Research Article

Study on the Biological Effects of Oligochitosan Fractions, Prepared by Synergistic Degradation Method, on Capsicum

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Chitosan with an initial molecular weight (Mw) of approximately 193 kDa was degraded into about 11.4 and 14.8 kDa by γ -ray irradiation of the solution containing 5% chitosan in 0.2 M acetic acid at 75 kGy and the solution of 5% chitosan in 0.2 M acetic acid supplemented with 1% H₂O₂ at 10 kGy, respectively. The synergistic degraded chitosan sample with Mw ~14.8 kDa was separated into 5 fractions by using ultrafiltration membranes. The analysis results from UV, FTIR, and NMR spectra indicated that the combined treatment of low irradiation dose and low H₂O₂ concentration did not cause any change in the molecular structure of degraded chitosan fractions. Separated chitosan fractions with Mw > 1 kDa inhibited the growth of *Colletotrichum capsici* *in vitro*. While all separated chitosan fractions remarkably enhanced fresh biomass (11–56%) and chlorophyll content (20–92%) of capsicum seedlings. In the field test, the treatment with oligochitosan fractions with Mw in a range of 1–3 kDa (F₂) to 3–10 kDa (F₃) gained 9.0–11.4% of the fruit yield and reduced 64.8–67.2% of the rate of anthracnose disease outbreak fruits caused by *C. capsici*. Thus, the F₂ and F₃ fractions in degraded chitosan product are the key fractions for the enhancement of both the growth promotion effect and defense respond activity against the infection of pathogenic *Colletotrichum* fungi causing anthracnose disease in capsicum.

1. Introduction

Chitosan is the second abundant natural polymer after cellulose. This copolymer consists of β -D-glucosamine and β -N-acetyl-D-glucosamine in molecules and has been applied in agriculture as an antimicrobial agent [1–4]. Degraded chitosan products are well known as promising bioagents for the promotion of the growth and development, enhancement of enzyme activity, and phytoalexin induction in plants [2, 3].

Degradation of chitosan has been carried out by chemicals [5–8] or enzymes [9–11] in conventional methods. The enzymatic hydrolysis of chitosan has been performed by

chitosanase, hemicellulase, lysozyme, etc., but some difficulties are still remaining for large-scale industrial production [9]. The chemical method has been reported to have more conveniences than the enzymatic method. However, some problems have been shown in a purification process as well as in causing environmental pollution [5, 8]. Irradiation has been reported as a useful and efficient tool for the degradation of polysaccharides such as chitosan, alginate, and carrageenan [12–14] due to several advantages such as the degradation reaction which can be performed at room temperature; the degraded products can be used without any purification, the simplicity of controlling the whole process, and ease large-scale application. Recently, the synergistic

degradation method using γ -ray irradiation in combination with hydrogen peroxide treatment has been found as a very efficient way for the preparation of low Mw of polysaccharides: alginate, chitosan, etc., with a low dose [15–22].

Radiation-degraded products of alginate and chitosan have been found to promote the growth and development of flower and crop plants. Especially, the fraction with Mw in the range of 1–3 kDa of oligoalginate (separated from irradiated alginate) and oligochitosan (separated from irradiated chitosan) was presumed to contain effective compounds for the growth promotion, to enhance activity of phytoalexin enzymes, and to increase the yield of crop plants [12, 23]. In this study, the synergistic degraded method using γ -ray irradiation in combination with hydrogen peroxide treatment was performed to reduce the irradiation dose as well as to enhance the bioactive fractions in degraded product for plant growth stimulant and defense respond activity against the infection of pathogenic *Colletotrichum* fungi causing anthracnose disease in capsicum. The bioactive fractions prepared by the synergistic degradation method may apply as a natural elicitor and growth promoter for red pepper production.

2. Material and Methods

2.1. Materials. The red pepper plant TN278 (*Capsicum frutescens* L.) used in this study was supplied by Trang Nong Co. Ltd. Fungal strain namely *Colletotrichum capsici* used for the study was a gift from Nong Lam University. The media Potato Dextrose Agar (PDA) was supplied by Merck Co. Ltd., Germany. Chitosan 8B (about 80% deacetylation degree), a product of Funakoshi Co. Ltd., Japan, was used without further purification.

2.2. Synergistic Degradation of Chitosan. To prepare the sample for degradation, 5 g chitosan was kept overnight in 100 ml of 0.2 M acetic acid solution with and without hydrogen peroxide (1%) at room temperature for swelling and then stirred for 5 h. The prepared solution was then irradiated by γ -rays from a ^{60}Co source (GC-5000, BRIT, India) with the absorbed dose range up to 150 kGy and a dose rate of 3 kGy/h for degradation.

2.3. Mw Estimation. Mw values of the irradiated chitosan samples were performed at 40°C by a gel permeation chromatography (GPC, Tosoh, Japan) equipped with TSK gel PWXL columns (G6000PWXL, G4000PWXL, G3000PWXL, and G2500PWXL; Tosoh) in combination with a TSK guard column. Chitosan (0.1%, w/v) samples were eluted with an acetate buffer containing 0.2 M acetic acid and 0.1 M sodium acetate solution at a flow rate of 1 ml/min and monitored by an RI-8020 differential refractometer. The Mw value was determined from a calibration curve using pullulan standard samples.

2.4. Fractionation of Degraded Chitosan. A stirred ultrafiltration cell (model 8400, Amicon Co., USA) was employed for fractionation of degraded chitosan. A series of cellulose ultrafiltration membranes (Millipore, Co. Ltd. USA), YM (Mw cut-off 1000 (YM1), 3000 (YM3), 10,000 (YM10) and

30,000 (YM30)), was used with 0.1 M acetic acid solution. The content of oligochitosan fractions was detected by a ninhydrin method [24]. Each fraction was further purified through precipitation with ethanol.

2.5. Characterizations. UV-visible spectroscopy of irradiated chitosan solution was performed at 25°C using a Shimadzu spectrophotometer UV-2401PC in the range of 200–600 nm to determine the absorbance. 0.025% (w/v) 0.1 M acetic acid solutions consisting of 0.025% chitosan were used for analysis. The FTIR (Fourier-Transform IR) spectra of chitosan samples were carried out by an FTIR spectrometer (FT-IR 8400S, Shimadzu, Japan). About 3 mg of dried sample was mixed well with 100 mg of KBr and was prepared as a disk. All the spectra were measured over 128 scans in the range of 450–4000 cm^{-1} , where the resolution was 4 cm^{-1} . ^1H and ^{13}C -NMR spectra of fractionated oligochitosan were carried out by a Fourier Transformation NMR (Ultrashield 500 plus, Bruker Bioscience Corporation, USA). Fractionated oligochitosan with Mw of 1–3 kDa was dissolved in D_2O and CD_3COOD (Cambridge Isotope Laboratories, Inc., USA) with a concentration of 5 mg/l. ^1H and ^{13}C spectra were measured at 500 MHz for ^1H and 125 MHz for ^{13}C under proton decoupling conditions with 10,000 scans.

2.6. Elemental Analysis. For analyzing the contents of elements, samples were precipitated by methanol to remove the free nitrogen and dried at 40°C in a vacuum oven. Elemental contents of C, N, and H were analyzed by the CHNS Analyzer 2400 series II (PerkinElmer, Norwalk, U.S.A.) using glycine (Sigma-Aldrich, USA) as a standard sample.

2.7. Antifungal Activity Test. The antimicrobial activities of native chitosan, irradiated samples, and separated fractions against *C. capsici* were tested by the inhibition of mycelia using a culture medium toxicity method. 4 mm discs of mycelia were removed from a well-grown colony of tested fungi which were placed on plates containing PDA (potato dextrose agar) medium supplemented with 500 mg/l oligochitosan. Stock solutions of chitosan samples were diluted in 0.5% acid acetic solution and added to sterile molten PDA by a 0.22 μ membrane (Sartorius Co., Germany) to obtain the desired concentrations. All cultural plates were incubated at 25°C for 10 days in dark conditions. The antifungal effects against *C. capsici* of tested samples were evaluated by measuring the diameter of the mycelial colony and calculated as follows: inhibition efficiency (%) = $100 \times D/D_0$, where D_0 and D are the diameters of the colony of the control and studied samples, respectively.

2.8. Growth Promotion Test. The growth promotion effect of unirradiated chitosan and chitosan fractions on pepper was evaluated using ten 14-day-old seedling plants. Each seedling plant was cultivated in 500 ml solution containing 0.1% hyponex and chitosan samples. The controls were performed under identical conditions without chitosan supplement. All cultures were cultivated in a standardized greenhouse at the Research and Development Center for Hi-Tech Agriculture of Ho Chi Minh City. The plant height and fresh biomass (root and shoot) were determined after 14 days of cultivation.

2.9. Chlorophyll Determination. To determine the content of chlorophyll, 5 g of tested seedling leaves was grinded in 100 ml ethanol (95%), centrifuging for 10 minutes at 4.000 rpm and quantifying on UV-Vis Lambda 25 machine (PerkinElmer, USA) at wavelengths of 648 and 664 nm. Total chlorophyll content was calculated by the following formula: chlorophyll (mg/g) = (chlorophyll a + chlorophyll b) $\times$ k/m, where chlorophyll a = $13.36 \times OD_{664} - 5.19 \times OD_{648}$; chlorophyll b = $27.43 \times OD_{648} - 8.12 \times OD_{664}$; OD_{664} and OD_{648} are in succession of the optical density at 664 and 648 nm; and k is the dilution factor and m is the initial leaf weight [25].

2.10. Ex Vitro Test. The *ex vitro* effect of oligochitosan fractions against anthracnose on pepper was evaluated by spraying 100 mg/l oligochitosan fractions on 65-day-old capsicum plants. The control plants were performed under identical conditions without chitosan supplement. Oligochitosan fractions were applied by foliage spraying 3 times for every 7 days. 24 hours after the third spray, 50 capsicum fruits in a plant were selected and infected by causing a wound on the fruit before applying conidial suspensions of 10^4 spores of *C. capsici*/ml. Conidial suspension of *C. capsici* was prepared by scraping the mycelium from 10-day-old pure cultures and suspending them in sterilized distilled water before filtering through paper and testing by a hemocytometer [26]. The incidence on capsicum fruit was determined after spraying for 14 days by counting the number of disease outbreak fruits from 50 infected fruits. The fruit weight was also determined by weighing 50 fruits after their color converting into red. The theoretical yield was calculated as follows: theoretical yield (kg/1000 m²) = the individual yield $\times$ 1600, where the individual yield is the average of the total fruit weight harvested from a plant and calculated from 50 plants, 1600 is the actual total of individual capsicums planted in 1000 m² greenhouse.

2.11. Statistical Analysis. All experiments were repeated three times with nine replicates. Data were statistically analyzed using the ANOVA test. The means were compared using the least significant difference (LSD) at a 5% probability level, and the standard deviations were calculated.

3. Results and Discussion

3.1. Change in Mw of Chitosan by Radiation Degradation. It can be seen from Figure 1 that irradiation of the chitosan in acetic solution by γ -rays produced a decrease in the Mw with the increase of irradiation dose. The Mw of the irradiated chitosan samples was rapidly decreased at irradiation doses up to 75 kGy and then slowly decreased with the increase of irradiation dose up to 150 kGy. The irradiation dose of 75 kGy provided a low Mw chitosan sample with 11.4 kDa.

According to Qin et al. [5], the treatments of chitosan with H₂O₂ led to a decrease in the Mw of chitosan, but this method also resulted in some changes in the chemical structure. On the other hand, Duy et al. [15] were also successful on the preparation of oligochitosan samples with Mw in the range of 5–10 kDa by γ -ray irradiation of 3% chitosan solutions in the presence of 0.25–1.0% H₂O₂ at doses less than

FIGURE 1: Change in the Mw of chitosan by gamma irradiation with and without the presence of H₂O₂.

FIGURE 2: Fraction content of chitosan irradiated in 5% in acid acetic solution at 75 kGy and in the presence of 1% H₂O₂ 10 kGy.

10 kGy. Ionizing radiation combined with oxidizing agents, such as hydrogen peroxide, acts synergistically to degrade chitosan, thereby permitting a reduction in the radiation dose used to degrade polysaccharides. In our experiment, a 5% chitosan solution containing 1% H₂O₂ was degraded by γ -ray irradiation. The results revealed that the addition of H₂O₂ led to a rapid decrease in the Mw of the chitosan product at a dose of 10 kGy, and the Mw of the degraded chitosan irradiated at this dose was found about 14.8 kDa (Figure 1). The Mw of this product was close to that of chitosan irradiated at 75 kGy without H₂O₂ treatment (Mw ~11.4 kDa). Clearly, the addition of 1% H₂O₂ to the chitosan solution reduced the required irradiation dose nearly by 85%. According to Kang et al. [18], hydroxyl radicals are powerful oxidizing species that can attack the β -1-4 glycosidic bonds of chitosan. Hence, the radiation treatment on chitosan in the presence of hydrogen

FIGURE 3: UV spectra of chitosan fractions.

FIGURE 4: FTIR spectra of chitosan fractions.

FIGURE 5: ^1H -NMR (a) and ^{13}C -NMR (b) spectra of irradiated chitosan fraction with a Mw of 1–3 kDa.

peroxide could reduce its molecular weight very effectively and the primary reactions might further occur as follows:

TABLE 1: The content of C, N, and H elements in chitosan fractions.

Sample	Elements' content (%)			N/C	H/C
	C	H	N		
Unirradiated chitosan	42.12	7.39	7.59	0.180	0.175
F ₅ (Mw > 30 kDa)	42.31	7.38	7.59	0.179	0.174
F ₄ (Mw: 10–30 kDa)	41.98	7.41	7.54	0.180	0.176
F ₃ (Mw: 3–10 kDa)	42.26	7.37	7.56	0.179	0.174
F ₂ (Mw: 1–3 kDa)	41.75	7.33	7.54	0.181	0.176
F ₁ (Mw < 1 kDa)	41.96	7.28	7.52	0.179	0.173

FIGURE 6: The growth of *C. capsici* after 10 days of incubation on PDA media supplementation with water chitosan fractions.

FIGURE 7: The growth of *C. capsici* mycelium after 10 days of incubation on PDA media supplementation with water (a); acetic acid (b); chitosan fractions: F₁ (Mw < 1 kDa) (c), F₂ (Mw ~1 ÷ 3 kDa) (d); F₃ (Mw ~3 ÷ 10 kDa) (e), F₄ (Mw ~10 ÷ 30 kDa) (f), F₅ (Mw > 30 kDa) (g); and unirradiated chitosan (h).

These results are also in good agreement with our previous results on alginate [16] and other reports on chitosan [15, 17, 18, 20, 21] and cellulose [19].

3.2. Fractionation and Characterization. In our previous study, the fractions with the Mw in the range of 1–3 kDa and 3–10 kDa were found as trigger fractions in degraded chitosan products for plant growth promotion activity and phytoalexin induction in crop plants [23]. In this study, the degraded chitosan products irradiated at 10 kGy in the presence of H₂O₂ (Mw ~14.8 kDa) and at 75 kGy without H₂O₂ (Mw ~11.4 kDa) were separated into 5 fractions: F₁: Mw < 1 kDa, F₂: Mw range of 1–3 kDa, F₃: Mw range of 3–10 kDa, F₄: Mw range of 10–30 kDa, and F₅:

TABLE 2: Effect of irradiated chitosan fractions on the growth of capsicum seedling after treating for 14 days.

Fractions	Plant height (cm)	Chlorophyll content (mg/g leaf)	Fresh biomass (g/plant)
Control	12.10 ^c	101.49 ^d	0.27 ^d
F ₁	15.10 ^b	122.42 ^{cd}	0.36 ^{bc}
F ₂	19.45 ^a	195.27 ^a	0.63 ^a
F ₃	18.30 ^a	174.12 ^b	0.42 ^b
F ₄	14.90 ^b	124.96 ^c	0.33 ^{cd}
F ₅	14.80 ^b	101.90 ^d	0.30 ^{cd}

The mean values followed by the same letter within a column are not statistically different according to Duncan's multiple range test at $P < 0.05$.

FIGURE 8: The growth of capsicum seedling plants after 14 days of treatment with irradiated chitosan fractions.

TABLE 3: Effect of irradiated chitosan fractions on the rate of disease outbreak and yield of fruit in capsicum.

Fractions	Rate of disease outbreak fruit (%)	Fruit weight (g/fruit)	Individual yield (g/plant)	Theoretical yield	
				kg/1000 m ²	Gain percentage (%)
Control	76.4 ^c	0.64 ^c	882.45 ^c	1411.9 ^c	0
F ₁	38.0 ^b	0.68 ^c	898.03 ^c	1436.9 ^c	1.8
F ₂	11.6 ^a	0.95 ^a	982.90 ^a	1572.6 ^a	11.4
F ₃	9.2 ^a	0.89 ^b	961.74 ^a	1538.8 ^a	9.0
F ₄	43.6 ^b	0.86 ^b	921.38 ^b	1474.2 ^b	4.4
F ₅	70.8 ^c	0.68 ^c	917.53 ^b	1468.1 ^b	4.0

The mean values followed by the same letter within a column are not statistically different according to Duncan's multiple range test at $P < 0.05$.

Mw > 30 kDa. The results in Figure 2 showed that the distribution of five separated fractions in each product was quite different. The contents of low Mw fraction (F₁) were decreased in the product degraded by the synergic method, while other fractions were increased. In addition, Figure 2 also indicates that the high bioactive fractions (F₂ and F₃) were significantly increased in synergistic degraded products and fraction F₂ was found with the highest content (25.9%).

The chitosan fractions induced by the irradiation of 10 kGy in the presence of H₂O₂ were used for characterizing the change in the molecule structure. The UV spectra in Figure 3 indicated that a new band appears at wavelengths of 270–290 nm with the intensity increased by the decrease of the Mw of the fraction. According to Quin et al. [5] and Andradý et al. [27], this new band might indicate the end groups of chitosan molecules.

The FTIR spectra of the mentioned chitosan fractions in Figure 4 also indicated that there are no new peaks that appeared among the spectra of chitosan fractions and unirradiated chitosan. In addition, ¹H-NMR and ¹³C-NMR spectra of chitosan fraction F₂ with the Mw in the range of 1–3 kDa in Figure 5 also confirmed the conservation of the molecular structure in the separated chitosan fraction.

On the other hand, Kang et al. [17] reported that the combined treatments with 10% H₂O₂ during gamma irradiation at a dose up to 100 kGy did not cause any change in the mass ratio of N/C and H/C of chitosan after degradation. In this study, the contents of C, N, and H elements in the initial chitosan and radiation-degraded chitosan fractions are enumerated in Table 1. It can be seen clearly that mass ratios of N/C and H/C in radiation-degraded chitosan fractions were not significantly different and almost the same as those in the native chitosan sample. These results again proved the unchanged structure in the molecule of the separated chitosan fractions.

3.3. Antifungal Activity Test. The separated fractions of chitosan sample irradiated at 10 kGy in the presence of 1% H₂O₂ were added into PDA media for testing the *in vitro* antifungal activity against *C. capsici*. The results from Figures 6 and 7 indicated that fraction F₁ showed no inhibition effect on the growth of tested fungus; the F₂, F₃, and F₄ fractions slightly inhibited the growth of this pathogenic fungus, while the F₄ and F₅ fractions inhibited remarkably the growth of *C. capsici* mycelium on PDA medium. Among the tested samples, the F₄ fraction with Mw > 30 kDa showed the strongest direct effect on the inhibition of the growth of the tested

FIGURE 9: The images of capsicum fruits treated by various chitosan fractions.

pathogeneous fungus with efficiency almost the same to that of unirradiated chitosan.

3.4. Growth Promotion Activity of Irradiated Chitosan Fractions. The effect of chitosan fractions on the growth and development of plants was reported to depend on the Mw of the product, and the fraction with the Mw in the range of 1–3 kDa was found as the most active fraction for growth stimulation in barley and soybean [23]. In this study, the mentioned irradiated chitosan fractions were also used for testing on capsicum plants and the results are shown in Table 2 and Figure 8. It can be seen that the F_2 and F_3 fractions displayed a strong stimulation on the development of plant height and fresh biomass of the tested plants. One of the reasons may be due to the increase of chlorophyll in the leaves of treated plants.

On the other hand, the results from Table 3 revealed clearly that the treatment with irradiated chitosan fractions F_2 and F_3 significantly prevented the damage caused by an infested pathogenic fungus in fruits. In particular, the rate of disease outbreak fruits was decreased from 76.4% in the control plot to 11.6 and 9.2% by the treatment of the F_2 and F_3 fractions, respectively. In addition, the results from Table 2 and Figure 9 also indicated that the application with the F_2 and F_3 chitosan fractions significantly increased the average weight of fruit from 0.64 g/fruit (in the control plot) to 9.5 and 8.9 g/fruit (in the plots treated with F_2 and F_3 fractions, respectively). These results are the key factor for the gain of individual and theoretical yields of capsicum fruit.

4. Conclusions

Synergistic degradation by γ -irradiation in combination with hydrogen peroxide was a very efficient method for the degradation of chitosan. The degradation product with a Mw of 14.84 kDa induced by irradiation of 5% chitosan

solution containing 1% H_2O_2 at 10 kGy had a rather high rate of fractions F_2 (Mw: 1–3 kDa) and F_3 (Mw: 3–10 kDa) in content. The F_2 and F_3 fractions had novel activities on the promotion of the growth of capsicum plants, enhancement of fruit weight and fruit yield, and prevention of the damage caused by infested pathogenic fungus (*C. capsici*) in fruits. The synergic degradation method using γ -ray irradiation in combination with hydrogen peroxide treatment is an efficient and promising method for the production of oligochitosan with a very low irradiation dose and high content of bioactive fractions.

Data Availability

The data used to support the findings of the chitosan fractions of this study have been deposited in [13, 15, 16, 23, 24]. The data used to support the findings of the UV, FTIR, and NMR spectra of chitosan fractions in this study have been deposited in [5, 13, 27].

Conflicts of Interest

The authors declare that they have no conflict of interest regarding the publication of this article.

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