Synthesis and Characterization of Chitosan Nanoparticles from the Shells of crabs (*Portunus pelagicus*) and Application as Antimicrobial

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Abstract. Chitosan was modified into nanoparticle chitosan in order to increase its absorptivity and solubility in water so that it can be applied in various fields. The objective of this research was to produce nanometer-sized chitosan, to characterize the functional groups and the particle size of chitosan as well as to test its antimicrobial activity. Chitosan nanoparticles were synthesized through ionic gelation method by reacting chitosan with tripolyphosphate ions through ionic cross-linking reaction. The functional groups of chitosan were determined from its FTIR spectrum, the particle size was obtained from its X-ray diffractogram and particle size analysis (PSA). Chitosan nanoparticles with the smallest size based on XRD were obtained at a concentration of 0.1% chitosan (CNPs 1) and chitosan 0.2% (CNPs 2) with the addition of 0.1 % tripolyphosphate ion. The sizes of chitosan nanoparticles, CNPs 1 and CNPs 2, obtained from the analysis of PSA were 224.68 and 204.32 nm, respectively. Polydisperse index value (pdi) of chitosan nanoparticles produced were 0.226 and 0.261 (pdi <0.5), respectively. The results indicate that chitosan nanoparticles synthesized have homogeneous particle size distributions. The test results against four types of pathogens showed that CNPs 2 had inhibitory activity against the three types of microbes, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Malassezia furfur*, but it was not active against the fungus of *Candida albicans*.

Keywords: Shells of crabs, Chitosan nanoparticles, Antimicrobials

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Introduction

Chitosan is a polymer-D-glucosamine composed of more than 5000 units of glucosamine with a molecular weight of higher than one million Dalton. It is a second dietary fiber after cellulose that can be eaten [1]. Chitosan is a product of deacetylation ion of chitinin crusta sea shells. One type crusta sea which has a high content of chitin in its shells and exist abundantly is Portunus pelagicus (small crab). The content of chitin or chitosan in crab shells could reaches about 22.66% [2].

Chitosan is hydrophilic; therefore, it can be used to improve the solubility of the drug in water. Chitosan has a positive charge that binds to the negative charge of a compound, so it can easily passes through the membrane. Chitosan is non-toxic, biocompatible, biodegradable, and bioadhesive. It is easily to be modified chemically and physically. Therefore, it has great potential for application in the pharmaceutical field[3] as well as an antibacterial agent[4,5].

Antibacterial mechanism occurs through two theories. The first theory is based on the presence of amine functional groups on the chitosan that can bind the bacterial cell wall; it caused leakage of intracellular constituents so that bacteria will dye. The second theory states that the mechanism begins with the damage the cell walls of bacteria, the chitosan perform intracellular binding, blocking mRNA, and inhibit protein synthesis[6].

On its utilization, chitosan still has some deficiencies due to its large molecular weight and low solubility in water that reduce its adsorption ability. Chitosan can only be dissolved in dilute organic acid. In addition, chitosan has an ability to hydrate and swell in acidic conditions[7]. Therefore, modifications are required to improve these limitations, so the use of chitosan that usually occurs in the acid environment can be changed into that in alkaline conditions.

Modification of chitosan has been widely carried out to change its chemical to increase the degree of deacetylation, or physically by changing the macro particle size in to nanoparticle size. Nanoparticles are granular or solid particles with the size range of 10–1000 nm[8]. Chitosan nanoparticles have particle sizes of 100-400 nm[9].

The simple and relatively easy method used in the preparation of chitosan nanoparticles is via ionic gelation method, in which the chitosan nanoparticle formats on mechanism is based on lectrostatic interactions between the cationicamine group(-NH₃⁺) on chitosan and cluster anions in ion tripolyphosphate(TPP) [9]. According to Yong mei and Yumin(2003), nanoparticle formation only occurs at a certain concentration of chitosan and TPP[10].

Chitosan nanoparticles have better ability to be used as adsorbent and antibacterial as well as antifungal compared to chitosan with a regular size[11]. Therefore, chitosan nanoparticle researches related to the synthesis, characterization and application as antimicrobial still need to be done. This research aimed to determine the smallest size of nanoparticles of chitosan that can be obtained from shell crab and to study the ability of nanoparticle chitosan as an antimicrobial.
Material and Methods

Materials

Materials used in this study were powder of crab (Portunus pelagicus) shells, NaOH pa (Merck), HCl pa (Merck), 0.5% NaOCl, sodium tripolyphosphate (Sigma), glacial acetic acid (Merck), tween80 (technical), 96% ethanol (Merck), Muller Hinton Agar (MHA) medium, a solution Mc. Farland, paper disk, spiritus, Whatman filter paper no. 40, deionized water and distilled water. Microbial test consists of two types of Staphylococcus aureus (gram positive) and Pseudomonas aeruginosa (gram negative) and two types of fungus, Malassezia furfur and Candida albicans.

The instruments used in this study were scale (OHAUS-Triple Beam Balance), analytical balance (Adventure ProAV264C), oven (Genlab LTD, Type: 2750C), Multiple heating magnetic stirrer (Velp Scientifica, AM4), magnetic stirrer (Cole-Parmer), spray dryer (LabPlant, SD-Basic), Enkas Büchner filter, petri dishes, oseneedles, glass speader, incubator (Memmerth 40050-IP 20), thermometer, tweezers, vacuum pump, pH meter (Horiba F-52), Fourier transformation Infrared instrument (Shimadzu Prestige-21 FTIR Spectroscopy), X-Ray diffraction instrument (Shimadzu XRD-7000), Particle Size Analyzer instrument (Shimadzu SALD-7101), as well as tools glass (Pyrex, Japan) which is commonly used.

Isolation of chitosan from crab shells (Portunus pelagicus)

Samples of crab shell powder (80-100 mesh) were processed into chitin through 3 stages, namely: (1) demineralization process conducted twice using 1.5 M HCl solution with the volume ratio of 1:10 for 1 hour at a temperature of 80 °C, (2) decolonization process using 0.5% NaOCl solution with the volume ratio of 1:10 for 1 hour at 80 °C, and (3) deproteinnization process using a strong alkaline solution of 5% NaOH with the volume ratio of 1:10 for 1 hour at a temperature of 80 °C. Chitosan was obtained by deacetylation of chitin using 50% NaOH solution at a temperature of 80-90 °C for 1.5 hours. The water, ash, N-total content in chitin and chitosan was then analyzed. The deacetylation degree (DD) of chitosan was determined by IR spectroscopy using a base line method proposed by Domzsy and Robert [12].

Synthesis of chitosan nanoparticles.

Ionic gelation of chitosan nanoparticles was synthesized by reacting chitosan solution with tripolyphosphate ions at a room temperature and stirring with a speed of 1500 rpm. Chitosan solutions at various concentrat ions (0.1; 0.2; 0.3; 0.4 and 0.5% (w/v)) were prepared by dissolving chitosan in a solution of acetic acid 2% (v/v). Triopolyphosphate solution with a concentrat ion of 0.1% (w/v) was prepared by dissolving sodium tripolyphosphate in deionized water.

To reduce the size of chitosan, 50 mL chitosan solution at various concentrat ions was stirred using a magnetic stirrer, at a speed of 1500 rpm for 60 minutes. Solution of emulsifier (0.2% Tween 80) as many as 25 mL was added and allowed to rotate for 30 minutes. After that, 10 mL of 0.1%
tripolyphosphate was added to maintain the stability of the particle size. The mixture was stirred for 30 minutes. The solution of nanoparticle chitosan was then dried with a spray dryer. Chitosan nanoparticles synthesized were characterized using FTIR spectroscopy, X-ray diffraction (XRD), and particle size analysis (PSA).

**Antimicrobial activity of chitosan nanoparticles with a paper disc diffusion method.**

Paper disc diffusion method is based on the ability of the compound tested antibacterial and antifungal in vitro. Test solution of chitosan nanoparticles with different concentrations (0.1%, 0.2%, 0.3%, 0.4% and 0.5%) was prepared by dissolving samples of nanoparticles in the distillate water sterile.

An appropriate amount of microbial cultures was grown on solid medium Muller Hinton Agar (MHA) in a petri dish and leveled using a glass speeder to dry on the test evenly distributed. Paper discs with a diameter of 5.5 mm moistened with a solution of chitosan nanoparticles at various concentrations, and then placed one of each petri dish containing solid media culture microbes. Then the petri dish was incubated in an incubator at 37 °C for 1x24 hours. The antimicrobial activity was shown by the emergence of clear zone around the paper disc. A positive control, 30 g of antibiotics chloram phenicol (for bacteria) and 20 mg/mL of nystatin (for fungi) were used for comparison. Diameter of inhibition was measured using amillimeters. Then the area of inhibition is calculated at each test microbe at each concentration.

**Results and Discussion**

**Isolation of Chitosan from shell crabs.**

As many as 15.5 grams of chitosan compound is obtained from 200 grams of crab shell. Chitosan has been isolated as a yellowish powder material and dissolved in 2% acetic acid at pH 4.0 to form a clear solution in tangible gel. This is consistent with a research of Sugita (2009), which states that chitosan is soluble in the organic acid/mineral dilute at a pH of less than 6.5. Good solvents for chitosan were formic acid, acetic acid, and glutamic acid. The solubility of chitosan was the best in 2% acetic acid solution[13].

Comparative characteristics of chitosan isolated with a standard of PROTAN biopolymers and commercial standards can be seen in Table 1.

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**Table 1. Characterization of chitosan from waste shell crabs (Portunus pelagicus)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chitosan Standard*</th>
<th>Chitosan Isolation</th>
<th>Chitosan Standard**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle form</td>
<td>flakes-powder</td>
<td>Powder</td>
<td>Flakes</td>
</tr>
<tr>
<td>Water content</td>
<td>≤ 10,0%</td>
<td>6,20%</td>
<td>&lt; 10%</td>
</tr>
<tr>
<td>Ash</td>
<td>≤ 2%</td>
<td>0,31%</td>
<td>&lt; 1%</td>
</tr>
<tr>
<td>Content of N-total</td>
<td>≤ 5%</td>
<td>5,67%</td>
<td>7 - 8,4%</td>
</tr>
</tbody>
</table>
Table 1 shows that the characteristics of chitosan obtained do not vary much with the standards issued by PROTAN biopolymers. Mineral content of resulted chitosan is 0.31% which is in accordance with the standard mineral content of ≤2%. Low levels of minerals indicate that demineralization process takes place effectively. The water content in chitosan obtained is 6.20% which fulfill the standard water content (≤10%). The water content was still high due to the drying process which was not perfect and the hygroscopic property of chitosan. The total N content of isolated chitosan is 5.67%. The value is not very different with the value of standard issued by PROTAN Biopolymer. The properties of isolated chitosan are also similar to that of the commercial chitosan. Therefore, the isolated chitosan can be commercialized. The FTIR spectrum of chitosan nanoparticles can be seen in Figure 1.

**Figure 1.** FTIR spectrum of Chitosan obtained from shell crabs (*Portunus pelagicus*)

An absorption band data wave number of 3446.79 cm\(^{-1}\) is a vibrational absorption band for-OH overlapped with vibrational absorption of-NH. Absorption band at wave number 2883.58 cm\(^{-1}\) is the C-H stretch vibration in aliphatic-CH\(_2\)-reinforced by the -CH\(_2\)-bend vibration absorption at a wave number of 1421.54 cm\(^{-1}\). Absorption band also appear at wave numbers of 1653.00 and 1597.06 cm\(^{-1}\),

<table>
<thead>
<tr>
<th>Degree of Deacetylation</th>
<th>≥ 70%</th>
<th>81.48%</th>
<th>80 – 90%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility(in 2% acetic acid)</td>
<td>soluble</td>
<td>soluble</td>
<td>Soluble</td>
</tr>
</tbody>
</table>

* PROTAN Biopolymer [14]
** Commercial [13]
which are for the bend NH vibration of NH$_2$. The absorption band of -CH$_3$ bend is in a wave number of 1379.10 cm$^{-1}$ with a weak in tensity, indicating the occurrence of deacetylation process that led to the loss of most of a methyl group, -CH$_3$. Vibration C-N is identified in a wave number of 1323.17 cm$^{-1}$ with a weak in tensity suggesting that there is still a little cluster of -NHCOC$_3$, so it can be concluded that there is still chitin in the chitosan sample. The degree of deacetylation (DD) of chitosan was obtained using a method derived from base line was 81.48%. The result is in parallel with the standard set by PROTAN biopolymer that is ≥70%. The high degree of deacetylation shows a high purity of the chitosan produced.[15]. The greater the degree of deacetylation, the more active the chitosan will be because more amino groups which replaces an acetyl group. The amino group is more reactive than the acetyl group because it has lone pair electrons on the nitrogen atom; thereby the chances of chitosan to interact with a negative charge in the cell walls of microorganisms increase[16].

**Synthesis of chitosan nanoparticles (CNPs)**

Figure 2 shows the FTIR spectra of chitosan-tripolyphosphate material compared to its raw materials. It is obvious that there are some changes in absorption bands after addition of TPP. Absorption bands for the presence of phosphate compounds can be indicated by absorption at wavenumbers of 1000-870 cm$^{-1}$ (P-O stretching), 1100-910 cm$^{-1}$ (P-OH

![Figure 2](image-url)
stretching) and 1300-1150 cm\(^{-1}\) (P=O stretching) (Silverstein, 1986). Peaks appearing at wave numbers of 1257.59, 1199.72, and 1155.36 cm\(^{-1}\) in Figure 2 belong to P=O stretching vibration, that at wave numbers of 1076.28, 1026.13, and 948.96 cm\(^{-1}\) are for P-OH stretching vibration and P=O stretching vibration is shown at 894.97 cm\(^{-1}\). The changes are caused by the presence of cross linking interaction between ionic charge of TPP and positive part of amino acid from chitosan (R-NH\(_3^+\)) [17].

In the FTIR spectrum of cross-linked chitosan-TPP, a peak at 1653.00 cm\(^{-1}\) disappears and two new peaks appear at wave numbers of 1656.58 and 1627.92 cm\(^{-1}\). The loss of the peak may be caused by the formation of a cross-link between phosphate and ammonium ions. The presence of a cross-link interaction is indicated by the existence of the peak at a wave number of 1155.36 cm\(^{-1}\).

The cross-linking reaction between the amino group and the tripolyphosphate (TPP) is supported by the reaction mechanism shown in Figure 3.

![Figure 3](image)

(a) Deprotonation chitosan; (b) Cross-linking ionic chitosan with tripolyphosphate (Bhumkar and Pokharkar, 2006)

X-ray diffraction patterns of chitosan and chitosan-tripolyphosphate are shown in Figure 4. X-ray diffraction pattern of the initial sample of chitosan shows a characteristic peak at 2\(\theta\) of 19.97\(^{\circ}\) indicating that the sample has a crystal line structure. At the diffraction patterns of chitosan-TPP samples, a peak at 2\(\theta\) of 19.97\(^{\circ}\) shifts to 2\(\theta\) of 19.54\(^{\circ}\), 19.56\(^{\circ}\), 19.30\(^{\circ}\), 19.20\(^{\circ}\), and 20.42\(^{\circ}\) for chitosan-TPP with the chitosan concentration of 0.1, 0.2, 0.3, 0.4, and 0.55, respectively. The shifted peak of the five chitosan-TPP samples shows that the change in crystal structure
occurred. Therefore, it is clear that there is the change in chitosan structure from crystalline to amorphous structures due to the cross-link occurred between TPP and chitosan.

![X-ray diffraction patterns](image)

Figure 4. X-ray diffraction patterns of a) chitosan powder; b) CNPs 1 (5:1); c) CNPs 2 (5:1); d) CNPs 3 (5:1); e) CNPs 4 (5:1); and f) CNPs 5 (5:1), respectively.

The crystal size of five samples of chitosan nanoparticles measured using Scherrer equation is given in Table 2.

Table 2. The size of crystal line chitosan nanoparticles

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume ratio</th>
<th>$2\theta$ (deg)</th>
<th>$\theta$ (deg)</th>
<th>$\text{Sin }\theta$</th>
<th>$\text{Cos }\theta$</th>
<th>$\beta_0$ (FWHM)</th>
<th>$d$ (nm)</th>
<th>$D$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan powder</td>
<td>-</td>
<td>19.97</td>
<td>9.987</td>
<td>0.173</td>
<td>0.984</td>
<td>1.912</td>
<td>1.336</td>
<td>7.364</td>
</tr>
<tr>
<td>CNPs 1</td>
<td>(5:1)</td>
<td>19.54</td>
<td>9.770</td>
<td>0.169</td>
<td>0.985</td>
<td>3.120</td>
<td>1.307</td>
<td>4.509</td>
</tr>
<tr>
<td>CNPs 2</td>
<td>(5:1)</td>
<td>19.56</td>
<td>9.780</td>
<td>0.169</td>
<td>0.985</td>
<td>2.396</td>
<td>1.308</td>
<td>5.872</td>
</tr>
<tr>
<td>CNPs 3</td>
<td>(5:1)</td>
<td>19.30</td>
<td>9.650</td>
<td>0.167</td>
<td>0.986</td>
<td>1.960</td>
<td>1.291</td>
<td>7.175</td>
</tr>
<tr>
<td>CNPs 4</td>
<td>(5:1)</td>
<td>19.21</td>
<td>9.605</td>
<td>0.167</td>
<td>0.986</td>
<td>1.448</td>
<td>1.285</td>
<td>9.710</td>
</tr>
<tr>
<td>CNPs 5</td>
<td>(5:1)</td>
<td>20.42</td>
<td>10.21</td>
<td>0.177</td>
<td>0.984</td>
<td>0.000</td>
<td>1.364</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Table 2 shows that the increase of the crystal size of chitosan is directly proportional to the increase in the concentration of chitosan. This shows that the higher the concentration of chitosan in a mixture, the greater the size of crystal line.
particles of chitosan produced. It is clear that the smallest crystal size is obtained in chitosan concentration of 0.1% and 0.2% with the addition of 0.1% TPP solution into each solution. This study is supported by Yong mei and Yumin (2003), who stated that then a nanoparticle formation only occurred at certain concentrations of chitosan and TPP.

The nanoparticle size distribution of chitosan produced in the two concentrations using PSA is shown in Figure 5.

![Figure 5. The particle size distribution of chitosan nanoparticles using PSA for the mixture of chitosana). CNPs 1 and b). CNPs 2.](image)

From the graph, the particle size of chitosan nanoparticles in chitosan concentration of 0.1% (CNPs1) and chitosan 0.2% (CNPs2) is 224.68 nm and 204.32 nm, respectively. Polydisperse index of CNPs 1 and CNPs 2a re 0.226 and 0.261, respectively showing that the degree of homogeneity of the two solutions of chitosan nanoparticles have good homogeneity (pdi < 0.5). Chitosan nanoparticles, CNPs 2 was chosen for antimicrobial test.

**Antimicrobial Test chitosan nanoparticles**

The inhibition zone of chitosan nanoparticles against several pathogenic microbes at various concentrations (CNPs 2) can be seen in Figure 6.

![Figure 6. Inhibition zone of chitosan nanoparticles for a). *Staphylococcus aureus* (gram positive bacteria), b). *Pseudomonas aeruginosa* (gram negative bacteria), c). *Malassezia furfur* and d). *Candida albicans* (fungi).](image)
The inhibition diameter caused by chitosan nanoparticle (CNPs 2) is shown in Table 3.

Table 3. Inhibition diameter of chitosan nanoparticles solution at various concentrations (24 hours and 48 hours observation).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inhibition diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. aureus</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>CNPs 2 (0.1%)</td>
<td>9.65</td>
</tr>
<tr>
<td>CNPs 2 (0.2%)</td>
<td>10.25</td>
</tr>
<tr>
<td>CNPs 2 (0.3%)</td>
<td>8.35</td>
</tr>
<tr>
<td>CNPs 2 (0.4%)</td>
<td>-</td>
</tr>
<tr>
<td>CNPs 2 (0.5%)</td>
<td>7.15</td>
</tr>
<tr>
<td>Positive control</td>
<td>14.95*</td>
</tr>
</tbody>
</table>

CNPs = Chitosan nanoparticles (concentration)
Positive control * bacteria = 30 µg of chloramphenicol
** fungus = 20 mg/mL of nystatyn

Table 3 shows that chitosan nanoparticles are more effective at inhibiting the growth of bacteria rather than fungus. This suggests that the chitosan nanoparticles are more effective as an antibacterial. Chitosan nanoparticles having the highest antibacterial activity is given by chitosan nanoparticles with the concentrations of 0.2% for *Staphylococcus aureus* and 0.3% for *Pseudomonas aurogenusa*. The smallest activity is shown by chitosan nanoparticle with the concentration of 0.5%. This may happen because the diffusion process in the paper disc become smaller with the increase of concentration. Antifungal activity of chitosan nanoparticles is only shown on skin fungus of *Mallasezia furfur*. The highest activity is performed by chitosan nanoparticles with the concentration of 0.5%, whereas the activity of the nanoparticles with other concentrations is smaller and it is clear that the activity at the concentration of 0.1% is the same as that at the concentration of 0.4%. Some experiments can not be measured at the concentration of chitosan nanoparticles of 0.2 and 0.3% because there was contamination with other kinds of microbes. Based on the inhibition ability of chitosan nanoparticles towards bacteria, the material is categorized as a bacteriostatic agent (a compound that has the ability to inhibit the growth or of bacteria in the usual dosage. Based on the long incubation time, the antimicrobial activity of chitosan nanoparticles decreases during 48 hours. The decrease in diameter inhibition zone of active substances may be caused by the growth of bacterial colonies which is great and solid after 24 hours. The decrease of the inhibition diameter after 24 hours is very small compared to after 48 hours. Therefore, the inhibition diameter at the time of 24 hours and 48 hours is not significantly different.
Conclusion

Chitosan isolated from the shells of crabs (*Portunus pelagicus*) have characteristics with consistent with the characteristics of standards stated by PROTAN biopolymer and can be used for commercialization. The deacetylation degree (DD) of chitosan obtained was 81.48%. Chitosan can be modified to increase the size of then nanoparticles for antimicrobial activity. Chitosan nanoparticles resulted from the synthesis have small size, 224.68 and 204.32 nm for CNPs 1 and CNPs 2, respectively. Polydisperse index (PDI) for the two nanoparticles, CNPs 1 and CNPs 2 are 0.226 and 0.261, respectively which indicates that both chitosan nanoparticles have a good homogeneity (pdi < 0.5). Chitosan nanoparticles (CNPs) show antimicrobial activity against bacterial strains of *Staphylococcus aureus* (gram positive bacteria), and *Pseudomonas aeruginosa* (gram negative bacteria), as well as the type of fungus *Malassezia furfur*.

Acknowledgement

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References


