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Preparation of chitosan-glucosamine derivatives (Maillard reaction products) by gamma Co-60 irradiation method and investigation of antibacterial activity

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Abstracts: The mixture solutions of glucosamine and chitosan with different molecular weights (123.5; 40.7 and 6.1 kDa) were irradiated by Co-60 gamma ray at dose of 50 kGy to prepare chitosan-glucosamine Maillard reaction products (MRPs). The formation of MRPs was determined by measuring UV absorbance (at 284) nm and browning (at 420 nm). The reaction efficiency was calculated based on the ratio of reacted glucosamine and total added glucosamine. The antibacterial activity of chitosan-glucosamine MRPs against *Escherichia coli* was also investigated. The obtained results showed that the chitosan-glucosamine MRPs exhibited strong antibacterial activity, in which chitosan-glucosamine MRPs prepared from 123.5 kDa chitosan could reduce up to 4 log CFU/ml in comparison with the control (45×10^6 CFU/ml). Therefore, the chitosan-glucosamine MRPs prepared by the Co-60 gamma irradiation method can be potentially applied as a natural preservative for food, cosmetics and substituted for banned chemical preservatives.

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

I. INTRODUCTION

Many types of food are perishable by nature, especially meat food group. Because of its abundant nutrient content and high moisture, food is the most preferred medium for the proliferation of bacteria and fungi. Besides causing undesirable reactions that deteriorate flavor, odor, color, sensory and textural properties of food, these microbial can potentially be responsible for foodborne illness [1]. In order to prevent the growth of spoilage and pathogenic microorganisms in food, various techniques of preservation such as heat treatment, salting, acidification, drying have been applied in the food industry [2]. In addition, use of preservatives is another way to prevent food spoilage. Because nowadays more and more consumers awareness and concern regarding synthetic chemical preservatives, these food additives must satisfy the stringent standards about permitted dosage. Therefore, the researches on the synthesis of new and safety preservatives are really essential for these day. The current and probably futuristic approaches towards to natural antimicrobial compounds can be applied in food preservation. These natural compounds can be essential oils from plants (e.g., oregano, cinnamon, garlic, etc.), enzymes from animal source (e.g., lysozyme, lactoferrin),

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bacteriocins from microbial source (nisin, natomycine), organic acid (e.g., sorbic, citric acid) and natural polymers (chitosan) [1].

Chitosan, a naturally occurring polysaccharide owning unique biological properties such as being non-toxic, biodegradable and highly biocompatible [4], is composed of two types of monomer, D-glucosamine and N-acetyl glucosamine, and is common prepared from chitin by deacetylation of shrimp/crab shells and/or squid pens in squeezed alkaline solution [5]. Among the naturally antimicrobial compounds, chitosan has received considerable attention because of its multidimensional application potential in biotechnology, material science, drugs and pharmaceuticals, agriculture, environmental protection and especially in food and nutrition. As a food component of natural origin, chitosan has been added to some meat and meat products not only to improve their qualities [4] but also to reduce the oxidation of meat and inhibit the growth of many spoilage and pathogenic microorganisms [6, 7] without causing undesirable side effects on sensory and textural properties of food. Unfortunately, the biological activities of chitosan depend on many factors such as: the degree of deacetylation, the molecular weight and especially the pH of chitosan solution [7]. In neutral and alkaline solutions (pH $\geq$ 6), chitosan is precipitated and reduced its biological activity as a result, therefore the application of chitosan is still limited in some fields.

The Maillard reaction, a non-enzymatic browning reaction, corresponds to a very complex reaction between the carbonyl-containing compounds, such as reducing sugars, aldehydes or ketones found, and the amino-containing compounds, such as amino acids, proteins or any nitrogenous compounds [14]. Many studies have reported that a myriad of products are formed by Maillard reaction, generally termed Maillard reaction products (MRPs), which possess the strong antioxidant and highly antibacterial properties [15, 16].

The amino groups in chitosan can react with the carbonyl groups contained in sugars and sugar derivatives such as glucose, fructose, maltose, glucosamine, etc., by Maillard reaction for forming MRPs [14, 17]. Among these sugars/ sugar derivatives, the MRPs formed from glucosamine and chitosan exhibit superior antibacterial activity even in pH 7 conditions.

Although there are many studies of chitosan-sugar MRPs, studies of the effects of chitosan molecular weights on antibacterial activity of MRPs are still limited. Moreover, very few studies on Maillard reaction by irradiation have been performed although this method is considered to possess many advantages such as: the process is reliable, carried out at room temperature, can apply in large scale without forming cytotoxic byproducts such as 5-hydroxymethylfurfural [18]. The aim of this study is to use gamma Co-60 irradiation for the MRPs formation in solutions of glucosamine and different-molecular-weight chitosan and study their antibacterial activity against *Escherichia coli*.

II. CONTENT

A. Materials and methods

- **Materials**: Chitosan from shrimp shell the weight 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 and cultivated and preserved at Biology Laboratory, VINAGAMMA, Ho Chi Minh City. The Luria- Bertani medium and agar plates used for bacteria incubation were

purchased from Himedia, India. Other chemicals such as: lactic acid, H_2O_2, … are used in analytical grade. Distilled water is used for all experiments.

- Methods:

Preparation of chitosan samples with different molecular weights

Chitosan samples with different molecular weights were prepared by the reference process published by Phu et al. (2017) with some modifications [19]. Briefly, chitosan (4 g) was swollen in 80 ml of 1% (w/v) H_2O_2 solution for 24h, followed by watching, drying and collected for "cut-off chitosan" sample. Another hand, chitosan (4 g) was dissolved in 80 ml of 2% (w/v) lactic acid solution, then 1.5 ml of hydrogen peroxide (30% H_2O_2) and 18.5 ml water were added to prepare 4% chitosan (w/v) solution containing 0.45 % H_2O_2 (w/v). This solution was irradiated at room temperature and under atmospheric pressure on gamma SVST Co-60/B irradiator at the VINAGAMMA Center up to the dose of 21 kGy, with dose rate of 1.12 kGy/h for forming chitooligosaccharide – COS sample. The M_w of the chitosan samples were measured by gel permeation chromatography (GPC) on a LC 20AB, Shimadzu with detector RI G1362A and the column ultrahydrogel models 250 from Waters (USA). Pullulans with different Mw was used as standards. The eluent was aqueous solution 0.25 M CH_3COOH/0.25 M CH_3COONa with the flow rate of 1ml min/1 and temperature at 30° C. IR spectra were taken on an FT-IR 8400S spectrometer (Shimadzu, Japan) using KBr pellets. The degree of deacetylation (DDA%) was calculated based on FT-IR spectra according to the following equation [18]:

$$A1320/A1420 = 0.3822 + 0.0313 \times (100 - DDA\%) \quad (1)$$

Where A1320 and A1420 are absorbance of chitosan at 1320 and 1420 cm^{-1}, respectively.

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 [18]. A 2% solution of chitosan in acetic acid (1%) was prepared. Similarly, a 2% solution of glucosamine was prepared in distilled water. Both solutions were mixed to obtain chitosan–glucosamine (1%) solution (CTS-GA solution). The chitosan–glucosamine solution was exposed to different doses of γ-irradiation (0–100kGy) in a Gamma-cell 5000 (BRIT, Mumbai, India) supplying a dose rate of 2.2 kGy/h.

The irradiated CTS-GA solutions were characterized by spectrophotometric analyses described by Chawla et al. (2009) [20]. The as-prepared CTS-GA solutions were appropriately diluted and absorbance at 284 nm (early Maillard reaction products) and 420 nm (late Maillard reaction products) were measured by a UV–vis spectrophotometer, Jasco-V630, Japan.

The glucosamine content irradiated CTS-GA solution was determined by high performance liquid chromatography (HPLC) method according to AOAC 2012 (2005.01) standard at the Quality Assurance and Testing Center 3 (QUATEST 3), Vietnam. Maillard reaction efficiency was expressed as the ratio of reacted glucosamine to total added glucosamine by the formula:

$$HS = \frac{M_0 - M_t}{M_0} \quad (2)$$

Where M_o and M_t are glucosamine content of CTS-GA solution before and after irradiated, respectively.

Antibacterial Tests

The antibacterial activity of CTS-GA MRPs was investigated against *Escherichia coli* 6538 in both qualitative and quantitative tests.

In qualitative test, the agar well diffusion method was used as described by Balouiri et al. (2016) [21]. The LB agar plates after being spread by *E. coli* (~ 10^3 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 derivatives from chitosan samples with different M_w were introduced to the wells respectively. Then the plates were incubated overnight at 37ºC and monitored colony formation.

In quantitative test, 1 ml of *E. coli* suspension (10^7 CFU/ml) was added into 19 ml of 0.04% CTS-GA MRPs solution in water. The mixture was shaken at 150 rpm for 4 hours and the survival cell density was determined by spread plate technique. The control sample only containing bacteria suspension was carried out simultaneously. 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 sample.

Table I: The characteristics of chitosan samples

Sample	Molecular weight (kDa)	Degree of deacetylation (%)
Raw CTS	123.5	93.3
Cut-off CTS	40.7	91.0
COS	6.1	88.6

Raw CTS: initial chitosan, Cut-off CTS: chitosan which was degraded partially by H_2O_2. COS: chitosan oligosaccharide.

B. Results and discussion

Preparation of chitosan samples with different molecular weight

According to the process mentioned above, three chitosan samples were prepared with the characteristics as shown in Table I.

Preparation of chitosan-glucosamine MRPs

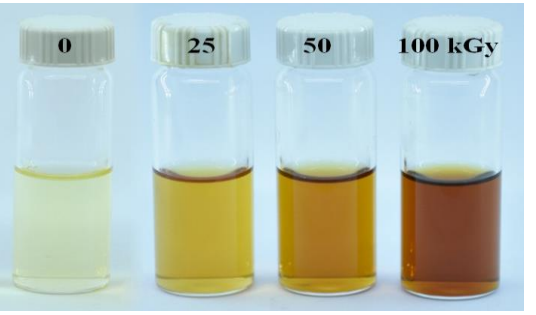

Fig. 1. The CTS-GA MRPs were prepared from Cut-off CTS with dose range of 0-100 kGy

CTS-GA solution from cut-off CTS sample was irradiated with the dose range of 0-100 kGy and measured light absorbance intensity. The Fig. 1 showed that during irradiation, there was a change in visual color of the CTS-GA solution, from colorless to dark brown. The same phenomenon occurred during irradiation of chitosan-glucose solution was also reported in the study of Rao et al. (2011) [18].

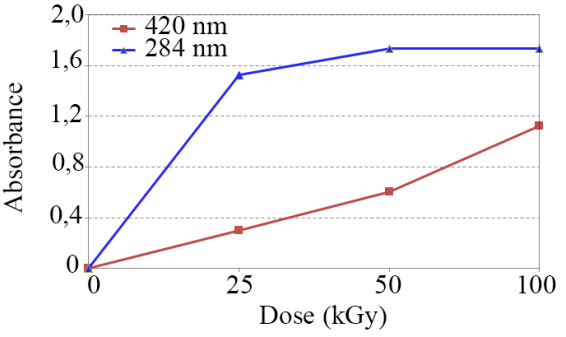

Fig. 2. UV absorbance (284 nm) and browning (420 nm) of irradiated CTS-GA solution at various irradiation doses

Increase in browning of CTS-GA solution can be observed by the rise of absorbance at 420 nm in Fig. 2. This result suggested that irradiation may lead to non-enzymatic browning reactions, similar to those induced by heating. On another hand, in Fig. 2 there was an increase in UV absorbance (284 nm) of CTS-GA solution with increasing irradiation dose. Maillard reaction is associated with development of UV-absorbing intermediate compounds, prior to generation of brown pigments [20, 22], thus this result revealed that intermediate compounds were produced to a great extent. Interestingly, the UV absorbance of CTS-GA solution increased dramatically in dose range of 0-25 kGy, then rose gently in 25-50 kGy dose range and finally was almost unchanged in 50-100 kGy dose range, whereas the browning went up continuously during irradiation. These results indicated that when CTS-GA solution was irradiated with the increasing dose of 0-100 kGy, the Maillard reaction products were formed, in which the formation of early MRPs was saturated at the dose of 50 kGy, while the late MRPs were produced continuously along with the dose up to 100 kGy.

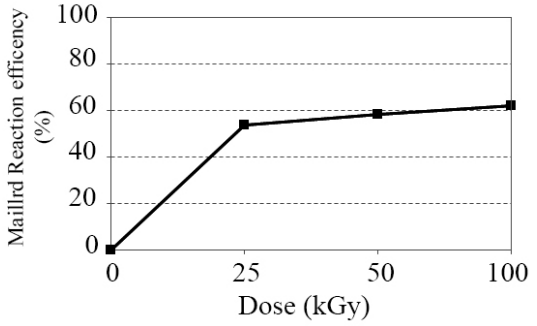

Fig. 3. The Maillard reaction efficiency versus irradiation dose

The Maillard reaction efficiencies expressed by the decreases in glucosamine content of cut-off CTS-GA solution after irradiated at different doses were described in Fig. 3. The obtained result showed that the Maillard reaction efficiency increased along with the irradiation dose, in which the highest rate of the increase is belong to the dose range of 0-25 kGy. This tendency is similiar to the increasing of UV absorbance. This 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 amount in the solution, while the late reactions just polymerized the intermediates, formed colored polymers [20, 22] and did not affect the glucosamine content.

According these results, the 50 kGy dose was chosen as a suitable dose for preparing MPRs from different chitosan samples.

Antibacterial Tests

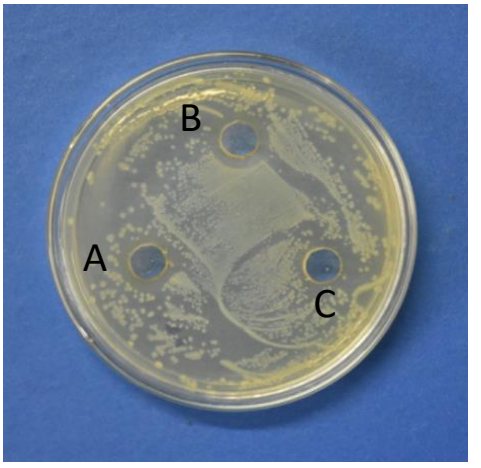

Fig. 4. The results of agar well diffusion test carried out by the MRPs of different chitosan samples (A, B, C are the MRPs of CTS, cut-off CTS, COS sample respectively)

In Fig. 4, all MRPs samples were able to form inhibition zone on *E. coli* plate, this indicated that these sample were all possessing antibacterial activity against *E. coli*. By comparing the diameters of inhibition zones formed on the plate by these samples, we may primarily predict the order of their antibacterial

effect [21]. Therefore, according to the result of this test, the antibacterial effect against *E. coli* of MPRs from COS sample was forecasted to be lowest.

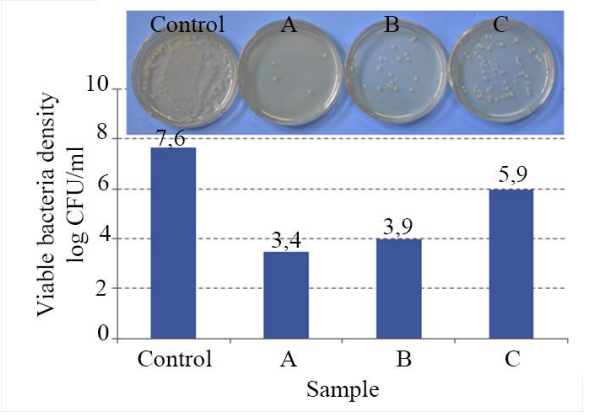

Fig. 5. Viable bacteria density of the mixture after exposing time of 4 hours

(A, B, C are the mixture containing MRPs of CTS, cut-off CTS, COS sample respectively)

The Fig. 5 showed that the bacterial densities of the mixtures after exposing time were significantly decreased in comparison with the density of control sample. The lower the viable bacteria density is, the higher antibacterial effect of MRPs is. Therefore, the antibacterial effect of MRPs from CTS (123.5 kDA) sample was highest (reduced up to 4 log CFU/ml) while antibacterial effect of MRPs from COS sample (6.1 kDa) was the lowest. This result is consistent with the prediction from qualitative test. Moreover, this test also suggested that the molecular weight of initial chitosan influenced significantly on the antimicrobial activity of the MPRs, namely in the range of molecular weight of 6 - 123 kDa, the chitosan with higher M_w could form MPRs with stronger antibacterial activity. In the study of Rao et al. (2011), the *E. coli* density of mixture after 24-hour shaking with chitosan-glucose MRPs was also decreased to 4 log CFU/ml compared to the control sample. This study also found that the chitosan-glucose MRPs exhibited the higher antibacterial effect than chitosan against both gram-positive and gram-negative bacteria in alkaline medium (pH 7.2) [18].

III. CONCLUSION

CTS-GA MRPs were efficiently synthesized by the Maillard reaction through gamma Co-60 irradiation technique. The 50 kGy dose was appointed to prepare CTS-GA MRPs from different chitosan samples. Among these as-prepared MPRs, the MPRs from 123.5 kDa chitosan exhibited the strongest antimicrobial activity against *E. coli* with the bacteria density reduction of ~ 4 log CFU/ml compared to the control sample. The antibacterial results also show that the CTS-GA MRPs prepared by gamma Co-60 irradiation is promising to be applied as an antibacterial agent for food, cosmetic and to substitute for banned chemical synthesis preservatives.

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