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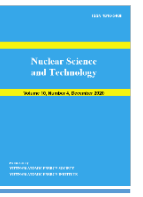

Maillard reaction products of chitosan and glucosamine: antibacterial and antioxidant activity

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Abstract: Maillard reactions between chitosan and glucosamine were induced by Co-60 gamma irradiation method and the antibacterial and antioxidant activities of resulting products were investigated. Briefly, a mixture of chitosan (1%) - glucosamine (0.5%) was irradiated with a dose range of 0-100 kGy. The Maillard reaction products of chitosan and glucosamine (CTS-GA MRPs) were analyzed by UV spectrophotometry, and residual glucosamine was determined by high performance liquid chromatography (HPLC). Antibacterial and antioxidant activities of the CTS-GA MRPs were investigated with radiation dose and pH by using directly contacted and $ATBS^{\bullet+}$ free radical scavenging methods. The results indicated that the CTS-GA MRPs formed at 25 kGy exhibited high antibacterial activity at both pH 5 and 7. On the other hand, antioxidant activity of CTS-GA MRPs increased with the increase of dose. The results also revealed that CTS-GA MRPs with high antimicrobial and antioxidant activities are potential candidates as preservative agents in food processing and cosmetics.

Keywords: *Chitosan, glucosamine, Maillard reaction, gamma Co-60, antibacterial, antioxidant.*

I. INTRODUCTION

In recent years, because of more and more consumer's awareness and concern regarding the safe of synthetic additives, number of publications on additives of natural origin has increased dramatically. Many natural compounds have been studied and used as safe additives because of their non-toxicity. These natural biomaterials are very diverse, including essential oils from plants, enzymes from animals, bacteriocins from microorganisms, organic acids and natural polymers from various sources [1]. Among of these compounds, chitosan has received considerable interest for commercial applications in medical, agricultural, chemical and food industry.

Chitosan, which is composed of D-glucosamine and N-acetyl-D-glucosamine, is a deacetylated derivative of the second most abundant biopolymer – chitin [2]. Chitosan is a well-known polysaccharide with nontoxic, biocompatible and biodegradable properties [3]. Therefore, chitosan and its derivatives have been intensively studied and applied in various field due to their antibacterial and antioxidative activities [4, 5]. In fact, chitosan has been approved as food additive in Japan and Korean since 1983 and 1995,

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respectively [6, 7]; and in 2001, shrimp-derived chitosan has archived a GRAS (Generally Recognized as Safe) for use in foods, including meat and poultry by US Food and Drug Administration [8].

The applications of chitosan as a preservative for many kinds of food have been widely reported in many studies, such as for fruit and vegetable [9, 10], seafood [11]; meat and meat products [2, 4, 8, 12, 13]. Unfortunately, the applications of chitosan are limited by its solubility, namely chitosan can only dissolve in acidic media while in neutral/alkaline media, chitosan is precipitated and reduced the biological activities as a result. Therefore, several studies have been carried out to improve the solubility and/or the biological activities of chitosan upon chemical and enzymatic modifications, in which chemical modification are generally not preferred in food applications [14].

The Maillard reaction, a non-enzymatic browning reaction, is a complex condensation reaction between carbonyl groups of reducing sugars, aldehydes or ketones, and amino groups of amino acids, proteins or any nitrogenous compounds [13]. Many studies have reported that a myriad of products are formed by Maillard reaction, generally termed Maillard reaction products (MRPs), exhibit strong antioxidant and antibacterial activities [15]. In addition, a MRP obtained by heat-induced Maillard reaction has been reported to have a relatively high antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* as compared with the native chitosan [16]. Therefore, formation of MRPs is a desirable strategy to modify chitosan with improved bioactivities. It also found that MRPs can be rapidly formed during gamma irradiation of chitosan-glucose admixture. This radiation condensation of MRPs does not produce any harmful by-product (5-hydroxymethylfurfural) like heat-induced Maillard reaction, as well as any other reagents [16]. However, up to now, there has been few reports on preparation of chitosan-glucosamine MRPs by gamma irradiation. The present study was carried out to investigate the formation of MPRs of chitosan and glucosamine by irradiation treatment. Radiation effect on efficiency of condensation reaction as well as antioxidant and antibacterial activities of resulting MPRs were also studied.

II. CONTENT

A. Material and methods

Materials: Chitosan from shrimp shell with the average molecular weight (Mw) of 123.5 kDa and degree of deacetylation of 93.3 % was supplied by a factory in Vung Tau province, Vietnam. Glucosamine was purchased by Merk (Germany). The *E. coli* ATCC 6538 was provided by Metabolic Biology Laboratory, University of Science, Ho Chi Minh City. The Luria- Bertani medium and agar plates used for bacteria incubation were purchased from Himedia, India. Ultra pure ABTS diammonium salt and potassium ferricyanide were products from Sigma-Aldrich. Other chemicals are in analytical grade. Distilled water is used for all experiments.

Preparation of chitosan-glucosamine MRPs

The preparation of chitosan-glucosamine MRPs solutions were carried out according to the method of Rao et al. (2011) with some modification [16]. A 2% solution of chitosan in acetic acid (1%) was prepared. Similarly, various solutions of glucosamine in distilled water were prepared with different contents of 1, 2 and 4 % respectively. The chitosan solutions were mixed to these glucosamine solutions with the ration 1:1 (v:v) separately in order to obtain three mixture solutions, namely A solution: chitosan 1% - glucosamine 0.5%; B

solution: chitosan 1% - glucosamine 1% and C solution chitosan 1% - glucosamine 2%. All solutions were exposured to γ-irradiation with doses in the range of 0–100 kGy by a Gamma-cell 5000 (BRIT, Mumbai, India) at the same dose rate of 2.2 kGy/h.

Spectrophotometric analyses

The irradiated solutions were characterized by spectrophotometric analyses described by Chawla et al. (2009) [18]. 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 content of irradiated solutions were determined by high performance liquid chromatography (HPLC) according to AOAC 2012 (2005.01) at Binh Duong Quality Control Centre, Vietnam. The efficiency of Maillard reaction was calculated as the ratio of reacted glucosamine to total added glucosamine as following:

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

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

Determination of antioxidant activity

Antioxidant activities of glucosamine, CTS and irradiated CTS-GA solutions were determined by $ATBS^{\bullet+}$ radical scavenging test described by Zhai et al. [19] and Chen et al. [20] with some modification. Briefly, $ATBS^{\bullet+}$ radical solution was prepared by mixing 7.4 mM ABTS and 2.6 mM $K_2S_2O_8$ in aqueous solution with the same volume and kept in the dark for 16h at room temperature, and then diluted by water to reach the optical density of 1 ± 0.1 as measured with UV-vis spectrophotometer at the wavelength of 734 nm (OD_{734}). 0.6 ml of each solution was thoroughly mixed with 1 ml $ATBS^{\bullet+}$ radical solution to obtain the desired concentrations. On another hand, 1 ml ABTS solution (without $K_2S_2O_8$) diluted with water was also added 0.6 ml of each solution with the same concentration for preparation of the blank samples. The OD_{734} measuring was carried out triplicate for each sample and the percentage of $ATBS^{\bullet+}$ radical scavenging was calculated as following equation:

$$ATBS^{\bullet+} \text{ radical scavenging (\%)} = (A_C - A_S) \times 100/A_C \quad (2)$$

Where A_C is the OD_{734} of the control ($ATBS^{\bullet+}$ radical solution and water) and the A_S is the OD_{734} of $ATBS^{\bullet+}$ radical solution and tested solutions.

Evaluation of antibacterial activity

The antibacterial activities of chitosan-glucosamine (CTS-GA) MRPs prepared by gamma irradiation at different doses were investigated against *Escherichia coli 6538* in both qualitative and quantitative tests.

In qualitative test, the agar well diffusion method was applied as described by Balouiri et al. [21]. The LB agar plates after being spread by *E. coli* (~ 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 with different irradiation doses of 0-100 kGy were introduced to the wells respectively. Then the plates were incubated overnight at 37ºC and monitored colony formation. The glucosamine solution was also tested by this method as the control.

The biological activities of chitosan, such as antibacterial activity, are highly

dependent on its solubility. Native chitosan only dissolves in acidic media and precipitates in neutral/alkaline media. Therefore in quantitative test, the antibacterial activity of CTS-GA MRPs against *E. coli* was investigated in both acidic and alkaline medium, namely at pH 5 and pH 7 respectively. Briefly, 1 ml CTS-GA MRPs solutions were simultaneously added into 19 ml *E. coli* suspensions (10^7 CFU/ml), in which the pH was already adjusted to 5 and 7 by lactic acid 0.5 % and/or NH_4OH 5% solution. Then the mixtures were shaken at 150 rpm for 4 hours and subsequently determined the survival cell density by spread plate technique. The control sample only containing bacteria suspension and water was carried out parallel. The antimicrobial activity of the CTS-GA MRPs was expressed by the reduction of bacteria density (log CFU/ml) in the testing mixture in comparison with the control.

B. Results and discussion

Formation of CTS-GA MRPs

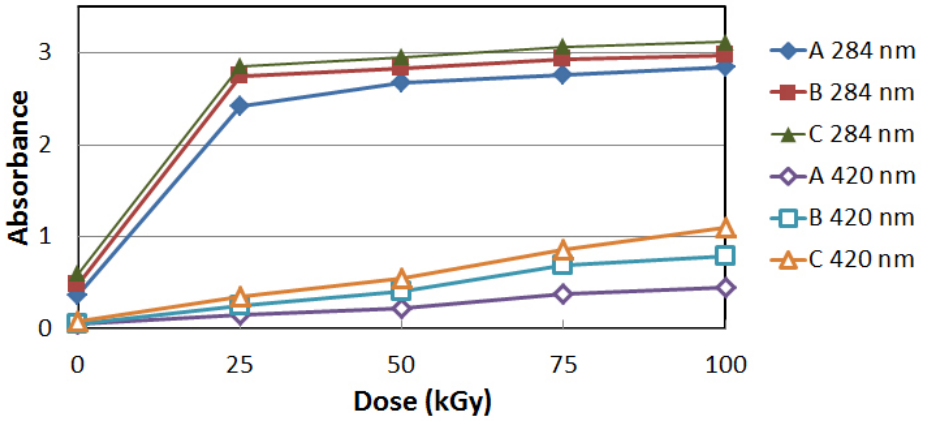

Fig. 1. UV absorbance (284 nm) and browning (420 nm) of irradiated CTS-GA solutions at various irradiation doses (A: CTS 1% - GA 0.5%; B: CTS 1% - GA 1% and C: CTS 1% - GA 2 %).

There was a change in visual color of the CTS-GA solutions from colorless to dark brown during irradiation process. Moreover, the increases in UV absorbance and browning intensity of CTS-GA solutions with irradiation dose were also observed as in Fig. 1. The same results were recorded in other studies where the CTS/sugar solutions were treated by heating [17] or irradiating [16]. In addition, although the CTS:GA ratio was different, the various solutions had a similar change in UV absorbance and browning intensity, namely 284 nm absorbance increased dramatically in dose range of 0-25 and then nearly steady up to the dose of 100 kGy while the 420 nm absorbance increased regularly with the increasing irradiation dose. In Maillard reaction, the UV absorbance intermediate compounds were developed prior to the generation of brown pigments. Therefore the results of spectrophotometric analyses indicate 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.

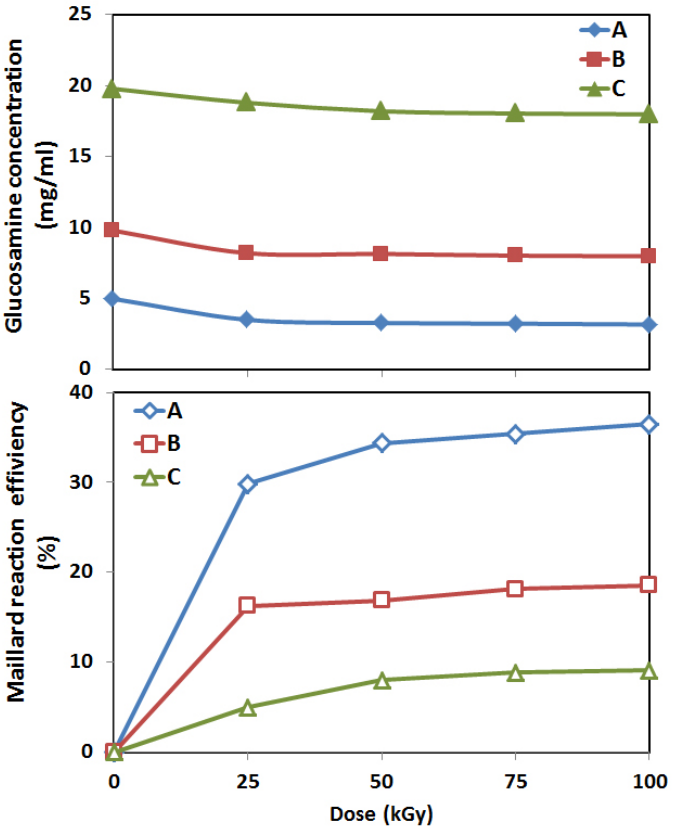

Fig. 2. The glucosamine concentration and Maillard reaction efficiency of CTA-GA solutions versus irradiation dose (A: CTS 1% - GA 0.5%; B: CTS 1% - GA 1% and C: CTS 1% - GA 2%).

The effects of glucosamine concentration in CTA-GA solutions and radiation dose on Maillard reaction efficiency were presented in Fig. 2. The results showed that the glucosamine concentration in the initial solution of CTA-GA decreased dramatically by gamma radiation in the dose range of 0-25 kGy and then almost steady up to 100 kGy. The presence of glucosamine in the remaining solution after irradiation implied that the glucosamine content in the initial was redundant for the condensation reaction of MRPs. The decrease of glucosamine in the CTA-GA solution irradiated at 100 kGy, was about 1.8 mg/ml (data not shown). This could be the suitable concentration of glucosamine for investigating Maillard reaction.

In addition, the results in Fig. 2 also showed that in all solutions, Maillard reaction efficiency increased along with the irradiation dose, in which the highest rate of the increase 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, only early reactions consumed glucosamine and caused the decrease of its concentration in the solution, while the late reactions just polymerized the intermediates, formed colored polymers [18, 22] and did not affect the glucosamine concentration.

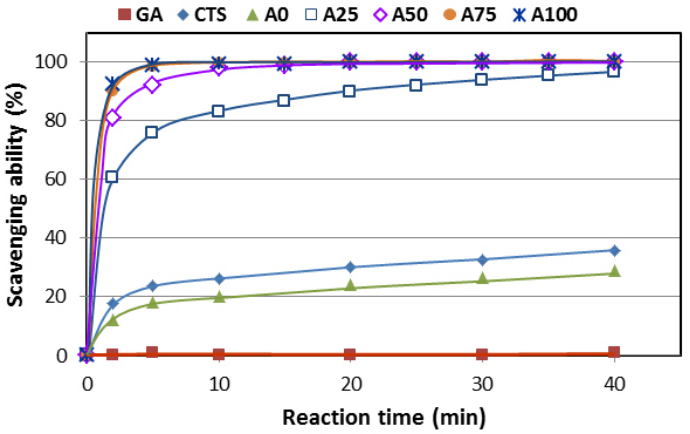

Fig. 3. The relationship of $ATBS^{\bullet+}$ radical scavenging ability versus reaction time (GA: glucosamine; CTS: chitosan; A0, A25, A50 and A100 were the A solutions irradiated with the dose of 0, 25, 50, 75 and 100 kGy respectively).

Determination of antioxidant activity

The results presented in Fig. 3 indicated that all chitosan- contaning solutions (CTS and CTS-GA solutions) exhibited $ATBS^{\bullet+}$ radical scavenging ability while GA solution did not. Moreover, the $ATBS^{\bullet+}$ radical scavenging ability of chitosan solution was higher than CTS-GA solution (A0 sample). This finding was suggested to be due to the obstacle of glucosamine over chitosan on scavenging $ATBS^{\bullet+}$ radical. Furthermore, the irradiated CTS-GA solutions manifested high ability on scavenging $ATBS^{\bullet+}$ radical in dependence on the irradiation dose and reaction time, namely the higher irradiation dose and/or reaction time the higher $ATBS^{\bullet+}$ radical scavenging capacity. The formation of antioxidant compound by heating sugar-amino solution has been reported [23]. This result indicated that MRPs formed upon irradiation of chitosan-glucosamine solution possessed significant antioxidant potential. In addition, the dose-dependent antioxidant activity of irradiation treating chitosan-glucose solution has also been recorded in other study [16]. As the above discussion, during irradiation treatment, the early MRPs was almost saturated at the dose of 25 kGy, while the late MRPs were produced continuously up to 100 kGy, so this results suggested that the formation of antioxidant compounds were mainly taken place at the late stage of Maillard reaction.

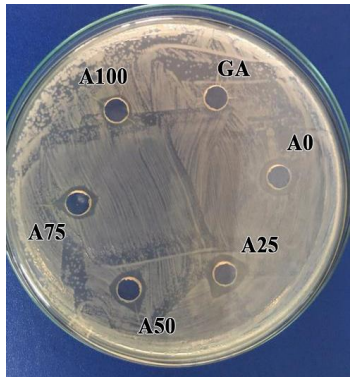

Fig. 4. The result of agar well diffusion test (GA: glucosamine; A0, A25, A50 and A100 were the A solutions irradiated with the dose of 0, 25, 50, 75 and 100 kGy respectively).

Evaluation of antibacterial activity

In Fig. 4, the A solutions prepared at different irradiation doses were able to form inhibition zone against *E. coli* while the GA sample was not. This meant that glucosamine did not exhibit the antibacterial activity in contrast to other A samples. Interestingly, around the well of A0 sample (a CTA-GA solution without irradiation), the presence of inhibition zone indicated that the antibacterial activity of this solution was due to the role of chitosan. The antibacterial ability of samples could be primarily compared through the diameters of their inhibition zones formed on the plate [21], therefore the result indicated that the antibacterial activity decreased obviously in A25, A50, A75 and A100 sample respectively.

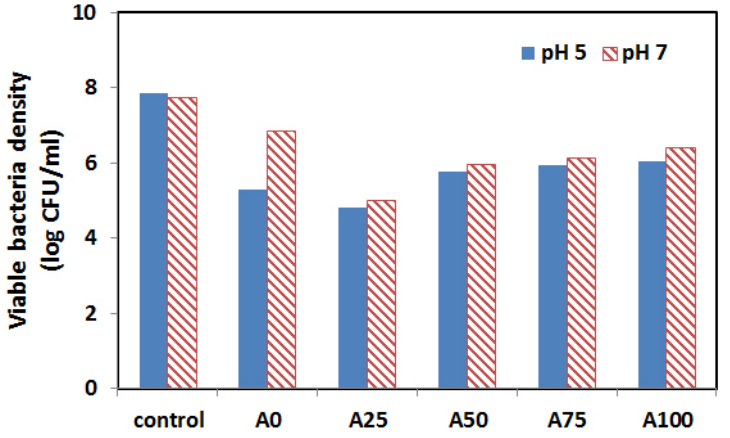

Fig. 5. Viable bacteria density of the suspension after exposing time (A0, A25, A50 and A100 were the A solutions irradiated with the dose of 0, 25, 50, 75 and 100 kGy respectively).

Modification of chitosan via Maillard reaction has been widely studied and it is suggested that MRPs produced from chitosan-sugar model system have been associated with the formation of compounds with high antibacterial [16, 22, 24]. However, there is no information of the influence of irradiation dose on the information of these compounds. Thus, in this study the antibacterial activities of MPRs prepared with different dose were examined and further compared with chitosan. Namely, after exposing time, the bacterial cell density of the suspensions at pH 5 and 7 was determined and described in Fig. 5. The result revealed that the antibacterial activity of A0 sample was affected deeply by the pH value, namely been high at pH 5 and low at pH 7. As above discussion, the antibacterial activity of A0 sample was mainly contributed by chitosan, which was precipitated and reduced its bioactivity at neutral or alkaline solution, hence the antibacterial activity of A0 sample at pH 5 was greater than at pH 7. This finding is totally in concurrence with other studies where the pH-dependent antibacterial activity of chitosan has been reported [17, 25]. In addition, the obtained results also indicated that all testing samples had the lower bacterial cell density in comparison with the control, this meant that these samples exhibited an effective antibacterial activity against *E. coli* at both pH 5 and 7. The lower viable bacteria density represented the stronger antibacterial activity. Therefore at pH 5 and 7, the antibacterial activity of irradiated samples decreased along with the increasing dose and A25 was the most antibacterial sample. This record completely matched with the results of agar well diffusion

test above. Furthermore, the higher antibacterial activity of chitosan-glucosamine derivatives prepared by heat-induced Maillard reaction than acid-soluble chitosan was also recorded in the study of Chung et al. (2005). In addition, because during irradiation treatment, the early MRPs were created prior to the formation of late MRPs so the results above suggested that the antibacterial activities of irradiated solutions were probably due to the role of early MRPs. Interestingly, the antibacterial activities of MRPs in irradiated solutions were maintained at high level at both pH 5 and 7. Hence, this result is one of the most demonstrations of Maillard reaction effectiveness in chitosan modification strategies.

III. CONCLUSIONS

This study demonstrated that the Maillard reaction can be easily occurred in the CTS-GA admixture solution by gamma irradiation. Moreover, the concentration of glucosamine suitable for Maillard reaction is much lower than the concentration of chitosan in the CTS-GA admixture solution, namely about 0.18% GA for 1% CTS (w/w), and as-prepared CTS-GA MRPs exhibited high antioxidant activity and strong antibacterial activity at pH 5 and 7. These findings indicated that CTS-GA MRPs can be used as potential natural products replacing for synthetic additives in food or cosmetic. Further studies are necessary to elucidate mechanism of compounds formed during irradiation treatment and their application in practice.

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