3268.15 cm-1 and 3107.11 cm-1, as well as loss absorption for vibration of C = O of secondary amide at wave number 1661.56 cm-1 and 1560.30 cm-1 as characteristic bands of N-acetylation were the result of a process of deacetylation. These fact were supported by the peak disappearance at 1320 cm-1 in chitosan as the characteristic of -OH, -NH2, and -CO groups of acetylation, while they were conserved at chitin spectra. The loss of CH3 symmetric of CH absorption at 1379.01 cm-1 was due to the process of deacetylation. According to the change of the group identified, it can be concluded that the powder obtained was chitosan.

3.4. The deacetylation degree of chitin and chitosan

The calculation of the deacetylation degree from infrared spectra of chitin and chitosan was done by comparing the absorbance at marker of wave number of -NHCO amide groups (1650 cm-1-1500 cm-1) to the absorbance at wave number for a primary amine group -NH2 (3500 cm-1 -3200 cm-1), respectively. In a complete deacetylation of chitin, absorbance value (A) for vibration amide group was 1.33 (Basttaman, 1989). The deacetylation degree of chitin calculated was 45.4% and the deacetylation degree of chitosan was 50.5%. Deacetylation degree below 50% was characterized as chitin, and deacetylation degree above 50% was characterized as chitosan (Fitri, 2005). Deacetylation degree of the chitosan isolated from mimi shell was 45.4% and 50.5% for chitin and chitosan, respectively.

4. Conclusion

The chitosan was isolated from mimi shells through several steps i.e. deproteination, demineralization and deacetylation. The optimum concentration of NaOH solution used in the deproteination process was 4.5% with a released protein concentration of 601 ppm. The optimum concentration of HCl solution used in demineralization step was 2.5 M with ash content remaining in the chitin of 0.821%. The differences between the infrared spectra of mimi shells, chitin and chitosan shifted secondary amide N-H stretching vibration absorption on chitin and lost the accordance absorption on chitosan, shifting C=O secondary amide vibration absorption on chitin and lost the accordance absorption on chitosan as the result of the deacetylation process. Deacetylation degree of chitin from the mimi shells obtained was 45.4% and 50.5% for chitosan.

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