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Study on the Minimum Bactericidal Concentration (MBC) of Maillard reaction products of Chitosan and Glucosamine prepared by Gamma-irradiation Method

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Abstract: The Maillard reaction products of chitosan and glucosamine (CTS-GA MRPs) were formed by gamma Co-60-irradiation method and determined their minimum bactericidal concentrations (MBC). The mixed solutions of chitosan - glucosamine were irradiated with a dose range of 0-100 kGy to induce the Maillard reaction products (MRPs). The formations of MRPs were determined by spectrophotometric analyses and the contents of remaining glucosamine were evaluated by high performance liquid chromatography (HPLC). The antibacterial activities of CTS-GA MRPs were tested by agar well diffusion test and MBC determination test against both gram negative (*Escherichia coli*) and positive bacteria (*Bacillus subtilis*) at acidic and alkaline pH was also carried out. By agar well diffusion test, irradiated CTS-GA solutions were able to form inhibition growth zones on *E. coli* plate whereas on *B. subtilis* plate, only CTS-GA MRPs irradiated at 25 kGy expressed this ability. The results of the MBC determination test indicated that CTS-GA MRPs formed at 25 kGy exhibited highly antibacterial activity in pH 5 and even pH 7. Therefore, this CTS-GA MRPs could be used as a promising preservative agent applied for meat and meat-product preservation.

Keywords: *chitosan, glucosamine, Maillard reaction, gamma Co-60, antibacterial, MBC.*

I. INTRODUCTION

Chitosan is a de-acetylated derivative of chitin, the second-most abundant biopolymer in nature and commercially prepared from shellfish-processing waste [1, 2]. Some interesting characteristics of chitosan such as being non-toxic, biocompatible and biodegradable allow it to be widely applied in different areas. Indeed, chitosan derived from shrimp has achieved a GRAS (Generally Recognized as Safe) in USA from 2001 and thus scale up its use in many fields including food applications [3]. In addition, chitosan has reported to exhibit excellent antibacterial and antioxidant activities and has therefore attracted tremendous attention as a potential food preservative of natural origin.

However, despite these wonderful properties, chitosan-related applications are limited by its poor solubility at neutral or basic pH. For this reason, numerous researches have been carried out to improve the solubility and/or the biological activities of chitosan upon chemical and enzymatic modifications, in which chemical modifications are generally not preferred in food application due to the formation of potential detrimental products [2].

Maillard reaction is generally known as a nonenzymic browning reaction between the

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carbonyl group and the amino group, which occurs with heat treatment or irradiation process. This is a very complex reaction and usually produces a wide range of products, named as Maillard reaction products (MRPs), with odors and colors, antioxidant, antiallergenic, antimicrobial and cytotoxic properties [4, 5]. This reaction is considered as a desirable strategy to create chitosan derivatives with enhanced biology properties. Indeed, in the recent decade, several studies have reported the effectiveness of chitosan-MRPs in increasing the nutritional value as well as in preserving the quality of foods such as on fish, seafood, fruits and meats [6-9]. In fact, MRPs exhibited lesser safety issues than chemically modified products, and non-toxic chitosan derivatives can be obtained by adding the functional ingredients and/or controlling the key factors of the reaction [10]. Remarkably, beside heat treatment, Maillard reaction could be induced by irradiation method with tremendous advantages such as faster reaction rate without the need of temperature control as well as the addition of other chemical reagents. Recently, the effectiveness of gamma-irradiation on enhancing the chitosan bioactivities by the MRPs formation has been reported [5, 11]. Among the MRPs of chitosan with different sugar, chitosan-glucosamine MRPs prepared by heat-induced Maillard reaction have exhibited more excellent antibacterial activity than the others as well as the native chitosan against *E. coli* and *B. subtilis* [1]. However, so far there have barely been reports on the irradiation of chitosan and glucosamine solution, the formation of MRPs as well as further studies on their biological activities. The aim of this study was therefore to apply gamma irradiation as an induced method to form chitosan-glucosamine MRPs and determine their minimum bactericidal concentration (MBC) against both gram-negative (*E. coli*) and gram-positive bacteria (*B. subtilis*).

II. CONTENT

A. Material and methods

Materials: Chitosan from shrimp shell with the average molecular weight (Mw) of 123.5 kDa and the degree of deacetylation of 93.3 % was supplied by Oligo Company Limited, Vietnam. Glucosamine was purchased by Merk (Germany). The *E. coli* and *B. subtilis* were provided by Research Institute for Aquaculture No.2, Ho Chi Minh City and cultivated and preserved at Biology Laboratory, VINAGAMMA, Ho Chi Minh City. The Mueller Hinton Broth (MHB) and Mueller Hinton Agar (MHA) media used for bacteria incubation were purchased from Himedia (India). Other chemicals such as lactic acid, HCl, NaOH,… are used in analytical grade. Distilled water is used for all experiments.

Preparation of chitosan-glucosamine MRPs

The preparation of chitosan-glucosamine MRPs solutions was carried out according to the method of Rao et al. (2011) [5] with some modifications. A 1% solution of chitosan in acetic acid (0.5%) was prepared. Similarly, a 1% solution of glucosamine was prepared in distilled. Both above solutions were mixed with the ratio 1:1 (v:v) in order to obtain the chitosan 0.5% - glucosamine 0.5% mixture solution. The chitosan-glucosamine (CTS-GA) solution was exposed to γ-rays with different doses particularly 0, 25, 50, 75 and 100 kGy by a Gamma-cell 5000 (BRIT, Mumbai, India) with a dose rate of 1.5 kGy/h.

Spectrophotometric analyses

The irradiated solutions were characterized by spectrophotometric analyses described by Chawla et al. (2009) [12]. The as-prepared solutions were appropriately diluted and the absorbance was measured at 284 nm (early Maillard reaction products) and 420 nm (late Maillard reaction products) for determining UV absorbance and browning

intensity, respectively by a UV–vis spectrophotometer, Jasco-V630, Japan.

Determination of glucosamine content

The glucosamine contents of irradiated solutions were determined by high performance liquid chromatography (HPLC) method according to AOAC 2012 (2005.01) standard at Binh Duong Quality Control Centre, Vietnam. Maillard reaction efficiency was expressed as the ratio of reacted glucosamine to total added glucosamine by the formula:

$$\text{Maillard reaction efficiency (\%)} = (M_0 - M_t) \times 100/M_0 \quad (1)$$

Where M_0 and M_t are glucosamine content of the CTS-GA solution before and after irradiation, respectively.

Evaluation of antibacterial activity

The antibacterial activity of CTS-GA MRPs prepared at different doses in the range of 0-100 kGy was investigated against *E. coli* and *B. subtilis* in both qualitative and quantitative tests.

In qualitative test, the agar well diffusion method was used as described by Balouiri et al. [13]. The MHA plates after being spread by *E. coli* or *B. subtilis* (~ 10^4 CFU/ml) on the surface were punched aseptically with a sterile tip to form wells with a diameter of 6 mm. 100 µl of CTS-GA MRPs prepared by different doses of 0-100 kGy were introduced to the wells respectively. Then the plates were incubated overnight at 37ºC before monitoring the colony formation. The glucosamine solution was also tested by the same method as the control.

In quantitative test, the antibacterial activity against *E. coli* or *B. subtilis* of CTS-GA MRPs prepared at the suitable dose was investigated by MBC determination method in both acidic and basic pH (pH 5 and pH 7 respectively) conditions. MBC is defined as the least concentration of antimicrobial agent required to prevent the growth of microorganisms after subculture on antibiotic-free media [14]. In the present study, a modification of the dilution technique described by Owuama et al. (2017) for the determination of MBC was used [15]. Briefly, Mueller Hinton broth was used as the culture medium for both *E. coli* and *B. subtilis* and pH of the broth was adjusted to 5 by 1 N HCl or controlled at pH 7 [16]. The CTS-GA MRPs (1%) solution was dispensed into the sterile broth in fifteen-milliliter test tubes by a two-fold serial dilution technique to obtain the dilution ratios in the range of from 2^{-1} to 2^{-8}. A tube containing only the nutrient broth without the MPRs was served as the control sample. The 0.1 ml bacteria suspension of about 10^8 CFU/ml (*E. coli* or *B. subtilis*) was aseptically inoculated into the test tubes containing 10 ml of various concentration of CTS-GA MRPs in the nutrient broth. The tubes were incubated at 37^0C for 24 h and thereafter tested for the bacteria growth by agar disk-diffusion method [13] with some modifications. In short, filter paper discs (about 5 mm in diameter) were dipped in the solution of each tube separately and then placed on a sterile MHA plate. The petri dishes were then incubated at 37^0C for 24 h and subsequently observed for the colony formation. The MBC was determined as the lowest concentration of CTS-GA MRPs required to completely kill the bacteria after the incubation.

B. Results and Discussion *Formation of CTS-GA MRPs*

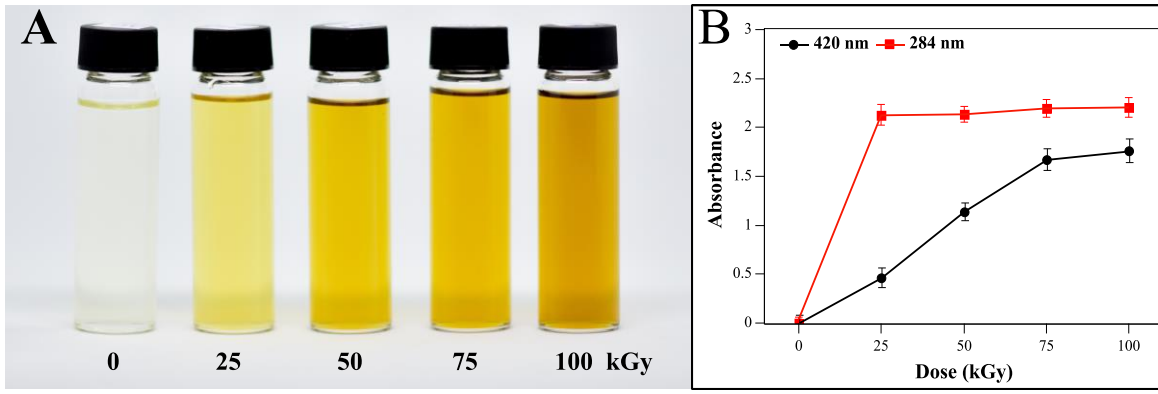

Fig. 1. The visual color (A) and the absorbance intensity (B) of the CTS-GA solutions irradiated in dose range of 0-100 kGy

During irradiation process, the visual color of the CTS-GA solution was changed from colorless to dark brown (Fig. 1A). This result was also confirmed by spectrophotometric analyses, namely there was the increase of absorbance intensity at 284 nm as well as 420 nm along with the increase of absorbed dose (Fig. 1. B). The same results were reported in other studies where the protein/sugar solutions were induced by heating [17, 18] or irradiating [11, 12, 19]. In Fig. 1B, the absorbance at 284 nm increased dramatically in dose range of 0-25 and then nearly steady up to the dose of 100 kGy while the absorbance at 420 nm increased regularly with the increasing dose. In Maillard reaction, the intermediate compounds that absorbed UV light (284 nm) were developed prior to the generation of brown pigments, named the late MRPs that absorbed 420 nm light [11]. Therefore, the results of spectrophotometric analyses indicated that during the irradiation process, the MRPs were formed, in which the formation of early MRPs were almost saturated at the dose of 25 kGy, while the late MRPs were produced continuously along with the dose up to 100 kGy.

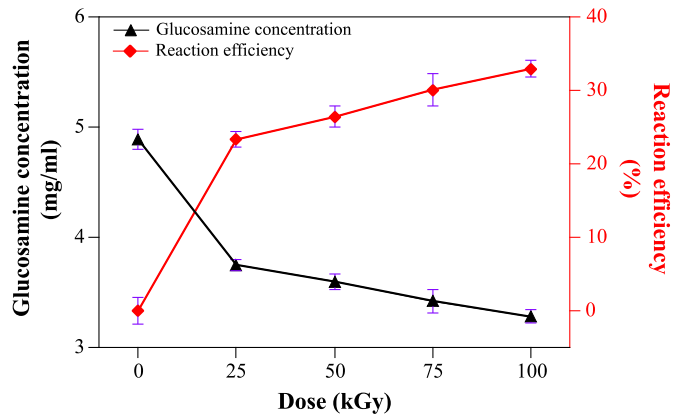

Fig. 2. The remaining glucosamine concentration and Maillard reaction efficiency of CTA-GA solutions irradiated in dose range of 0-100 kGy

The glucosamine concentration and Maillard reaction efficiency of CTA-GA solution prepared at different doses were expressed in Fig. 2. The results indicated that the glucosamine concentration of the CTS-GA solution decreased dramatically in the dose range of 0-25 kGy and then almost steady up to the dose of 100 kGy. The presence of glucosamine in the solution after irradiation process revealed that the initial glucosamine content was redundant for the Maillard reaction with 0.5 % chitosan solutions.

In addition, the Fig. 2 also showed that, Maillard reaction efficiency increased continuously along with the dose, in which the highest rate of the increase in efficiency was belong to the dose range of 0-25 kGy. This tendency was similar to the increasing of UV absorbance. Therefore, the result suggested that the as-calculated efficiency could be represented for the formation of the early MRPs because during irradiation, most of early reactions consumed glucosamine and caused the decrease of its concentration in the solution, while the late reactions mainly polymerized the intermediates, formed colored polymers [11, 12] and slightly affect the glucosamine concentration.

Evaluation of antibacterial activity

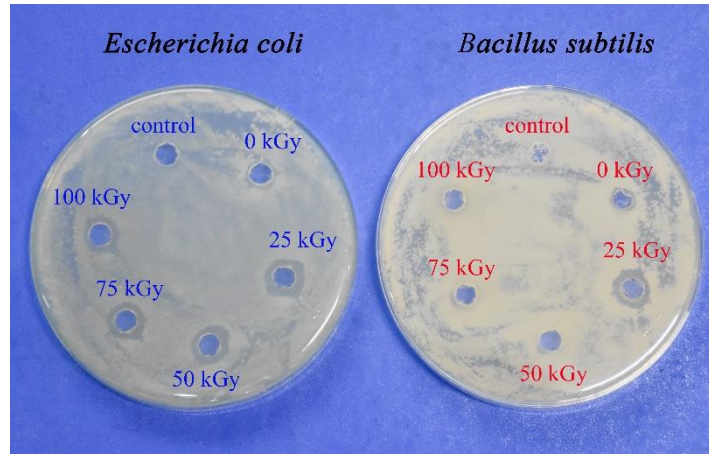

Fig. 3. Agar well-diffusion test of CTS-GA solutions irradiated in dose range of 0-100 kGy

In Fig. 3, the antibacterial activity of the CTS-GA solutions irradiated at different doses was investigated against *E. coli* and *B. subtilis* by agar well diffusion method. In the plate of *E. coli*, all CTS-GA solutions were able to form inhibition growth zones but the control (GA solution) was not and the zone diameter of irradiated solution were obviously larger than the unirradiated solution. In another hand, in the plate of *B. subtilis*, only CTS-GA solution and CTS-GA solution irradiated with 25 kGy presented the ability to form inhibition growth zones in contrast to other solutions. Interestingly, although GA solution did not show the antibacterial activity, the CTS-GA solution exhibited the activity against both the bacteria. This result proved that the antibacterial ability of CTS-GA solution was due to the role of chitosan which had been reported in many studies [16, 20 and 21]. The antibacterial activity of different antibiotics could be compared relatively by diameter of their inhibition growth zones formed on the plate, namely the larger diameters represent the higher antibacterial activity. Hence, results in Fig. 3 indicated that among the irradiated

solutions, 25 kGy-irradiated CTS-GA solution exhibited the highest antibacterial activity, even higher than the native CTS-GA solution. This result also revealed that the antibacterial activity of chitosan was significantly improved by irradiation-induced Maillard reaction. Furthermore, by the comparative result of the inhibition growth zones on the plates, *E. coli* were suggested more sensitive to chitosan and/or CTS-GA MRPs than *B. subtilis*. Rao et al. (2011) have reported the higher antibacterial activity of chitosan-glucose MRPs induced by gamma irradiation against *E. coli* than *Bacillus cereus*, another gram-positive bacterium belonging to genus *Bacillus* [5]. Chitosan glucose complex, prepared by heat-induced Maillard reaction, has also been reported to have a higher antibacterial effect on *E. coli* than *B. cereus* [2]. Finally, according to agar well diffusion test, the CTS-GA solution irradiated with 25 kGy, the highest antibacterial activity solution in this study was selected for further antibacterial investigations.

MBC determination test

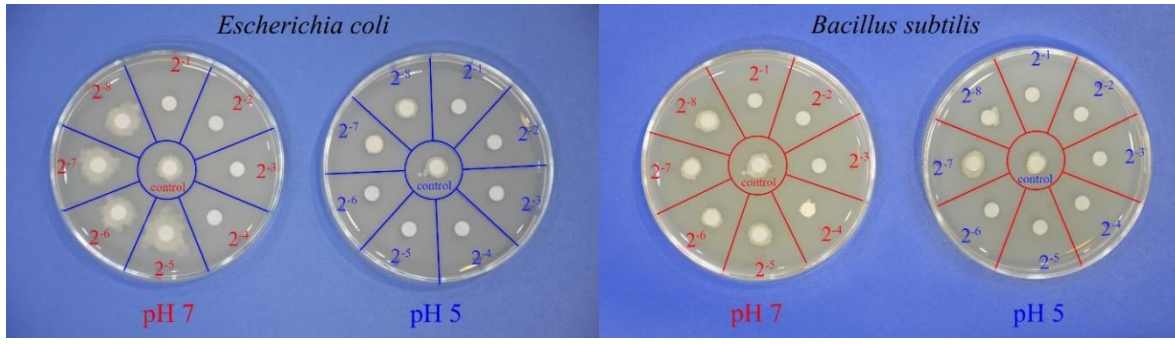

Fig. 4. Agar disk-diffusion test of the *E. coli* and *B. subtilis* suspensions in the serial dilution tubes

Table I. Minimum bactericidal concentration (dilution ratio and ppm) of CTS-GA MRPs solution prepared at the dose of 25 kGy against *E. coli* and *B. subtilis* at pH 5 or pH 7

	pH condition	*E. coli*	*B. subtilis*
Dilution ratio	pH 7	2^{-4}	2^{-3}
	pH 5	2^{-6}	2^{-6}
CTS-GA MRPs concentration (ppm)	pH 7	625	1250
	pH 5	156	156

The antibacterial activity of chitosan as well as its derivatives has been widely studied. Generally, there is a strong correlation between the antibacterial activity and the cationic amino group (NH_3^+) [1]. Table I listed the MBC of CTS-GA MRPs solution prepared at dose of 25 kGy against *E. coli* and *B. subtilis* at pH 5 or pH 7. At pH 7, the antibacterial of activity of the CTS-GA MRPs solution against *E. coli* was higher than that of *B. subtilis*. However, the equal effects on both the bacteria were true at pH 5 which recorded by the clear of the colony around the 2^{-6}-dilution dishes in Fig 4. The results again indicated that *E. coli* was more sensitive to chitosan and/or CTS-GA MRPs than *B. subtilis*, especially at pH 7. Moreover, the antibacterial of activity of the solution at pH 5 were greater than at pH 7. The same effect was also recorded for the chitosan solution (data not shown). Chung et al. (2005) have also reported the chitosan-glucosamine Maillard derivatives expressed higher antibacterial

activity of at pH 5 than at pH 7 by minimum inhibitory concentration (MIC) determination test, and the antibacterial activity was obviously greater than that of native chitosan at both pH [1]. The reason may be due to the fact that more amino groups (NH_3^+) are formed at pH 5 than pH 7, as determined from the pK_a (6-6.5) of the amino group in chitosan and its derivative [22]. According to results of the MBC determination test, the CTS-GA MRPs solution prepared by gamma irradiation method exhibited the effectively antibacterial activity at both acidic and alkaline pH. Thus it appeared to be a promising candidate for food applications as a natural preservative agent.

III. CONCLUSION

In conclusion, ionizing radiation, especially γ-rays from a Co-60 source, can be successfully applied to induce Maillard reaction in chitosan-glucosamine solution. The results of the present study demonstrated that the formation of CTS-GA MRPs together with the antibacterial activity improvement after the CTS-GA solution was irradiated. To the best of our knowledge, this is the first report to demonstrate the MBC of CTS-GA solution prepared by irradiation-induced Maillard reaction against gram-negative (*E. coli*) and gram-positive (*B. subtilis*) in both acidic and alkaline pH. However, more researches are need to determine the MBC of CTS-GA MRPs against other common food spoilage and pathogenic bacteria in order to demonstrate the potential application of these products as a natural preservative agent in food industry.

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