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Study on gamma-irradiation degradation of chitosan swollen in H_2O_2 solution and its antimicrobial activity for E. coli

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Abstract: Degradation of chitosan in swollen state with hydrogen peroxide solution (5% w/v) by γ-irradiation was investigated. Molecular weight (M_w) of irradiated chitosan was determined by gel permeation chromatography (GPC). Fourier transform infrared (FT-IR) and ultraviolet-visible (UV-vis) spectrawere analyzed to study the structure changes of degraded chitosan. The results showed that the chitosan of low Mw ~30-45 kDawas efficiently prepared by γ-irradiation of chitosan swollen in hydrogen peroxide solution at low dose less than 20kGy. The main structure as well as the degree of deacetylation of the degraded chitosan was almost no significant change. Furthermore, the radiation degradation yield (G_s) was remarkably enhanced by the presence of H_2O_2. The obtained low Mw chitosan revealed high antimicrobial activity for *E. coli*that can be used for food preservation and other purposes as well.

Keywords: *Chitosan, degradation, E. coli, gamma-irradiation, hydrogen peroxide.*

I. INTRODUCTION

Chitosan, a biodegradable polymer, is generallyprepared by deacetylation of chitin from crab, shrimp shells and squid pens.Chitosan consists of glucosamine and N–acetylglucosamine units linked by β (1–4) glycosidic bonds. Chitosan is extensively applied in agriculture,pharmaceuticals and environmentaltreatment due to itsunique such as antibacterial,antifungal, antioxidant activity, plant growth promotion, inhibition effect against tumor cell [1] and so on. Recently, low molecular weight chitosan and oligochitosan have been gained considerable attentionsdue totheir goodsolubility compared to that of ordinary chitosan, so that the applications of chitosan can be improved.Therefore, it is necessary to prepare low M_w chitosan and its oligomerfor further studies.Most of previous research works on radiation degradation of chitosan was focused on irradiation of chitosan in powder/flake form and/or in solution [2-4]. Irradiation degradation of chitosan in gelated state with acetic acid using H_2O_2 as a radiation sensitizer was also reported by Kang et al. [5] and El-Sawy et al. [6].However, it was rather difficult to collectthe degraded chitosan product in powder product from irradiated mixturebecause of gelation of chitosan. According to Hien et al., low Mw chitosan prepared byγ-irradiation of

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chitosan in powderform required a dosehigher than 100 kGy, and the $G_s$value wasrather low about 0,1 μmol/J [3,4]. Thus, the high dose for degradation of chitosan may be not convenient to apply to large scale because of high production cost.

In this work, degradation of chitosan in swollen state with 5% H_2O_2 solution by gamma Co-60irradiationat low dose to prepare low Mw chitosanwas investigated. The G_s values of chitosan irradiated at different absorbed doses from 5-20 kGy were calculated. Furthermore, degraded low Mw chitosan powder product was easily collected by drying irradiated chitosan in forced air oven or even in open air at ambient temperature.The antimicrobial activity of resultant low Mw chitosan against *E. coli* was also tested.

II. EXPERIMENTAL

Materials

Chitosan from shrimp shell with M_{w0} of 91.7kDa (Polydispersity index, PI~2.26) and degree of deacetylation (DDA) of 91.3% was purchased from Newgreentechvn JSC., Vung Tau province, Vietnam. Before use, chitosan was dried in a forced air oven at 60°C to constant weight in order to remove moisture. Hydrogen peroxide was of reagent grade supplied by Merck, Germany. The Luria-Bertani (LB) medium for bacteria incubation was purchased from Himedia, India. The *Escherichia coli ATCC 6538* was provided by University of Medicine-Pharmacy, Ho Chi Minh city. All the other chemicals were of reagent grade and used as received without any further purification. Distilled water was used in all experiment.

Degradation of chitosan

Chitosan samples (2g) in powder form were swollen in 10 ml aqueous H_2O_2 solution with concentration of 5% (w/v) for 30 min. The ratio of chitosan and H_2O_2solution of 1/5 (w/v) was selected owing to the fact that this ratio almost reached to the saturated capacity of water binding of chitosan [7]. Then, these swollen samples were irradiated by ^{60}Co gamma rays on the Gamma Chamber 500, BRIT, India at the Nuclear Research Institute, Da Lat with the dose range of 0-20 kGy and the dose rate of 3.6 kGy/h at ambient temperature. For clarification of the influence of H_2O_2 in enhancement ofG_s ofradiation degradation process, a series of chitosan samples swollen in water (H_2O) at the similar ratio as forH_2O_2solution was also simultaneously irradiated. Then,the irradiated samples of chitosan were dried at 60°C in a forced air oven and ground into fine powder for characterization.

Characterizations

The M_w of degraded chitosan was measured by gel permeation chromatography (GPC)on an Agilent 1100 instrument equipped a detector RI G1362A using two columnsultrahydrogel model 250 and 500 from Waters (USA). The standards used to calibrate the column were pullulan (M_w 780-380.000). The eluent was aqueous solution containing 0.25M CH_3COOH/0.25M CH_3COONa with flow rate of 1.0 $ml.min^{-1}$and temperature at 30°C [8]. The concentration of chitosan sample was ca. 0.1% (w/v), and the injection volume was 50 μl.

IR spectra were taken on a Shimadzu FT– IR 8400S spectrophotometer in the range between 4000 cm^{-1} and 400 cm^{-1}usingKBr pellets . The DDA% of the degraded chitosan was calculated based on FT–IR spectra according to the following equation [9]:

$$A_{1320}/A_{1420} = 0{,}3822 + 0{,}0313(100 - DDA) \quad (1)$$

Where A_{1320} and A_{1420} were absorbances of chitosan at 1320 cm^{-1} and 1420 cm^{-1}, respectively.

UV-vis absorption spectra of chitosan samples were obtained using V630 UV-Visible spectrophotometer, Jasco (Japan) at the range of 200–600nm [10]. The concentration of chitosanwas 0.1% (w/v) and acetic acid of 0.05% (w/v) was used as reference sample.

Investigation of antimicrobial activity chitosan with different M_w

The different Mw chitosan powder was dissolved in 0.5 % acetic acid solution for the stock solutions with the final concentration of 1% (w/v) chitosan. The *E. coli ATCC 6538* was incubated overnight at 37°C in LB [11]. The culture obtained with the *E. coli* concentration of about 10^8 CFU/ml was used as the inoculated suspension in antibacterial test. Antimicrobial activity of chitosan with different Mw against E. coli was carried out as follows: 0.1 ml of chitosan stock solutions with different Mw particularly 30, 45, 60 and 91 kDa was added into sterilized water for the final volume of 10 ml with concentration of chitosan of 100 mg/l. The same volume of 0.5% acetic acid was used for the control sample. These samples were inoculated with 1 ml *E. coli* suspension of 10^8CFU/ml and shaken by vortex mixer in 5 min. After that, the surviving *E. coli* cells in each sample were evaluated at Quality Assurance and Testing Center 3 following the test method ISO 16649-2:2001. The antimicrobial efficiency (η%) was calculated based on following equation [12]:

$$\eta, \% = 100 \times (N_0 - N_i)/N_0 \quad (2)$$

Where N_0 and N_i are the cell forming unit per 1 ml (CFU/ml) of *E. coli* in the control and chitosan samples, respectively.

III. RESULTS AND DISCUSSION

Reduction of chitosan M_w

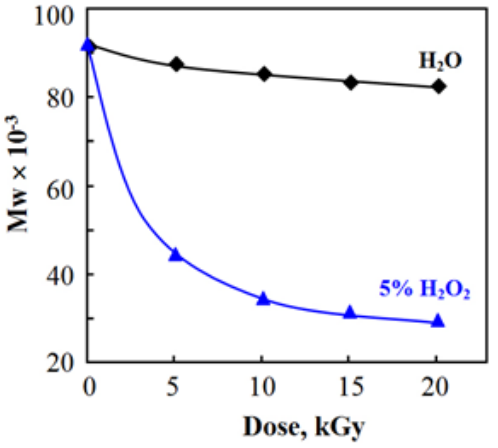

Fig. 1. The molecular weight of chitosan versus treatment dose (the dose rate 3.6 kGy/h)

Results in Fig. 1 showed the relationship of chitosan $M_w$versus treatment dose. Accordingly, the M_w of chitosan decreased with the increase of the absorbed dose. It was obvious from Fig. 1 that for the chitosan in swollen state with H_2O_2solution, the rapid drop of M_w was observed at the dose range from 0 to 10kGy, and then it slowed down gradually up to 20 kGy. Whereas the M_w of the irradiated chitosan sample swollen in water decreasedonly slightly. This can be explained that hydroxyl radicals (•OH) as powerful oxidizing agentwere formed by the radiolysis of water and H_2O_2as follows [13]:

$$H_2O \xrightarrow{\gamma\ ray} e^-_{aq}, H^\bullet, {}^\bullet OH, H_2O_2, H_2, H_3O^+ \quad (3)$$

$$H_2O_2 \xrightarrow{\gamma\ ray} {}^\bullet OH \quad (4)$$

Furthermore, e^-_{aq} and $H^\bullet$ can react with H_2O_2 forming an additional amount of •OH during the irradiation as follows:

$$e^-_{aq} + H_2O_2 \longrightarrow OH^- + {}^\bullet OH \quad (5)$$

$$H^\bullet + H_2O_2 \longrightarrow {}^\bullet OH + H_2O \quad (6)$$

The •OH radicals attack the chitosan molecule causing breakage of the β–1–4 glycosidic bonds. This means that •OH radicals were main agent for degradation process for chitosan in solution and/or in swollen state [14]. Consequently, γ-irradiation of chitosan swollen in H_2O_2 solution could efficiently reduce its Mw. The dose for degradation of chitosan to prepare low Mw chitosan was less than 20 kGy. Results in Fig. 1 also showed that the dose of less than 10 kGy should be effective due to most of the existing H_2O_2content decomposed at low dose and subsequently the resultant content of •OH radicals for the degradation of chitosan was less at higher dose.

Assuming that chitosan swollen in hydrogen peroxide solution is supposedly as a solution, then the G_s (mol/J) of chitosan can be calculated based on the following equation [15,16]:

$$(1/M_w - 1/M_{w0}) = G_s Dd/2C \quad (7)$$

Where M_{w0} and $M_w$are the weight-average molecular weight of polymer before and after irradiation, d is the solution density (kg/dm^3), D is the absorbed dose [Gy (J/kg)], and C is the concentration of polymer in solution (g/dm^3). In this swollen mixture of chitosan and H_2O_2solution, d was measured to be of 0.705 kg/dm^{3}and C was of 117.65 g/dm^3. The calculated G_s values were presented in Table I.

Table I: $G_s$values of irradiated chitosan at the different dose

Dose (kGy)	G_s(μmol/J)	
	γ-ray/H_2O	γ-ray/5% H_2O_2
5	0.031	0.741
10	0.024	0.591
15	0.022	0.454
20	0.019	0.375

Results in Table I indicated that G_s values of chitosan in swollen state with water were from 0.03 to 0.02 μmol/J in the dose range from 5 to 20 kGy, whereas $G_s$value of chitosan in swollen state with 5% H_2O_2 solution were drastically higher from 0.741 to 0.375μmol/J. Thus, G_s value for the radiation degradation of chitosan swollen with 5% H_2O_2solution was about twenty times higher than that of chitosan swollen with water. It was also recognized that the G_s values decreased as the increase of dose. Duy et al. (2011) studied on the synergistic degradation by γ-irradiation of 3% chitosan solution in the presence of H_2O_2 with the different concentration from 0-1% [2].They also reported that G_s values decreased with the increase of dose. Particularly, G_s values of irradiated chitosan in solution with the presence of 1% H_2O_2at dose of 4, 8, 12 and 20 kGy were 2.322, 1.400, 1.042 and 0.855 μmol/J, respectively [2].The G_s value for 5% chitosan solution without H_2O_2wasof 0.10 μmol/J [2]. Thus, the presence of H_2O_2 during irradiation can cause significant enhancement ofG_s, typically in swollen state. Therefore, degradation of chitosan in swollen state with H_2O_2 solution by γ-irradiation at low dose to prepare low Mw chitosan (30-45 kDa) can be potentially applied on large scale.

FT-IR spectra

Infrared spectroscopy has been extensively applied to characterize the structure and calculation DDA of chitosan owing to its simplicity. Fig. 2 described the FT-IR spectra of initial and degraded chitosan in swollen state with H_2O_2solution irradiated at 5, 10, 15 and 20kGy. It was obvious in Fig. 2 that the spectra of degraded chitosan exhibited most of the characteristic bands as the initial chitosan. The bands in the range of 1158 – 890 cm^{-1} corresponded to the characteristics of its polysaccharide structure. Peaks at 3450, 1650 and 1250 cm^{-1}were assigned to the hydroxyl, carbonyl, methyl and C–O–C groups, respectively [17]. The FT-IR indicated that there was no change in main structure of degraded chitosan compared to that of initial one. Peaks at 2920, 2872, 1423, 1265 cm^{-1} indicated the D-glucopyranose ring CH_2 group' symmetric and asymmetric vibration [9] were not altered. It indicated that there was no ring opening reaction. Furthermore, the absence of carboxyl groups as a transient product of glucopyranose ring cleavage–peak at 1730 cm^{-1} [2] and no simultaneous increase of peak at 1375 cm^{-1}that assigned to methyl group formed after ring–opening reaction [5]. The results of DDAin Table II also showed that the DDA of degraded chitosan was not significantly different from initial chitosan.

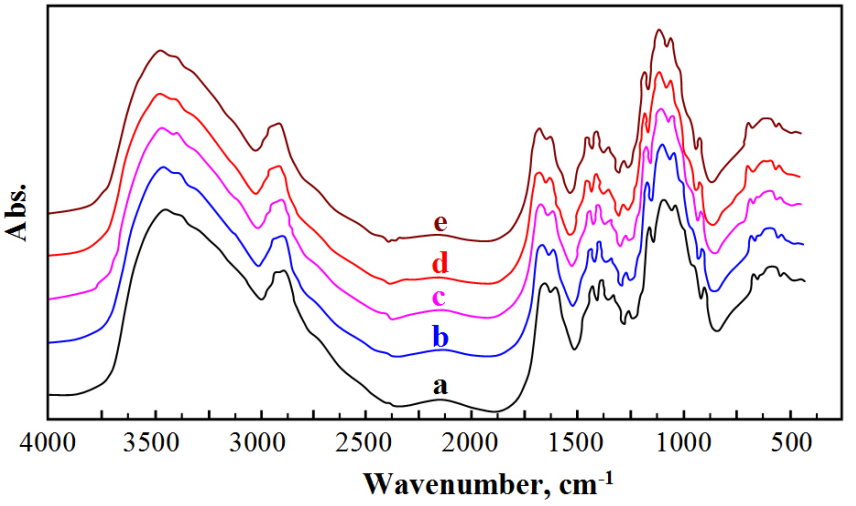

Fig. 2. FT-IR spectra of the initial (a) and degraded chitosan in swollen state with H_2O_2 solution at 5kGy(b), 10kGy (c),15kGy(d),and 20 kGy(e) absorbed dose

Table II: DDA% of initial chitosan and degraded chitosan in swollen state with H_2O_2 solutionat the different dose

Samples	Initial chitosan	Degraded chitosan			
		5 kGy	10 kGy	15kGy	20 kGy
DDA%	91.3 ± 0.3	92.1 ± 0.4	91.2 ± 0.3	90,4 ± 0.5	90.9 ± 0.7

UV-vis spectra

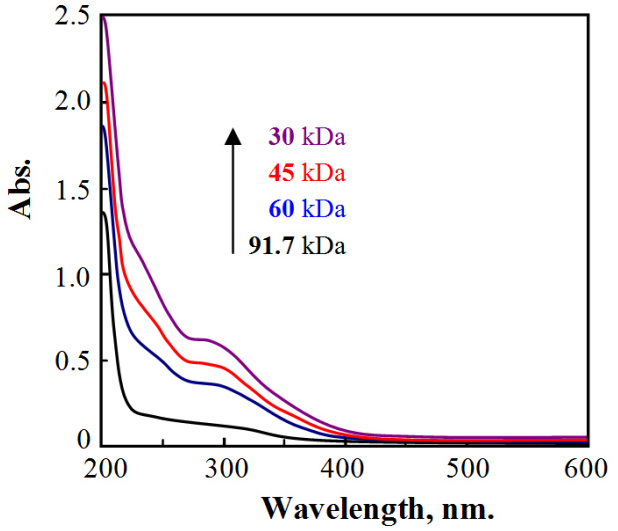

Fig. 3.UV-vis spectra of 0.1% w/v chitosan solution with the different M_w in 0.05% acetic acid solution

The UV-vis spectra of chitosan with the different M_w were showed in Fig. 3. It indicated that chitosan with M_w of 91.7 kDa (initial chitosan) had almost no absorbance in the wavelength range of 240-320 nm. Whereas irradiated chitosan had peak at 299 nm ascribed to the $n \rightarrow \pi^*$ transition for carbon-oxygen double bonds [17] that was evidence of the presence of carbonyl groups (C=O) of the irradiated chitosan [17,18]. It was likely that these carbonyl groups were the new side groups of degraded chitosan.

Antimicrobial activity of chitosan with different Mw

The antimicrobial activity of chitosan was reported to be influenced by its Mw,DDA%, concentration and pH of the medium [11, 12, 19-22]. Chitosan has been applied for improvement of quality and shelf life of foods mainly due to its antimicrobial activity [22]. Therefore, preparation of chitosan with high antimicrobial activity is of great interest.

Table III: Antimicrobial activity of chitosan with different Mw against *E. coli*

Samples	Control	Cts 91 kDa	Cts60kDa	Cts 45kDa	Cts 30 kDa
E. coli, CFU/ml	2.9×10^8	1.8×10^8	7.0×10^2	7.8×10^3	9.7×10^2
η, %	-	44.8276	99.9998	99.9973	99.9997

Results in table III indicated that chitosan Mw of 60 k Daprepared from 2.5 kGy irradiated chitosan swollen in 5% H_2O_2 (data not shown) exhibited the highest antimicrobial activity against *E. coli* with η~ 99.9998%.Lee et al. [21] and Qin et al. [11] also reported that the optimum of antimicrobial action was found for chitosan Mw of 50-60 kDa. Thus, the resultant low Mw chitosan can be used as antimicrobial agent for food preservation and for other purposes as well.

IV. CONCLUSIONS

Chitosan in swollen state with H_2O_2 solution was efficiently degraded by γ-irradiation. Low Mw chitosan (~30 kDa) could be obtained at low dose. FT-IR spectra indicated that there wasalmost no change ofthe DDA of degraded chitosan. UV-vis spectra suggested that the carbonyl groups were formed in the radiation degraded chitosan product. The G_s was remarkably enhanced in the presence of H_2O_2.The degraded chitosan with Mw ~ of 30-60kDawithhigh antimicrobial activity could be obtained by mild degradation of native chitosan. Thus, degradation of chitosan in swollen state with H_2O_2 solutionbyγ-irradiation isfeasibly applicable on large scale.

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