Preparation of Chitosan with Various Molecular Weight and Its Effect on Depolymerization of Chitosan with Hydrogen Peroxide using Conventional Technique

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**Abstract**: Depolymerization of chitosan with hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) using conventional techniques has been carried out by varying the molecular weight of chitosan. This study was aimed to determine the optimum conditions of chitosan depolymerization and the effect of molecular weight on the preparation of oligochitosan. Chitosan was produced with different molecular weights in 5\% acetic acid with variations of heating time for 2, 12, 24 and 24 h. The molecular weight of chitosan was determined by the viscometry method. Chitosan produced from commercial chitin had the molecular weight of 13.30x10\textsuperscript{4} (CC-2), 12.02x10\textsuperscript{4} (CC-12), 7.75x10\textsuperscript{4} (CC-24) and 4.35x10\textsuperscript{4} g / mol (CC-120) for 2, 12, 24, and 120 h of heating time, respectively. The results indicated that the properties of produced chitosan are affected by heating time. The longer the heating time, the lower the molecular weight of chitosan would be. Furthermore, chitosan was depolymerized by varying the concentration of H\textsubscript{2}O\textsubscript{2}, temperature, and depolymerization time using conventional techniques. The depolymerization process of chitosan with H\textsubscript{2}O\textsubscript{2} was influenced by H\textsubscript{2}O\textsubscript{2} concentration and heating temperature where the optimum conditions of depolymerization of chitosan using H\textsubscript{2}O\textsubscript{2} were obtained for CC-12 when the process was conducted using H\textsubscript{2}O\textsubscript{2} concentration of 4\%; at 30°C, for 4 h to give the corresponding oligochitosan with the molecular weight of 4.2x10\textsuperscript{3} in 97.57\% yield.

**Keywords**: chitosan, hydrogen peroxide, molecular weight, oligochitosan.

I. Introduction

In Indonesia, shrimp is generally exported as frozen product which has been removed from the head, tail and skin. The skin contains protein (25-40\%), chitin (15-20\%) and calcium carbonate (45-50\%). Chitin consists of monomer of 2-acetamido-2-deoxy-β-D-glucose which is associated to 1,4-glycosidic bond\textsuperscript{2}. Chitin (Figure 1) is not soluble in water, which limits its application. By modifying the chemical structure of chitin, the chitin derived products, such as chitosan, which possesses better chemical properties, will be obtained\textsuperscript{3}.
Chitosan (poly-2-amino-2-dioxy-β-D-glucose) can be obtained via partial or complete deacetylation of chitin. The properties of chitosan (Figure 2) depend not only on the changes in structure but also on molecular weight. The applications of chitosan are still not optimal since the length of the chain with high molecular weight leads chitosan to be difficult to dissolve in water4. In fact, the solubility is one of important characteristics for chitosan. The increase of solubility will facilitate chitosan to be used more widely in the fields of food, agriculture, pharmacy, and other technical applications. High molecular weight results in chitosan with low solubility in water. This has led to the development of several attempts to cut chitosan bonds to give shorter oligomers called oligochitosan.

Figure 1. Structure of chitin

Oligochitosan can be produced from chitosan via the biochemical depolymerization of chitosan using enzymes5,6 or chemical depolymerization using acids such as HCl, H2SO4, and CH3COOH7,8,9. Additionally, the chitosan depolymerization process can be also carried out in the presence of oxidant such as O3, NaNO2, and H2O210 - 17. It has been also reported that sodium hypochlorite (NaClO) can be used to produce chitosan with lower molecular weights or oligochitosan18. Among the reagents aforementioned, hydrogen peroxide is more widely used because it is easy to handle, easy to obtain, and more environmentally friendly4,13. In order to effectively and efficiently get oligochitosan, some researchers have combined hydrogen peroxide using microwave irradiation19,20, gamma ray radiation21, ultraviolet light22, ultraviolet-irradiated oxygen23, heteropoly acid24,25, and ultrasonic26.

The reported studies put more attention on the effect of H2O2 concentration, time and temperature of depolymerization without seeing the effect of chitosan properties such as molecular weight and degree of deacetylation. In fact, by understanding the influence of molecular weight and deacetylation degree of chitosan, the use of oligochitosan will be more applicable. In this study, the molecular weight of chitosan, which was converted from commercial chitin, would be varied. The chitosan would be depolymerized into oligochitosan with H2O2 using conventional techniques. The purposes of this study were to determine the optimum conditions of chitosan depolymerization and the effect of chitosan molecular weight on depolymerized chitosan.

2. Experimental

2.1. Chemical and Instruments

Chemicals used in this study were commercial chitin (Sigma Aldrich), CH3COOH p.a (E. Merck), NaOH p.a (E. Merck), HCl p.a (E. Merck), H2O2 p.a (E. Merck), ethanol p.a (E. Merck), K3Fe(CN)6 p.a (E.
Merck), Na$_2$CO$_3$ p.a (E. Merck), D-glucosaminehydroxide (GAH) (Sigma Aldrich), distilled water and filter paper Whatman No. 42.

The instruments used in this study included UV-Vis spectrophotometer (PD-3035) and FTIR spectrophotometer (Prestige 21 Shimadzu).

2.2. Experimental Procedures

2.2.1. Conversion of chitin into chitosan

The conversion of chitin into chitosan was carried out using No and Meyer method with slight modification. Commercial chitin powder (30 g) was mixed with 300 mL of 50% aqueous NaOH solution. The mixture was stirred and heated at 100 °C for 6 h with a ratio of chitin and solvent of 1:10 (m/v). Then, the mixture was filtered and washed with distilled water until the pH was neutral. Residue of chitosan was dried in the oven at 65 °C for 24 h then weighed. The obtained chitosan was characterized by FTIR spectrophotometer.

2.2.2. Preparation of chitosan with various molecular weights

Chitosan (5 g) was dissolved in 95 mL of 5% acetic acid solution. The mixture was then heated at 50 °C with variations in heating time of 2, 12, 24 and 120 h. Next, the mixture was cooled and centrifuged at 5000 rpm for 20 min. The supernatant was added with 4 N NaOH until the pH was neutral. The precipitate was filtered and washed with distilled water, then dried in the oven at 50 °C. The molecular weight of chitosan was determined by the viscometry method and deacetylation degree was determined by UV-Vis spectrophotometry method. Then, the chitosan was characterized by FTIR spectrophotometer.

2.2.3. Determination of molecular weight of chitosan using viscometry method

As much as 0.1 g of various molecular weights of the prepared chitosan was dissolved in 100 mL of 0.01 M HCl solution. The concentration was varied to give the concentration of 0.01%, 0.02%, 0.03%, 0.04% and 0.05%. Then, 5 mL of each solution was put into the viscometer and the flow time was determined. Chitosan molecular weight was calculated based on Equations 1 and 2 with K and a values for chitosan with 0.01 M HCl solvent at 30 °C were 5.48 $\times 10^{-4}$ and 0.72, respectively.

\[
\eta = \frac{\eta_p}{c} \quad \text{...(1)}
\]
\[
[\eta] = K M^a_\eta \quad \text{...(2)}
\]

Determination of deacetylation degree of chitosan

Determination of deacetylation degree (DD) of chitosan was carried out using Liu et al. method with slight modification. As much as 3 mg of chitosan was dissolved in 50 mL of 0.01 M acetic acid solution. The absorbance of the solution was measured by UV-Vis spectrophotometer at wavelength of 201 nm. The degree of deacetylation is calculated based on Equations 3 and 4.

\[
DA = \frac{16.1 \times 1.4 \times V - 0.0218 M}{3.3615 M - 42.1A V} \quad \text{...(3)}
\]
\[
DD = (1 - DA) \times 100\% \quad \text{...(4)}
\]

Preparation of oligochitosan from chitosan using H$_2$O$_2$

Into 1.0 g of chitosan was added 20 mL of 2.0% acetic acid solution. The mixture was heated with H$_2$O$_2$ concentration of 4, 5 and 6% with the heating time of 2, 4 and 6 h at 30, 40, 50, and 60 °C. Then, the solution was cooled, neutralized with 10% NaOH solution. The residue was separated by filtration, and the filtrate was added with 2 parts by volume of ethanol. Water-soluble chitosan (oligochitosan) will be obtained after being left overnight. It was dried in the oven at 50 °C, and was weighed. The yield of oligochitosan was calculated based on Equation 5.
\[
\text{% yield} = \frac{\text{weight of water-soluble chitosan}}{\text{weight of crude chitosan}} \times 100\% \quad \cdots (5)
\]

**Determination of molecular weight of oligochitosan using the end group analysis**

Determination of molecular weight of oligochitosan using the end group analysis was performed using Shao et al method\(^\text{19}\) with slight modification. The coloring reagent was prepared by dissolving 0.5 g of K\(_3\)Fe(CN)\(_6\) in 1 L of 0.5 M Na\(_2\)CO\(_3\) and was then stored in a brown reagent bottle. The standard solution was prepared by dissolving 1 g of D-glucosamine hydrochloride (GAH) in 100 mL of distilled water. Preparation of standard curves was done by mixing 0.2; 0.4; 0.6; 0.8 and 1 mL of standard GAH solution, respectively with 2 mL of color reagent into the test tube, then the volume was adjusted to 5 mL with distilled water. The test tube was closed, heated in boiling water for 15 min, and cooled to room temperature. Then, the absorbance was measured by UV-Vis spectrophotometer at the wavelength of 420 nm with distilled water as blank. For the oligochitosan sample, 0.1 g of sample was added by 2 mL of the coloring reagent and put into a test tube. The volume was adjusted to 5 mL with distilled water, heated in boiling water for 15 min, cooled and measured by UV-Vis spectrophotometer at wavelength of 420 nm. The average molecular weight (\(M_n\)) was calculated based on Equation 6.

\[
\bar{M}_n = \frac{W_1}{W_2} \times 215.5 \quad \cdots (6)
\]

**3. Results and Discussion**

**3.1. Conversion of chitin into chitosan**

First of all, chitin was converted into chitosan via the deacetylation reaction. The deacetylation process was aimed to remove the acetyl groups (-COCH\(_3\)) by using high concentrated alkaline solutions to generate the amine groups (-NH\(_2\)). Chitin has a long crystalline structure with strong hydrogen bonds between nitrogen atoms and carboxylic groups in adjacent chains\(^\text{32}\).

This deacetylation process can be detected by evaluating the on both chitin and chitosan FTIR spectra at certain characteristic wavenumber. The functional groups which were characteristic for the chitin and chitosan can be seen in the FTIR spectra(Figure 3).

![Figure 3. FTIR spectra of (a) commercial chitin and (b) chitosan.](image)

Figure 3 showed that most of the absorption bands of chitin were similar with those of chitosan. The band shifts were observed including NH(s) from 3242 to 3280-3105, NH(b) from 1563-1538 to 1556 cm\(^{-1}\), and C=O from 1668-1645 to 1668-1645 cm\(^{-1}\) and from 1061-1010 to 1053 cm\(^{-1}\). The shift of some absorption bands indicated that the deacetylation of chitin occurred to give chitosan.

**3.2. Preparation of chitosan with various molecular weights**
Solubility of chitosan in acetic acid indicated that the deacetylation process had been taken place. The solubility of chitosan was caused by the presence of hydrogen atoms of free amine which may facilitate interaction with water through hydrogen bonds. The presence of carboxyl groups in acetic acid will facilitate the solubility of chitosan via the formation of hydrogen bond between carboxyl groups and amine groups of both. Chitosan were prepared by dissolving in 5% of acetic acid solution with the variation of heating time of 2, 12, 24 and 120 h, so that chitosan with various molecular weights will be produced. The prepared chitosan were then symbolized by CC-2, CC-12, CC-24, and CC-120 for heating times of 2, 12, 24 and 120 h, respectively.

The viscous chitosan solution in acetic acid obtained after heating with various heating time, was then added 4 M of NaOH solution to form a white lump and the solution formed will coagulate. The lump was filtered and neutralized using distilled water, then dried at 50°C. The results showed that heating the chitosan for 2, 12, 24 and 120 h gave the product in 10.56, 16.78, 23.98 and 32.61 g, respectively. The molecular weight of chitosan was calculated using the viscometric method based on Equations 1 and 2 and the degree of deacetylation was determined based on Equations 3 and 4, and symbols were shown in Table 1.

Table 1. Molecular weight and deacetylation degree of chitosan

<table>
<thead>
<tr>
<th>Term of Prepared Chitosan</th>
<th>Molecular Weight (g/mol)</th>
<th>Deacetylation Degree (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC-2</td>
<td>1.3299 x 10^5</td>
<td>79.11</td>
</tr>
<tr>
<td>CC-12</td>
<td>1.2021 x 10^5</td>
<td>80.77</td>
</tr>
<tr>
<td>CC-24</td>
<td>7.7492 x 10^4</td>
<td>88.64</td>
</tr>
<tr>
<td>CC-120</td>
<td>4.3542 x 10^4</td>
<td>95.72</td>
</tr>
</tbody>
</table>

Table 1 showed that the longer the heating time led the increase of the molecular weight and the decrease of the deacetylation degree. The results were in accordance with previous studies, where the produced chitosan had the molecular weight of 9x10^6 g/mol with deacetylation degree of 76% and 9x10^4 g/mol with deacetylation degree of 92%. When compared with previous research by Liu, et al., the molecular weight of chitosan prepared with heating time of 2 - 145 hours ranged between 15.5x10^4 - 5.5x10^4 Da and the same degree of deacetylation of 80% ± 0.29. The difference was presumably due to the method used for the determination of different molecular weights and deacetylation degrees.

The prepared chitosans were then analyzed using FTIR spectrophotometer (Figure 4) and have absorption bands displayed in Table 2.

Figure 4. FTIR spectra of (a). CC-2, (b). CC-12, (c). CC-24, and (d). CC-120
Table 2. FTIR absorption profile of the prepared chitosan

<table>
<thead>
<tr>
<th>Term of Prepared Chitosan</th>
<th>Functional Group (wave number, cm⁻¹)</th>
<th>NH (s)</th>
<th>CH</th>
<th>C=O</th>
<th>NH (b)</th>
<th>C-O</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC-2</td>
<td></td>
<td>3155.54-3116.97</td>
<td>3093.82-2873.94</td>
<td>1658.70-1643.35</td>
<td>1566.20-1552.70</td>
<td>1041.56-1031.92</td>
</tr>
<tr>
<td>CC-12</td>
<td></td>
<td>3271.27-3234.62</td>
<td>2881.65</td>
<td>1665.50-1651.07</td>
<td>1556.55-1537.27</td>
<td>-</td>
</tr>
<tr>
<td>CC-24</td>
<td></td>
<td>3296.35-3217.27</td>
<td>2881.65</td>
<td>1665.50-1651.07</td>
<td>1556.55-1537.27</td>
<td>1060.85-1012.63</td>
</tr>
<tr>
<td>CC-120</td>
<td></td>
<td>3294.42-3194.12</td>
<td>2883.58</td>
<td>1665.50-1651.07</td>
<td>1556.55-1537.27</td>
<td>1072.42-1035.77</td>
</tr>
</tbody>
</table>

Based on Table 2, it can be shown that the FTIR spectra of chitosans with variations in heating time displayed similar pattern. For example, the absorptions of NH (s), C = O, NH (b), and CO were not shifted. While CC-12, CC-24 and CC-120 had the same absorption for NH (b) functional groups, CC-12 and CC-24 displayed the same absorption for CO groups.

3.3. Depolymerization of chitosan to produce oligochitosan

The depolymerization of chitosan, which was performed using H₂O₂, generated oligochitosan with a reaction mechanism according to Tian et al.¹⁴ which begins with an equilibrium system in Reactions (7) and (8) and a total reaction as in Reaction (9).

\[
\text{R-NH}_2 + H^+ = R-NH_3^+ 
\]  
\[
\text{H}_2\text{O}_2 = H^+ + \text{HOO}^- 
\]  
\[
\text{R-NH}_2 + \text{H}_2\text{O}_2 + H^+ = R-NH_3^+ + \text{HOO}^- + H^+ 
\]  

Anion \(\text{HOO}^-\) is very unstable and very easily decomposes into highly reactive hydroxyl radicals (Reactions 10 and 11).

\[
\text{HOO}^- \rightarrow \text{OH}^- + O^• 
\]  
\[
\text{H}_2\text{O}_2 + \text{HOO}^- \rightarrow \text{HO}^• + \text{O}_2^- + \text{H}_2\text{O} 
\]

Hydroxyl radical(\(\text{HO}^•\)) is strong oxidants. The main chemical action of this radical with a polysaccharide is very fast as shown in Reactions (12) and (13). The hydroxyl radical attracts one H atom and forms water so that it forms a water-soluble oxidation product with a low molecular weight. Depolymerization of chitosan using \(\text{H}_2\text{O}_2\) gave the products with the yields as displayed in Table 3.

Table 3. Optimum conditions of depolymerization of chitosan, weight, yields and average molecular weight (\(M_n\)) of oligochitosan

<table>
<thead>
<tr>
<th>Term of Prepared Chitosan</th>
<th>Optimum Conditions</th>
<th>Weight Oligochitosan(g)</th>
<th>Yields (%)</th>
<th>(M_n) (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC-2</td>
<td>6%, 30 °C, 4 hours</td>
<td>1.00035</td>
<td>58.31</td>
<td>2404.37</td>
</tr>
<tr>
<td>CC-12</td>
<td>4%, 30 °C, 4 hours</td>
<td>1.00040</td>
<td>97.57</td>
<td>4187.31</td>
</tr>
<tr>
<td>CC-24</td>
<td>4%, 40 °C, 4 hours</td>
<td>1.00045</td>
<td>63.70</td>
<td>3909.13</td>
</tr>
<tr>
<td>CC-120</td>
<td>5%, 30 °C, 4 hours</td>
<td>1.00050</td>
<td>50.03</td>
<td>2992.79</td>
</tr>
</tbody>
</table>

Optimization of process conditions was carried out for each preparation time based on the results of the study by taking into account to the concentration, temperature and time during the depolymerization process. Based on the yield of oligochitosan in Table 3, the optimum conditions of chitosan depolymerization process using \(\text{H}_2\text{O}_2\) were obtained when using \(\text{H}_2\text{O}_2\) concentration of 4% at 30 °C for 4 h for CC-12 which produced oligochitosan with molecular weight of \(4.2 \times 10^3\) g/mol and yield of 97.57%. When compared with research by
Duet et al\textsuperscript{31} which varied H$_2$O$_2$ concentration, time, and heating temperature, it indicated that each factor had a significant effect on oligochitosan results. The optimum conditions of chitosan depolymerization can theoretically be determined using Response Surface Methodology (RSM)\textsuperscript{19} at H$_2$O$_2$ concentration of 5.5\% at 42.8 °C for 3.5 h in a yield of 93.5\% of oligochitosan product.

3.4. Determination of molecular weight of oligochitosan using the end group analysis

Oligochitosans obtained from depolymerization were characterized for their average molecular weight by the end group analysis method using UV-Vis spectrophotometer. The results can be seen in Table 3. The molecular weight of chitosan significantly decreased after depolymerization with H$_2$O$_2$. The molecular weight of chitosan CC-2 had decreased from 1.32986x10$^5$ to 2404.37 g/mol. Similarly, the molecular weight of chitosan CC-12, CC-24, and CC-120 also decreased. When compared with research conducted by Julianti\textsuperscript{26} using 30\% H$_2$O$_2$, the molecular weight of chitosan decreased from 1.49x10$^5$ to 3106.705 and 630.957. The difference was probably due to the use of ultrasonic bath in the degradation process of chitosan.

Based on Table 3, for the optimum conditions of chitosan CC-2 with a high molecular weight of chitosan, the concentration of H$_2$O$_2$ used was higher with lower temperature and longer heating time. When compared with the low molecular weight of chitosan, the concentration of H$_2$O$_2$ used was small while the time and temperature were higher to break the β-glycosidic bond. Thus, the weight of the oligochitosan molecule produced for each molecular weight of chitosan preparation decreased. The decrease in molecular weight was due to the small size of the polymer chain, so the rate of movement of the transition was fast and the molecular weight became low\textsuperscript{37}.

4. Conclusion

Based on the results and discussion, it can be concluded that:

1. The optimum conditions of depolymerization of chitosan using H$_2$O$_2$ were obtained for CC-12 when the process was conducted using H$_2$O$_2$ concentration of 4\%; at 30°C, for 4 h to give the corresponding oligochitosan with the molecular weight of 4.2x10$^3$ in 97.57\% yield.

2. The molecular weight of chitosan preparation used for depolymerization has an effect on the molecular weight of oligochitosan, leading to the significant reduction in molecular weight of chitosan.

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