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Synthesis and Characterization of Low Molecular Weight Irradiated Chitosan in Various Water Levels and Gamma-Ray Doses

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Abstract
Chitosan is a biopolymer derived from marine animal shell waste that exhibits numerous pharmacological activities. However, its high molecular weight limits the application in various fields due to its low solubility. Therefore, this study aims to synthesize low molecular weight chitosan using varying water content and doses of gamma irradiation. To initiate chitosan degradation, H_2O (5 and 10 mL) was added, followed by gamma ray irradiation at doses of 5 and 10 kGy. The Molecular Weight (MW) of degraded chitosan was determined using Gel Permeation Chromatography (GPC), while Fourier Transform Infrared Spectroscopy (FTIR) was used to characterize the functional groups and degree of deacetylation of chitosan. The study found that the molecular weight of irradiated chitosan decreased as the irradiation dose and H_2O addition increased. The addition of 10 mL of water and gamma irradiation at a dose of 10 kGy has been found to reduce the molecular weight of chitosan to 118 kDa, with a high deacetylation degree of 86.78%. The FTIR analysis showed no significant changes in the functional groups, indicating that gamma irradiation did not affect the structure of chitosan.

Keywords: chitosan; degree of deacetylation; gamma irradiation; low molecular weight; water

1. INTRODUCTION

Chitin, poly (β(1-4) N-acetyl-D-glucosamine), a natural polysaccharide that is very widely available in nature after cellulose[1]. It is found in the exoskeleton of crustaceans, where it can make up to 20-30% of the shell[2]. Chitosan is the main derivative compound of chitin, obtained through the several processes of N-deacetylation of chitin such as alkaline hydrolysis, enzymatic, compression method and etc[2,3].

The physicochemical and biological activities of chitosan depend heavily on its degree of deacetylation (DD) and molecular weight (MW). DD represents the percentage of acetyl groups that have been replaced by an amine[4]. Commercial chitosan typically has a DD value of 70-90%[5,6]. The low molecular weight and high DD of chitosan increase its solubility, making it a promising material for use in the pharmaceutical and biomedical fields[7].

Chitosan with a high molecular mass (> 250 kDa) has been widely used for coagulation in wastewater, flocculation of bioplastics and biofiber applications[8]. However, its poor solubility at neutral pH and high viscosity limit its application in the pharmaceutical field. The molecular mass of chitosan in the range of 50 – 250 kDa has good activity against *Streptococcus aureus* bacteria[9]. It also has good solubility in neutral pH and low viscosity[10]. Accordingly, it is imperative to modify chitosan with a low molecular mass in order to facilitate its utilization in the pharmaceutical field.

Various methods have been used to modify the molecular weight of chitosan, including gamma rays, infrared waves, microwaves, and ultrasonic-assisted[11–13].

Gamma-ray radiation is particularly effective in reducing the molecular weight of chitosan, as it can cleave the main chain in polysaccharides without affecting the backbone structure[14]. Chitosan was significantly degraded by low dose of γ-ray irradiation[11].

Gamma irradiation helps the formation of OH radicals through radiolysis of water (H_2O). This causes the molecular weight of wet chitosan to be degraded at a higher rate than dry chitosan[15]. In previous research, the addition of hydrogen peroxide (H_2O_2) was carried out to increase the amount of OH radical formation, apart from water. Mahmud et al.[16] carried out chitosan degradation using gamma rays (6 kGy) with the addition of 1%-5% H_2O_2 to the chitosan-water mixture. A significant decrease in chitosan molecular weight only occurred at 1% H_2O_2, from 79.2 kDa to 13.62 kDa. At high level of H_2O_2, the degradation of chitosan became less effective. This is due to the over formation of hydroxyl radicals which are going to attack or react each order to form stable molecules such as water or oxygen gas during propagation reaction[16].

Therefore, this research aims to synthesize degraded chitosan in various levels by soaking chitosan in distilled water without the addition of H_2O_2. The effect of radiation on molecular weight was determined by carrying out Gel Permeation Chromatography (GPC) to characterize degraded chitosan. Fourier Transform Infra-Red (FTIR) was used to identify functional groups in chitosan and calculate the degree of deacetylation. There are several methods that can be used to measure the degree of deacetylation, such as electrolyte titration, thermal analysis, alkalimetry, gas chromatography, ultraviolet spectroscopy and infrared spectroscopy[17]. In analyzing the degree of deacetylation, the FTIR method is often used in functional group analysis because it has high sensitivity which can identify the functional groups of a compound only through specific absorption peaks at certain wave numbers[18].

2. RESEARCH METHODS

Tools and Materials

The equipment used in this study was basic laboratory glassware, analytical balance (Acculab Sartorius group), pH meter (Mettler Toledo), hotplate (IKA C-MAG HS7), magnetic stirrer, centrifuge (Cole-Parmer), mortar (Agate), vortex mixer (Wisemix), incu-shaker (Benchmark), ultrasonic sonicator (Cole-Parmer) and other equipment. The instruments used were Gel Permeation Chromatography (GPC, Tosoh EcoSEC Elite 8420 (Tosoh Corporation, Tokyo, Japan) equipped with a refractive index (RI) detector (HLC-8420GPC). a UV detector (UV-8420), and column (TSK-Gel G3000PWXL-CP, 7.8 mm × 30 cm, particle diameter 10 m, Tosoh Corporation, Tokyo, Japan), and Fourier Transform Infra-Red (FTIR, Prestige 21 Shimadzu). The material used for this study is chitosan powder (Sigma-Aldrich), sodium hydroxide (Merck), absolute ethanol (Merck), distilled water, glacial acetic acid 100 % (Merck), sodium acetate (Merck), potassium bromide (KBr, Wako).

Chitosan Degradation and Purification of Degraded Chitosan[16]

Chitosan powder (1 g) was added with 5 and 10 mL of distilled water in a plastic pot and stirred constantly for 24 hours. Chitosan samples were irradiated at 5 and 10 kGy. All samples were irradiated at Gamma Cells at the BRIN irradiation facility (Pasar Jumat, Jakarta) with a dose rate of 3.5 kGy/hour at room temperature.

Acetic acid solution (2 M) was added carefully to the irradiated chitosan suspension until it reached pH 4–5.5 and stirred constantly with a magnetic stirrer for 24 hours. Then sodium hydroxide (2 M) was added to precipitate chitosan. The precipitate was separated from the supernatant solution by washing using absolute ethanol (96%) and distilled water. Deposits in the form of gels were dried by lyophilization method which is a freeze-drying technique carried out rapidly through sublimation under vacuum conditions[19].

Determination of Molecular Weight by Gel Permeation Chromatography (GPC)[20]

The molecular weight of the degraded chitosan was characterized by GPC using Pullulan (Lot: BCCB4132) as standard to obtain the calibration curve. Chitosan sample preparation was carried out by dissolving chitosan (1 mg) in 1 ml of acetate buffer (0.5 M CH_3COOH/0.5 M CH_3COONa) for one night, then the samples were mixed using vortex, incubated (50°C, 60 mins) then centrifuged at 10.000 rpm for 10 mins. Chitosan samples (80 µL) injected into the column using an acetate buffer eluent with a flow rate of 1.0 mL min^{-1} at 40°C. The molecular weight was determined from the peak in GPC estimated in the pullulan standard sample.

Determination of Functional Groups and Degree of Chitosan Deacetylation by FTIR

Characterization using the FTIR with Diffuse Reflectance Spectroscopy (DRS) technique was done at room temperature where chitosan samples were mixed with dry KBr. Characterization was carried out in the wavenumber range of 500 – 4000 cm^{-1} with a spectral resolution of 2 cm^{-1} for 20 seconds.

The measurement of the degree of deacetylation for each sample is calculated according to equations 1-3[21]. The values of AB, AC, DF_2, DE are obtained from transmitting intensity through IR Solution software and refer to the research of Zainol et al[21].

$$DD=100-\left[\left(\frac{A_{1655}}{A_{3450}}\right)x115\right] \quad \text{..... (1)}$$

Where:

$A_{1655}=Log(DF_2/DE)$ ..... (2)

$A_{3450}=Log(AC/AB)$ ..... (3)

notes:

A_{1655} = absolute peak absorbance of the amide group at wavenumber 1655 cm^{-1}

A_{3450} = absolute peak absorbance of hydroxyl group at wavenumber 3450 cm^{-1}

AC = %transmittance at baseline

AB = %transmittance at the minimum peak of the hydroxyl group

DF_2 = %transmittance at baseline

DE = %transmittance at the minimum peak of the amide group

3. RESULTS AND DISCUSSION

Chitosan Degradation Results and Purification of Degraded Chitosan

Commercial chitosan has an MW range of 50–2000 kDa, with an average DD up to 90%. Chitosan was irradiated in the wet condition by adding distilled water (5 and 10 mL). Gamma radiation in 5 and 10 kGy is known to be able to break the bond chain of 1,4-β-glycosides in chitosan resulting in low molecular weight chitosan (oligochitosan)[22].

Figure 1 shows the structural differences between chitosan and oligochitosan based on the number of monomers. Chitosan consists of 2 monomer compounds, while oligochitosan, which only has 1 monomer compound, is produced by breaking the 1,4-β-glycosidic bond chain. Oligochitosan has a lower molecular weight due to its shorter polymer or monomer chain. The shorter the polymer chains in chitosan, the lower its molecular weight. This reduction in molecular weight increases the solubility properties[23].

Chitosan, which has been irradiated and purified with acetic acid to ensure complete solubility, undergoes a process of leaching (maceration) using absolute ethanol (96%) and distilled water to separate the gel precipitate obtained from the addition of NaOH from the supernatant solution. This washing process reduces the activity of water molecules and binds acetic acid, preventing chitosan from dissolving (coagulating) and producing a more effective precipitation. This precipitation mechanism allows the solubility of chitosan to produce a more effective product that does not contain acid. Because the solubility of chitosan in acetic acid aims to understand chitosan in the absence of air so that it can dissolve completely because chitosan can only dissolve in organic acids such as acetic acid. The reaction for purifying chitosan is depicted in Figure 2.

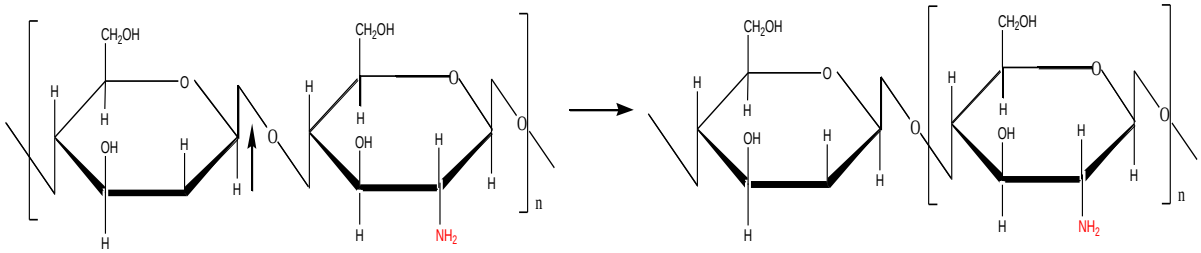

Figure 1. Chitosan chain breaking reaction at 1,4-β-glycosidic bonds becomes oligochitosan

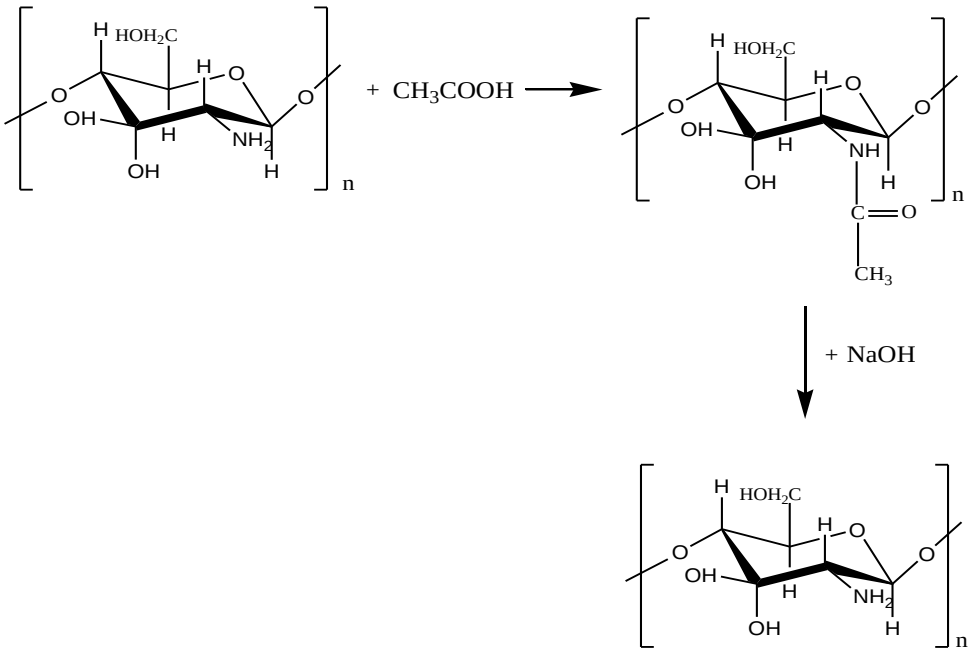

Figure 2. Chitosan purification reaction[24]

The chitosan purification reaction is an acid-base reaction, where chitosan is dissolved in an acidic medium through protonation. In this study, acetic acid was used as a proton donor. The amino group ($–NH_2$) accepts a proton (H^+) from acetic acid and becomes a positively charged ammonium group ($–NH_3^+$). As a result, cationic chitosan polymers with higher charge density become more polar and soluble in water[2]. To achieve the conservation process and induce the deposition of chitosan macromolecules on the surface of the core material, sodium hydroxide (NaOH) was added with the aim of increasing the pH of the reaction medium. Solid chitosan is converted by neutralizing the ammonium groups back into neutral amino groups.

The gel precipitate is dried using the lyophilization method, which is a freeze-drying technique carried out rapidly through sublimation under vacuum conditions[25]. Once the chitosan sample has been dried, it is stored in a sealed vial in a desiccator.

Figure 3 shows that chitosan changes color to brown when exposed to gamma-ray irradiation. This is due to degradation during the radiation process. The ionizing radiation causes photochemical reactions in chitosan molecules, generating free radicals or reactive forms that break the glycosidic bond in the chitosan backbone, thereby increasing the formation of double bonds (C=O groups) and causing a brownish color change[21]. The degree of color change is proportional to the radiation dose. The results indicate that radiation can cause the formation of free radicals, which can cleave the polymer chains and lead to discoloration of the polymer.

The molecular weight can be determined by using GPC, which provides the average molecular weight number (Mn) and average molecular weight (Mw). The average molecular weight amount (Mn) expresses the distribution of the length of the polymer chain that makes up the polymer. It is obtained from the calculation of the weight fraction of each species multiplied by its molecular weight. The average molecular weight (Mw) states the molecular weight distribution of the constituent polymer components. It is obtained from the calculation of the number of molecules of each weight in the sample[26].

The Poly Dispersity Index (PDI) is calculated from the average Mw/Mn and indicates the type of distribution of polymer molecules based on the values of Mn and Mw[26]. PDI expresses the width of the average molecular mass distribution. According to International Standards Organizations (ISO), the Mw/Mn value should be less than 0.05 for samples with a narrow particle size distribution (mono dispersion), while it should be greater than 0.7 for a wide particle size distribution (polydispersion).

Table 1 describes the distribution of Mw and Mn in chitosan samples with varying chitosan levels and irradiation doses. The results of lower Mn and Mw values than the initial chitosan, indicating that gamma radiation causes changes in the average molecular weight of chitosan. The radiation process causes a random reaction in which gamma rays break the bonds of chitosan polymer chains, resulting in non-uniform chain sizes and molecular weights that produce different distribution values (Mw/Mn). Increasing the irradiation dose (10 kGy) showed a higher Mw reduction in chitosan. Chitosan with an initial molecular weight of

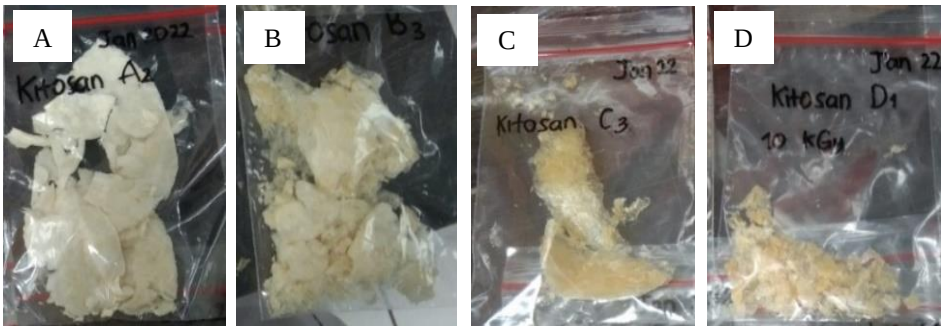

Figure 3. Discoloration of chitosan A (5 kGy, 5 mL), B (5 kGy, 10 mL), C (10 kGy, 5 mL), and D (10 kGy, 10 mL)

Table 1. Molecular Weight of Non-Irradiated and Irradiated Chitosan

Samples of chitosan	Mn (gram/mol)	Mw (kDa)	Mw/Mn	Mw percentage decrease (%)
Non radiation	115081	654.975	5.691	-
A (5 kGy, 5 mL)	74304	231.016	3.109	64.72
B (5 kGy, 10 mL)	101728	149.595	1.471	77.16
C (10 kGy, 5 mL)	79032	138.861	1.757	78.80
D (10 kGy, 10 mL)	50358	118.877	2.361	81.85

654 kDa, decreased to 231 and 149 kDa when irradiated with 5 kGy, and to 138 and 118 kDa when exposed with 10 kGy gamma rays. Based on these results, molecular weight reductions of 64.72%, 77.16%, 78.80%, and 81.85% were obtained, respectively.

This study produced a higher percentage of chitosan molecular weight reduction compared to the study by Puspitasari et al[4]. The reduction was only 52% at the 25 kGy dose and 73% at the 50 kGy dose. The difference in results is attributed to the condition of the irradiated chitosan samples[4]. The sample was in a dry state without the addition of H_2O. However, the addition of H_2O to chitosan before radiation is beneficial in the degradation process and leads to a higher percentage decrease in molecular weights. During irradiation, interactions occur between the radiation and the samples (chitosan) as well as the water molecules (water radiolysis)[11].

Table 1 shows that the radiation process of chitosan powder with conditions containing water molecules can reduce chitosan with a high molecular weight (654 kDa) to chitosan with a medium molecular weight (118 kDa). Additionally, the results indicate that adding 2 times the volume of H_2O in chitosan did not cause a 2-fold decrease in molecular weight at the same irradiation dose.

Radiation breaks the long chain of chitosan polymers at the 1,4-β-glycosidic bond, resulting in shorter molecular chain chitosan. Figure 4 shows the reaction mechanism for breaking the chitosan polymer chain.

Figure 4 explains that exposure to gamma-ray radiation can cause the formation of radicals at positions C_1, C_4, and C_5 followed by a process of rearrangement or fragmentation[17]. Radicals in each of these positions will break the chain of polymer or produce the opening of the chitosan ring followed by the formation of carbonyl compounds. The increase in C=O groups is a product of the chitosan fragmentation process. Radicals at positions C_1 and C_4 will initiate chain breaking in the (1,4)-β-glycosidic bond, while the presence of radicals at position C_5 will initiate the opening of chitosan pyranose rings. Breaking of (1,4)-β-glycosidic bonds causes shortening of the chitosan polymer chain and a decrease in the chitosan molecular weight. The mechanism of chain breakage in chitosan compounds during radiation exposure is shown in Equations 4-7.

where R–N and $R–NH_2$ are chitosan macromolecules, and $R^{\bullet}(C_n)$ are chitosan macroradicals located on carbon atoms Cn and $F_1^{\bullet}$, F_2 is a fragment of the main chain after breaking[23]. This chitosan chain breaking mechanism, where gamma rays hit the chitosan chain to produce chitosan macroradicals and hydrogen radical atoms (Equations 4 and 5), then the (1,4)-β-glycosidic chitosan radical bond breaks into two fragments (Equation 6). Chitosan macromolecules react with hydrogen radicals to produce chitosan macroradicals and ammonia compounds[27] (Equation 7).

If the sample is dry, polymer chain breaking is caused by the direct effects of ionizing radiation[28]. However, when a sample containing water is irradiated, most of the radiant energy is absorbed by the water. In this case, the process occurs mainly through indirect effects. Direct effects occur when ionizing radiation hits atoms contained in molecules, causing chain bonds to break. Indirect effects occur when radiation hits water molecules, which are the main components in cells, resulting in a radiolysis process in water molecules and the formation of free radicals[29].

The percentage value of molecular weight reduction in this study was higher than the previous research[20], due to the addition of H_2O. Meanwhile, the percentage value of molecular weight loss carried out in this study was lower than the study of Hien et al.[11] which used the addition of H_2O_2. Therefore, it was found that the addition of hydrogen peroxide is more effective than H_2O only in degrading chitosan at comparable gamma irradiation dose.

Functional Group Analysis and Degree of Chitosan Deacetylation

There were no significant structural changes in chitosan after exposure to radiation rays (5 and 10 kGy), as confirmed by the FTIR spectrum in Figure 5. This is consistent with the findings of Cheng et al.[30], where the FTIR results showed that the structure of chitosan produced after deacetylation is similar to commercially chitosan. Therefore irradiation does not cause deformation of chitosan compounds.

FTIR spectra from non-irradiated chitosan and irradiated chitosan show the functional groups of -OH and amine (-NH) in the range of ~3400-3600 cm^{-1}. The functional groups of C-H aliphatic in symmetric and asymmetric stretching showed at 2885-2942 cm^{-1}. The absorption of C=O from amide showed at 1663-1673 cm^{-1}. In addition, absorption bands at 1150-1157 cm^{-1} is associated with stretching of C-O-C bridging. The results of the chitosan spectrum obtained are in line with the previous results[31]. Most chitosan samples have the same band fingerprint area at 898 cm^{-1} that corresponding

$$R–H \rightsquigarrow R^{\bullet} (C_4–C_6) + H^{\bullet} \quad (4)$$
$$R–H + H^{\bullet} \rightarrow R^{\bullet} (C_1–C_6) + H_2 \quad (5)$$
$$R^{\bullet} (C_1, C_6) \rightarrow F_1^{\bullet} + F_2 \text{ (scission)} \quad (6)$$
$$R–NH_2 + H^{\bullet} \rightarrow R^{\bullet} (C_2) + NH_3 \quad (7)$$

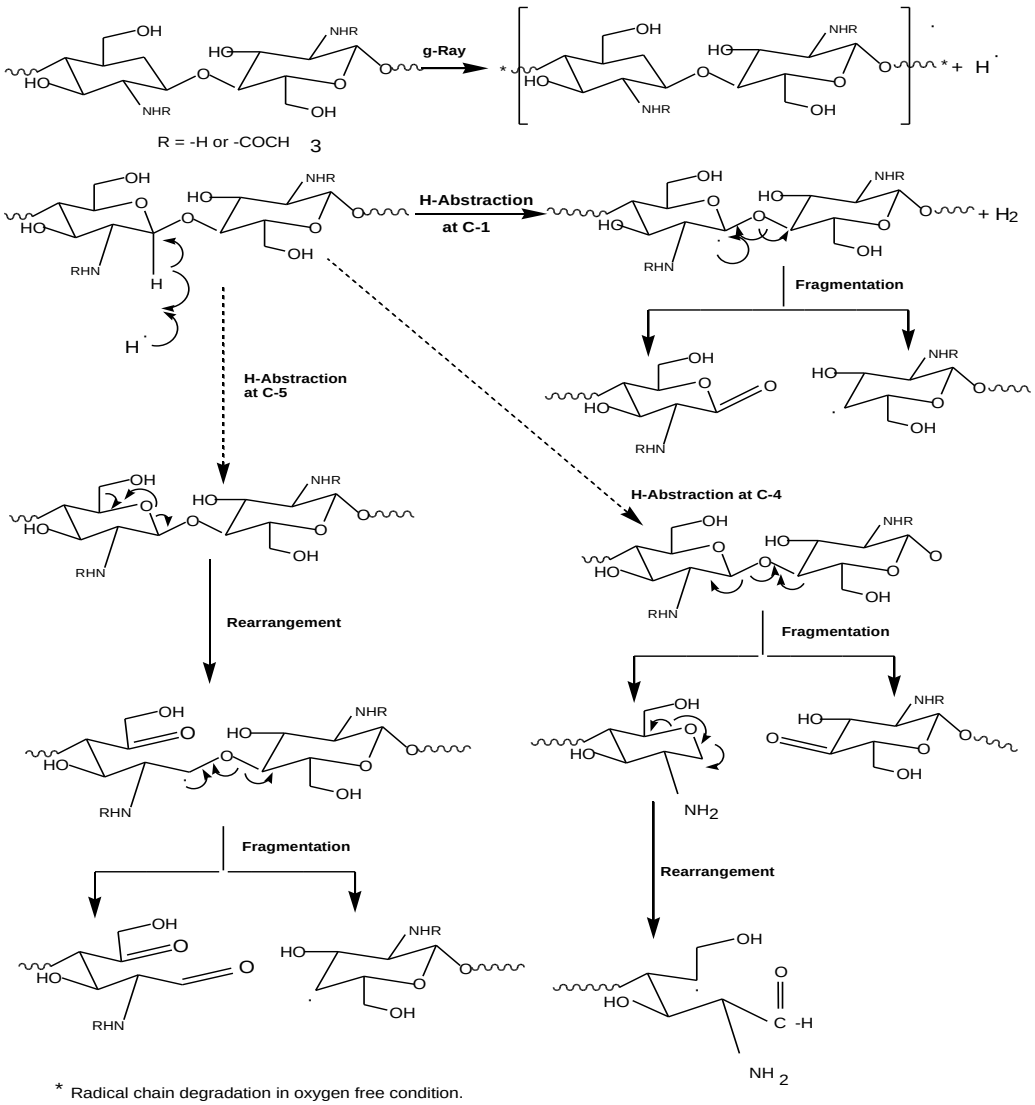

Figure 4. Chitosan polymer bonding termination mechanism[17]

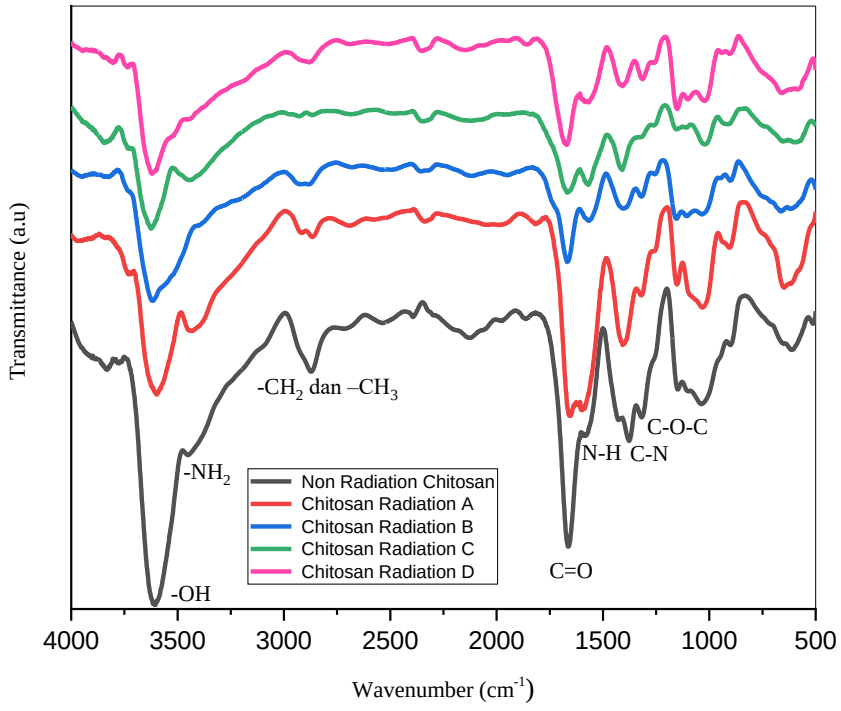

Figure 5. FTIR Spectrum of non-irradiated and irradiated chitosan

Table 1. Wavenumber of Non-Irradiated and Irradiated Chitosan

Functional groups	Wavenumber (cm^{-1})				
	Non-Irradiated Chitosan	Irradiated chitosan			
		A	B	C	D
O-H stretching	3608	3594	3624	3628	3621
–NH stretching	3451	3441	3394	3445	3431
C-H aliphatic (-CH_2 dan –CH_3)	2885	2913	2942	2936	2882
C=O amide bending	1663	1663	1666	1663	1673
N-H bending (NH_2)	1564	1585	1561	1568	1561
C-N bending	1380	1401	1401	1415	1408
C-O-C stretching	1150	1150	1157	1157	1154
β-1,4 glycosidic stretching (pyranose ring)	898	898	892	912	898

Table 3. The Value of the Degree of Deacetylation of Chitosan at Each Radiation Dose

Chitosan	Degree of Deacetylation (%)
Chitosan Non Irradiated	80.32
Chitosan A (5 kGy, 5 mL)	80.96
Chitosan B (5 kGy, 10 mL)	81.08
Chitosan C (10 kGy, 5 mL)	86.40
Chitosan D (10 kGy, 10 mL)	86.78

mainly to β-1,4-glycosidic. Peak absorption about 898-1150 cm^{-1} confirmed the ring of pyranose and saccharide structure in chitosan[5]. The details of wavenumber absorption peaks are shown in Table 2.

Based on Table 3, it can be seen that the degree of deacetylation increases gradually with the increasing of irradiation dose, as reported before[11]. However, there is no significant difference between the degree of deacetylation of non-irradiated and radiated chitosan results.

The radiation dose greatly affects the degree of deacetylation of chitosan, especially with 10 kGy that resulted in high degree of deacetylation (> 85%)[11]. All of the dose results met the quality standards of industrial chitosan according to Qingdao Yunzhou Biochemistry Co., Ltd which is > 80 %[32]. The degree of deacetylation rises due to the number of acetyl groups reduced because NH_2 functional groups protonation on the C_2 position of the repeating unit D-glucosamine[11]. Free amino groups in the form of NH_2 or in the protonated state of NH_3^+ group at those positions can affect the biological activity of chitosan.

4. CONCLUSIONS

The addition of H_2O to chitosan irradiated with gamma rays can reduce the molecular weight of chitosan up to 118 kDa. Gamma irradiation did not significantly affect the structure of chitosan. This is also supported by the results of functional group analysis between non-irradiated chitosan and irradiated chitosan that show the same functional groups. The irradiated chitosan has a degree of deacetylation (DD) in the range of 80-86%, that meets the standard of commercial chitosan quality.

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