



Effect of Gamma Irradiation on Antibacterial Properties of Sea Crab Shell Chitosan

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Authors' contributions

This work was carried out in collaboration between all authors. FCKO designed the study, performed the statistical analysis, wrote part of the protocol, and wrote part of the first draft of the manuscript. AAG also wrote part of the protocol and part of the first draft. EAQ helped in the irradiation of the chitosan solution. YSA, DKA and CO managed the analyses of the study. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Effect of gamma irradiation on antibacterial activities of chitosan is described. Chitosan was prepared from crab shells via demineralization, deproteinization, decoloration and deacetylation. Chitosan solutions (2%) were prepared in 1% acetic acid and irradiated at 0, 5, 15 and 25 kGy. The degree of deacetylation and viscosity-average molecular weight of the chitosan were determined. Susceptibility tests of *E. coli* and *S. paratyphi* against the chitosan were determined. *E. coli* was more susceptible to lower concentrations of chitosan solutions. Irradiated chitosan in solutions exerted a slightly faster inhibition on both *E. coli* and *S. paratyphi* than the unirradiated chitosan solution, but there was no difference observed between irradiated and unirradiated chitosan in solutions after 48 hours of incubation. The degree of susceptibility of both *E. coli* and *S. paratyphi* to irradiated chitosan in solutions was not significantly affected by the irradiation dose.

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1. INTRODUCTION

Chitosan, an amino polysaccharide, has received much attention as a functional biopolymer for many diverse applications in food, pharmaceutical and cosmetics (Kumar, 2000; Shahidi et al., 1999). In many of these applications, specific molecular weights (Mw) of polysaccharides are required. Chitosan with an average Mw in the range of 5–10 kDa possesses strong bactericidal and superior biological activities (Kittur et al., 2003). Chitosan of 20 kDa prevents progression of diabetes mellitus and exhibits higher affinity for lipopolysaccharides compared to 140 kDa chitosan (Kondo et al., 2000). Chitoooligomers have special antimicrobial activity (Begona and Ruth, 1997; Zheng and Zhu, 2003) and antitumour activity (Qin et al., 2002).

Recently, the antimicrobial and antioxidant activities of chitosan and its derivatives have attracted attention. It is of great interest to degrade chitosan into low molecular weight fragments under appropriate conditions, as these low molecular weight chitosans possess useful biological activities. The antibacterial effects of chitosan and chitosan oligomers are reported to be dependent on the molecular weight, degree of deacetylation (DD), and the type of bacterium (Uchida et al., 1989; Jeon et al., 2001; No et al., 2002; Tsai et al., 2002). The mechanism of antimicrobial activity of chitosan and derivatives is still yet to be elucidated. However, chitosan molecules are reported to be stacked over the microbial cell surface, blocking the nutrients (Shon, 2001) or bind to DNA as such inhibiting transcription or permeability of the microbial cell wall (Tharanathan and Kittur, 2003). Kumar et al. (2007) reported of higher bactericidal activity against *Bacillus cereus* and *Escherichia coli* for homogeneous low molecular weight chitosans (LMWC) of molecular weight 9.5–8.5 kDa, obtained by pronase catalyzed non-specific depolymerization (at pH 3.5, 37°C) than native chitosan.

Low-molecular weight chitosan can be prepared by chemical, radiation, or enzymatic degradation of the high-molecular weight polymer. Radiation can provide a useful tool for degradation of different polymers. In the reaction, no other chemical reagents are introduced and there is not a need to control the temperature, environment or additives (Feng et al., 2008). Specifically, radiation can induce reactions such as chain scissions of the 1-4 glycosidic bonds which cause a reduction in molecular weight of the polymer and negligible cross-linking (Lim and Tung, 1997). Recent work by Feng et al. (2008) proved that lowering the molecular weight of chitosan increased antioxidant activity. However, research is necessary to determine the antimicrobial activity of chitosan as the molecular weight is reduced. The objective of this study was to assess the effect of gamma irradiation on the antibacterial activity of chitosan.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Sample collection and preparation

Crab shells were purchased from fishermen in Accra, Ghana. The shells were washed and then dried in the oven at 60°C overnight. The dried shells were ground in a moulinex

blender, sieved to a particle size of 90 µm and then packaged in polyethylene bag for storage at ambient temperature until used.

2.1.2 Reagents and media

All reagents used in the study were from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). These reagents were used without any further purification. Microbiological media were from Oxoid, United Kingdom.

2.2 Isolation/Production of Chitosan

Chitosan was produced from crab shells using the methodology of No et al. (1989) with some modification.

2.2.1 Demineralization

Crab shells were demineralized with 1N HCl for 30 minutes at ambient temperature with a solid to solvent ratio of 1: 15 (w/v) (No et al., 1989) with constant stirring and then filtered under vacuum. The retentate was washed for 30 minutes with tap water and oven-dried.

2.2.2. Deproteinization

Demineralized shells were deproteinized with 3.5 % (w/w) NaOH solution for 2 hours at 65⁰C with constant stirring at a solid to solvent ratio of 1: 10 (w/v) (No et al., 1989). The sample was filtered under vacuum, and the retentate washed with tap water for 30 minutes and oven-dried.

2.2.3 Decoloration

Demineralized and deproteinized Crab shells (crab chitin) were decolorized with acetone (1:10) for 10 minutes and dried for 2 hours at ambient temperature, followed by bleaching with 0.315 % (v/v) sodium hypochloride (NaOCl) solution (containing 5.25% available Chloride) for 5 minutes at ambient temperature with a solid to solvent ratio of 1: 10 (w/v), based on dry shell (No et al., 1989). Samples were then washed with tap water and dried under vacuum for 3 hours until the powder was crispy.

2.2.4 Deacetylation

Crab chitin was refluxed for 6 hours at 100⁰C using 50% concentrated sodium hydroxide solution (NaOH) with a solid to solvent ratio of 1: 15 (w/v). The resulting chitosan was washed to neutrality with tap water, rinsed with hot distilled water (90⁰C), filtered, and dried at 60⁰C for 24 hours in the oven.

2.3 Radiation

Samples of chitosan in solutions were irradiated at the Gamma Irradiation Facility at the Radiation Technology Centre (RTC) of the Ghana Atomic Energy Commission using a Co-60 source. Samples of 2g each were dissolved in 1% (v/v) acetic acid solution (100 mL), packaged in plastic container and samples irradiated at 0, 5, 15 and 25 kGy at a dose rate of 1.9846 kGy/hour.

2.4 Analysis

2.4.1 Degree of deacetylation

Degree of deacetylation was determined for unirradiated chitosan. Film prepared from the sample was used to study the degree of deacetylation (DD). The film was prepared by casting 1.0% w/v chitosan in 1% acetic acid solution, followed by drying in a vacuum air for 12 hr. The film was then deprotonated by washing 3 times with methanol and kept in a desiccator for 12hr, then placed in sealed plate before scanning. The spectra of the chitosan was obtained using a FTIR (Fourier Transform Infrared Spectroscopy) (FTIR-8400S CE, Shimadzu Corporation, Japan) with a frequency range of 4000 – 400 cm^{-1} . The degree of deacetylation of the chitosan was calculated using the baseline developed by Sabnis and Block (1997):

$$DD = 97.67 - [26.486 \times (A_{1655} / A_{3450})]$$

where A_{1655} and A_{3450} are the absorbance at 1655 cm^{-1} of the amide-I band (a measure of the N-acetyl group content) and 3450 cm^{-1} of the hydroxyl band as an internal standard to correct for film thickness.

2.4.2 Viscosity-average molecular weight (Mv) determination

Five chitosan concentrations of 0.20, 0.10, 0.05, 0.025 and 0.0125% (w/v) were prepared in 1% acetic acid for each of the irradiated chitosan samples (0, 5, 15 and 25 kGy). The relative viscosity measurement was performed by using an Ubbelohde capillary viscometer (size 1, Poulsen, Selve & Lee Ltd, England) at $25 \pm 1^\circ\text{C}$. The intrinsic viscosity is defined as:

$$[\eta] = (\eta_{red})C^{-1} \text{ at } C \rightarrow 0$$

Which is the value of the reduced viscosity (η_{red}) at zero concentration obtained from the linear plot of intrinsic viscosity against concentration. The viscosity molecular weight was calculated based on Mark Houwink equation (Chen and Hwa, 1996):

$$[\eta] = KM^a \text{ or } \log [\eta] = \log K + a \log M$$

where, $[\eta]$ is intrinsic viscosity, M is viscosity average molecular weight (in Dalton), K and a are empirical volumetric constants given by $8.93 \times 10^{-4} \text{ cm}^3/\text{g}$ and $a = 0.71$ respectively.

2.4.3 Antibacterial property of chitosan samples

2.4.3.1 Bacterial culture

Stock culture of *Escherichia coli* (*E. coli*) and *Salmonella paratyphi* (*S. paratyphi*) were reactivated on Eosin Methylene Blue Agar and Xylose Lysine Deoxycholate Agar, respectively, to obtain 24 hr cultures. An inoculum concentration between 10^7 - 10^9 cfu/ml was made by inoculating colonies into sterile trypticase soya broth (TSB, Oxoid) and used for susceptibility test.

2.4.3.2 Susceptibility test

Susceptibility test was done by the tube dilution method described by Sugumar et al. (2010) with some modification. Stock solutions were 2% chitosan in solutions irradiated at 0, 5, 15 and 25 kGy. Varying concentrations of 0.02, 0.04, 0.06 and 0.2% were prepared for each dose treatment and the final pH adjusted to 6.8. One millilitre (1 ml) of the inocula was added to 9 ml of each chitosan solution prepared and incubated at 37°C. One millilitre (1 ml) of the incubated mixture was taken into 9 ml of TSB at intervals of 0, 6, 12, 24 and 48 hrs for pour plating using Eosin Methylene Blue Agar (Oxoid, UK) for *E. Coli* and Xylose Lysine Deoxycholate Agar (Oxoid, UK) for *S. paratyphi*. Plates were incubated at 37°C and counts were made between 18 – 24 hrs. For each chitosan solution, triplicate plating was undertaken.

2.5 Statistical Analysis

ANOVA was performed on the average-molecular weight data using MINITAB 14 (Minitab Inc., USA). The level of significance used was $p < 0.05$ at 95% Confidence Intervals. Microsoft excel 2000 was used for graphical representation.

3. RESULTS AND DISCUSSION

3.1 Degree of Deacetylation

The degree of deacetylation as determined by FTIR was 80% (result not shown), which is comparable to values of 56 – 96% reported by No and Meyers (1995). Values ranging from 70 – 95% have also been reported (Canella and Garcia, 2001; Fernandez-Kim, 2004; Emi-Reynolds et al., 2007). Ocloo et al. (2011) have equally reported values of degree of deacetylation to be 76% for shrimp chitosan and 82% for commercial crab shell chitosan.

3.2 Viscosity-Average Molecular Weight (M_v) Determination

The molecular weight (M_v) of chitosan in solutions decreased significantly ($p < 0.05$) with irradiation dose (Fig. 1) as a result of degradation. A sharp decrease was observed when irradiation dose was increased from zero (0) to 5 kGy. Similar findings were reported by Pasaphan et al. (2010).

3.3 Antibacterial Property of Chitosan Samples

The 1% Acetic acid without chitosan had no effect on the populations of both *E. coli* and *S. paratyphi* after 48 hours incubation (Tables 1 and 2). Also, unirradiated chitosan solutions of 0.02, 0.04 and 0.06% concentrations reduced the population of *E. coli* by 1 to 2 log units after 24 to 48 hours incubation (Table 1). However, the 0.2% concentration of unirradiated chitosan solution reduced the population of *E. coli* by 2 to 6 log units after 6 to 48 hours (Table 1). Chitosan solutions of 0.02, 0.04 and 0.06% concentrations from irradiated samples (5, 15 and 25 kGy) reduced populations of *E. coli* by up to 3 log units after 12 to 48 hours but the solution of 0.2% concentration greatly reduced the population of *E. coli* by 2 to 6 log units.

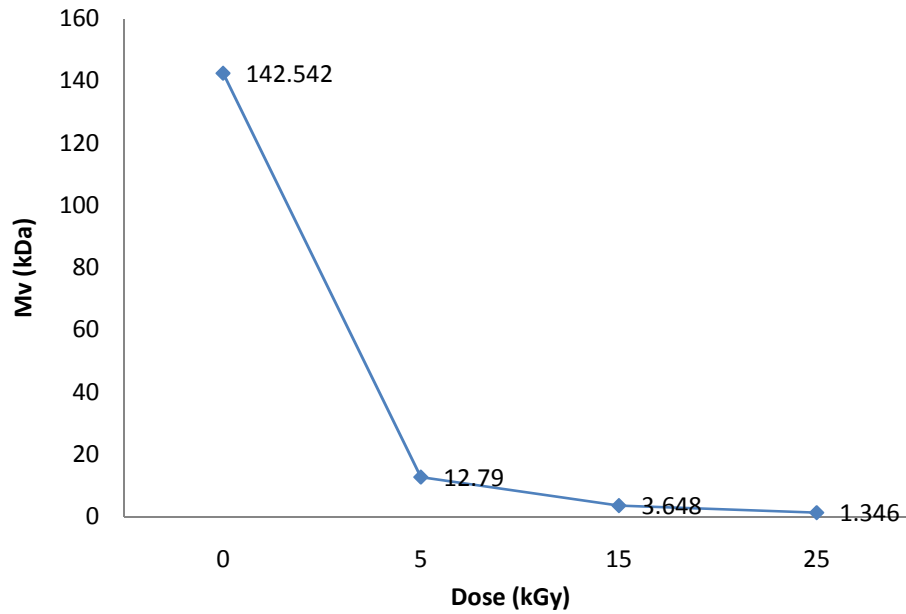


Fig 1: Effect of irradiation on viscosity-average molecular weight (Mv) of chitosan solutions

Table 1. Susceptibility of *E. coli* to irradiated chitosan solution at different concentrations

Dose (kGy)	Concentration of Chitosan (%)	0 hr	6 hrs	12 hrs	24 hrs	48 hrs
0	Control (1 % acetic acid)	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶
	0.02	> 10 ⁶	> 10 ⁶	3.0 x10 ⁵	7.3 x10 ⁴	4.2 x10 ⁴
	0.04	> 10 ⁶	> 10 ⁶	> 10 ⁶	5.2 x10 ⁶	2.3 x10 ⁵
	0.06	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶	3.1 x10 ⁴
	0.20	> 10 ⁶	1.7 x10 ⁴	1.3 x10 ⁴	0	0
5	Control (1 % acetic acid)	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶
	0.02	> 10 ⁶	> 10 ⁶	7.5 x10 ⁵	8.3 x10 ⁴	6.0 x10 ⁴
	0.04	> 10 ⁶	> 10 ⁶	4.8 x10 ⁶	8.2 x10 ⁵	4.3 x10 ³
	0.06	> 10 ⁶	4.0 x10 ⁵	1.8 x10 ⁴	3.4 x10 ⁴	6.7 x10 ³
	0.20	> 10 ⁶	4.9 x10 ⁵	1.2 x10 ⁴	0	0
15	Control (1 % acetic acid)	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶
	0.02	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶	6.3 x10 ⁵
	0.04	> 10 ⁶	> 10 ⁶	3.0 x10 ⁵	4.9 x10 ⁵	3.8 x10 ⁴
	0.06	> 10 ⁶	6.2 x10 ⁴	1.1 x10 ⁴	8.7 x10 ⁴	1.9 x10 ³
	0.20	> 10 ⁶	1.5 x10 ⁵	3.0 x10 ¹	0	0
25	Control (1 % acetic acid)	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶
	0.02	> 10 ⁶	> 10 ⁶	1.2 x10 ⁶	> 10 ⁶	8.1 x10 ⁴
	0.04	> 10 ⁶	> 10 ⁶	1.9 x10 ⁴	3.5 x10 ³	7.6 x10 ³
	0.06	> 10 ⁶	4.0 x 10 ⁶	1.3 x10 ⁵	1.5 x10 ⁴	8.3 x10 ³
	0.20	> 10 ⁶	7.5 x 10 ⁶	4.0 x10 ⁴	5.4 x10 ²	0

Each value is the average of 3 counts

On the other hand, unirradiated chitosan solutions of 0.02 and 0.04% concentrations had no effect on the population of *Salmonella paratyphi* after 48 hours incubation (Table 2). While the 0.06% concentration of unirradiated chitosan solutions gradually reduced population of *S. paratyphi* by 3 log units from 12 to 48 hours, the 0.2% concentration of unirradiated chitosan solution also reduced the population by 5 to 6 log units. Solutions of 0.02% concentration from irradiated chitosan (5, 15 and 25 kGy) had no inhibitory effect on the populations of *S. paratyphi* after 48 hours of incubation; however 0.04% concentration of chitosan solution irradiated samples (15 and 25 kGy) slightly reduced populations of *S. paratyphi* 1 to 2 log units after 24 to 48 hour. Solutions of 0.06% concentration from irradiated chitosan (5, 15 and 25 kGy) reduced populations of *S. paratyphi* by 1 to 3 log units after 24 to 48 hours. In the case of 0.2% concentration from irradiated chitosan in solution at 25 kGy, the population of *S. paratyphi* was reduced greatly by 1 to 6 log units from 6 to 48 hours.

Generally, all the irradiated chitosan solutions exerted a slightly faster inhibition (within 12 hours) on both *E. coli* and *S. paratyphi* than the unirradiated chitosan solution. However, there was no observable difference between the irradiated and unirradiated chitosan solutions after 48 hours of incubation.

The results of this study have shown that different concentrations of unirradiated and irradiated chitosan in solutions had varying degrees of inhibition against *E. coli* and *S. paratyphi*. However, 1% Acetic acid solution did not exert any noticeable effect on the population of the test isolates. These observations have demonstrated the antimicrobial activity of chitosan against *E. coli* and *S. paratyphi*, confirming results reported by Chen et al. (1998), Rhoades and Roller (2000), Roller and Covill (2000) and Tsai et al. (2000).

The study has also shown that the degree of microbial inhibition of chitosan was dependent on its concentrations in solutions. This observation supports the findings of Seo et al. (2008). The study further showed that the degree of inhibition of the various chitosan solutions is dependent on the duration of incubation.

Despite the fact that there are several intrinsic and extrinsic factors that affect the antimicrobial activity of chitosan, the viscosity-average molecular weight has been identified as vital factor. Various studies have confirmed that irradiation of chitosan reduces the viscosity-average molecular weight (Yoksan et al., 2004; Feng et al., 2008) and that chitosan with lower molecular weight (of less than 10kDa) have greater antimicrobial activity than native chitosans (Uchida, 1989). In this study also, an irradiation dose of 5 kGy significantly decreased molecular weight of chitosan but this did not proportionately enhance the antimicrobial activity. Although both *E. coli* and *S. paratyphi* were found be susceptible to irradiated and unirradiated chitosan solutions, the rate of inhibition of these test isolates was marginally increased by irradiation.

Generally, the study suggests that *E. coli* was more susceptible to lower concentrations of chitosan compared to *S. paratyphi* and that the degree of susceptibility of *E. coli* and *S. paratyphi* to irradiated chitosan in solutions was not significantly affected by the irradiation dose.

Table 2. Susceptibility of *Salmonella paratyphi* to irradiated chitosan solution at different concentrations

Dose (kGy)	Concentration of Chitosan (%)	0 hr	6 hrs	12 hrs	24 hrs	48 hrs
0	Control (1% acetic acid)	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶
	0.02	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶
	0.04	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶
	0.06	> 10 ⁶	> 10 ⁶	4.4 x 10 ⁴	1.9 x 10 ⁴	6.4 x 10 ³
	0.20	> 10 ⁶	> 10 ⁶	6.3 x 10 ⁵	2.0 x 10 ¹	0
5	Control (1 % acetic acid)	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶
	0.02	> 10 ⁶	> 10 ⁶	4.6 x 10 ⁶	> 10 ⁶	> 10 ⁶
	0.04	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶
	0.06	> 10 ⁶	4.0 x 10 ⁶	8.6 x 10 ⁵	1.3 x 10 ⁵	3.6 x 10 ³
	0.20	> 10 ⁶	6.4 x 10 ⁵	2.5 x 10 ²	1.0 x 10 ¹	0
15	Control (1 % acetic acid)	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶
	0.02	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶
	0.04	> 10 ⁶	> 10 ⁶	> 10 ⁶	4.7 x 10 ⁵	7.3 x 10 ⁴
	0.06	> 10 ⁶	> 10 ⁶	4.1 x 10 ⁶	6.2 x 10 ³	1.8 x 10 ³
	0.20	> 10 ⁶	2.3 x 10 ⁵	4.5 x 10 ²	4.0 x 10 ¹	0
25	Control (1 % acetic acid)	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶
	0.02	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶	> 10 ⁶
	0.04	> 10 ⁶	> 10 ⁶	7.6 x 10 ⁶	4.1 x 10 ⁶	8.6 x 10 ⁵
	0.06	> 10 ⁶	> 10 ⁶	2.7 x 10 ⁴	5.0 x 10 ³	2.2 x 10 ³
	0.20	> 10 ⁶	3.1 x 10 ⁵	3.1 x 10 ⁴	4.2 x 10 ²	0

Each value is the average of 3 counts

4. CONCLUSION

Microorganisms such as *E. coli* and *S. paratyphi* are susceptible to chitosan solution and this could be explored to improve food safety and stability. *E. coli* was more susceptible to lower concentrations of chitosan solutions and the degree of inhibition of both *E. coli* and *S. paratyphi* was marginally increased by irradiation. Irradiation decreased molecular weight of chitosan but the degree of susceptibility of *E. coli* and *S. paratyphi* to irradiated chitosan solutions was not significantly affected by the irradiation dose (0, 5, 15 and 25 kGy).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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