Antimicrobial and antioxidant capacity of glucosamine-zinc(II) complex via non-enzymatic browning reaction

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Abstract Coordination compounds play an important role in the life process, and have been widely used in food, cosmetics and pharmaceutical industry. Herein, we have developed a novel kind of glucosamine-zinc(II) complex (GlcN-ZC) for food additive using non-enzymatic browning reaction. The GlcN-ZC was characterized by FTIR and XRD. Moreover, UV absorbance changes, browning intensity, fluorescence changes, antioxidant activity and antimicrobial assessment of GlcN-ZC were also evaluated. Results showed the GlcN-ZC intermediate compounds were accumulated in non-enzymatic browning while prolonging heating time and melanoidins were produced in the final stage. The fluorescence changes confirmed that fluorophores were formed during the non-enzymatic reaction and fluorescence intensity reached a maximum at 60 min. The highest radical scavenging activity of GlcN-ZC formed after 180 min of heating was 79.2%. Furthermore, GlcN-ZC exhibited excellent antibacterial activity against *E. coli* and *S. aureus*. Therefore, GlcN-ZC can be used as a novel promising additive in the food industry.

Keywords Glucosamine · Non-enzymatic browning · Antioxidant · Antimicrobial · Complex

Introduction

Most of the compounds in nature exist in the form of chelates. Complexes play an important role in the life activities of animals, plants and human beings. The essential trace elements such as Fe, Zn, Cu, I, Co, Se, Mn, Mo, and so on are present as the form of complexes, in which metal ions are the central atoms and biological macromolecules (proteins, nucleic acids, etc.) are as ligands [1, 2]. Trace elements are key components of various enzymes involving in energy metabolism of protein, carbohydrates and fat, such as carbonic anhydrase (containing Zn) and superoxide dismutase (containing Zn and Cu). Moreover, many drug such as insulin therapy for diabetes (zinc complex) and vitamin B12 (cobalt complex) are also coordination compounds containing metal elements [3]. Therefore, it is crucial to develop a novel kind of complex for life science and medicine.

The non-enzymatic browning reaction, also named as Maillard reaction (MR), is a chemical reaction involving the condensation between carbonyl group of reducing sugars, aldehydes or ketones, and amino group of amino acids, proteins or nitrogenous compound [4, 5]. The reaction occurs widely in food processing, storage and cooking process [6, 7]. Generally, the Maillard reaction is divided into three distinct stages: initial stage, intermediate stage and final stage [8]. In the initial stage, carbonyl-amino group condensation produces a Schiff base and Amadori rearrangement product. Because of unstable at ambient temperature, Amadori compound undergo enolization, deamination, dehydration, fragmentation and Strecker degradation steps to generate keto-aldehydes, dicarbonyls, reductones, and N-heterocyclic flavor compounds in the intermediate stage. The colourless or yellow products of the initial and intermediate stages of the Maillard reaction

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were attributed to the highly coloured compounds known as melanoidins formed from aldol condensation and aldehyde-amine polymerisation in the final stage. Melanoidins are reported to exhibit antioxidative effect [9], antibacterial activity [10], desmutagenic effects, metal-chelating properties [11], scavenging activity against reactive oxygen species [12]. Therefore, it can be estimated that melanoidin might be effective for the prevention of stomach cancer and can be used as additive for functional foods [13].

Glucosamine (GlcN) is an amino monosaccharide used as a dietary supplement for the relief of osteoarthritis symptoms, which are currently produced by acid hydrolysis of chitin extracted from crab and shrimp shells. The molecular structure of GlcN contains an amino group at the C2 position next to the carbonyl group forming an aldose-amine structure that can resemble an Amadori rearrangement product [14, 15]. Due to its reactivity, GlcN has been conducted in non-enzymatic browning reaction in order to generate novel functional food ingredients. For instance, Hrynets et al. studied the non-enzymatic browning of GlcN in the presence of NH_3 incubated at 37 °C in phosphate buffer from 0.5 to 12 days. The authors demonstrated that GlcN produced two major dicarbonyl compounds including glucosone and 3-deoxyglucosone, which were important intermediates in flavor generation and also possessed antimicrobial properties [15]. Hong et al. developed a flavour enhancer formulation through the isoelectric solubilisation precipitation process glycosylated with GlcN at moderate temperatures (37/50 °C). The results illustrated that GlcN treatments can increase savouriness [16]. Meanwhile, they found Maillard reaction products (MRPs) derived from GlcN showed a high efficacy in scavenging DPPH and ABTS radicals in accordance with the increasing browning intensity [14]. These phenomena suggest that GlcN browning can be modulated according to the specific desired properties to produce multifunctional food additives. Moreover, the participation and activity of metal ions play an important role in non-enzymatic browning reaction. Metals can cause a catalytic effect on browning progress [17, 18]. The antioxidant activity of MRPs can be enhanced in retarding metal catalyzed lipid oxidation reactions through chelation of metal ions [19–22]. Also, many of these complexes show appealing antibacterial activity [11, 23]. The objective of this study was to develop a novel glucosamine-zinc(II) complex for food additive via non-enzymatic browning reaction. The kinetics of the browning reaction were monitored by measuring UV absorbance, browning intensity and fluorescence changes over time. Furthermore, the antioxidant (DPPH radical scavenging, reducing power) and antibacterial activity (against *Staphylococcus aureus*, *Escherichia coli*) of GlcN browning solution produced at different incubation times were evaluated. This work has the potential application of GlcN

browning products with specific properties in food industry.

Materials and methods

Materials

D-Glucosamine hydrochloride and 2,2-diphenyl-1-picrylhydrazyl (DPPH·) were purchased from Sigma-Aldrich. All other chemicals used were of analytical grade obtained from Sinopharm Chemical Reagent Co. Ltd.

Preparation of GlcN-zinc complex (GlcN-ZC)

Glucosamine hydrochloride (1 g) and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.667 g) were dissolved in 100 mL H_2O in a beaker. After adjusting the pH value to 9.0 using sodium hydroxide (1 M), the mixture was then transferred to a flask and refluxed in an oil bath at 100 °C for 5 h. In order to study the non-enzymatic browning reaction between GlcN and Zn(II), aliquots (8 mL) were withdrawn at 0, 60, 120, 180, 240 and 300 min and placed in an ice-water bath for analysis. For comparison, GlcN from non-enzymatic browning reaction was respectively heated under the same experimental conditions as controls.

Characterization of GlcN-ZC

Maillard reaction products from GlcN and GlcN-ZC were freeze-dried and grounded to powder. FTIR spectra of GlcN and GlcN-ZC were recorded with KBr discs in the range of 4000–400 cm^{-1} on Nicolet MX-1E spectrophotometer. X-ray diffraction (XRD) spectra of ZnSO_4 , GlcN and GlcN-ZC were collected on German Bruker D8-Advance X-ray diffractometer over the 2θ range of 5–40°.

Spectrophotometric analysis

The heated browning solutions were appropriately diluted with distilled water for spectrophotometric analysis and antioxidant activity. UV–vis absorbance and browning intensity of GlcN-ZC were recorded using Hitachi-3310 ultraviolet and visible spectrophotometer at 294 and 420 nm, respectively. The fluorescence intensity was measured at an excitation wavelength of 343 nm and an emission wavelength of 415 nm on a Hitachi-F4500 fluorescence spectrophotometer.

DPPH radicals scavenging activity and reducing power

The DPPH radical scavenging activity of GlcN-ZC at different time was estimated according to the methods described by [11, 24]. An aliquot (0.1 mL) of GlcN-ZC was added to 2.0 mL of 0.1 mM DPPH $\cdot$ in ethanol. After being mixed vigorously and being left to stand for 30 min at room temperature in the dark, the absorption was measured using Hitachi UV-3310 at 517 nm. The percentage of DPPH radical scavenging activity was calculated as follows:

$$\text{Scavenging activity (\%)} = [1 - (A_s - A_r)/A_0] \times 100 \quad (1)$$

where A_0 was the absorbance of 0.1 mM DPPH $\cdot$, A_s was the absorbance of 0.1 mM DPPH $\cdot$ with the solutions, and A_r was the absorbance of ethanol with solutions.

The reducing power of GlcN-ZC was also determined according to the method of [25]. An aliquot of the solution (2.5 mL) was mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide, and then incubated at 50 °C for 20 min. Tri-fluoroacetate (10%, 2.5 mL) was added to the mixture and centrifuged at 10,000 $\times g$ for 5 min. The supernatant (2 mL) was mixed with 2 mL of water and 1 mL of 0.1% ferric chloride and the absorbance was measured at 700 nm. There were three replicate samples in each group for the measurements of free radical scavenging and reducing power.

Antibacterial activity

The antibacterial activity of GlcN-ZC at different time against *S. aureus* and *E. coli* was determined using the disk diffusion method. Cell suspension (10^7 cfu/mL, 50 μ L) was added onto agar plates before paper disks (diameter of 6.0 mm) containing test solutions were placed on plates. Inhibition zones were observed and measured after incubation at 37 °C for 36 h. All experiments were carried out in triplicate, and average values with standard deviation errors were reported. A statistical difference at $p < 0.05$ was considered significant.

Results and discussion

Characterization of the complex

FTIR spectroscopy has been used for the analysis of the derivatives formed by the non-enzymatic browning reaction. Figure 1 showed the FTIR spectra of GlcN(a), GlcN-ZC (240 min) and GlcN-ZC (300 min). In the spectrum of

GlcN, A peak at 3282 cm^{-1} is assigned to be from N–H and hydrogen-bonded OH stretching vibrations. The bands between 856 and 912 cm^{-1} are typical of the C–H out-of-plane bend. The shoulder peak stretching at 1089 and 1093 cm^{-1} were reflected the C–O bond of the C3. The two characteristic peaks at 1537 and 1618 cm^{-1} are corresponding to the N–H bending of GlcN [26, 27]. The FTIR spectrum of GlcN-ZC (240, 300 min) exhibits many alterations from that of GlcN. The major differences are observed to be the appearance of new sharp peaks at 890 cm^{-1} (Zn–OH) and 1650 cm^{-1} (C=N). The changes suggest that significant complex formation occurred between the GlcN and Zn(II).

The X-ray diffraction spectra of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, GlcN and GlcN-ZC (at reaction time 300 min) were shown in Fig. 2. The diffraction spectrum of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ exhibited two major crystalline peaks at $2\theta = 14.96^\circ$ and 19.96° , while GlcN consisted of two typical crystalline peaks at $2\theta = 16.70^\circ$ and 24.22° . GlcN browning solution chelating with Zn(II) showed broader peak at 21° , and the crystallinity sharply decreased, indicating that the non-enzymatic browning reaction can significantly reduce the crystallinity of the reactants.

UV–vis absorbance and browning intensity

UV–vis analysis is a useful tool to monitor the progress of GlcN-ZC non-enzymatic browning reaction. Absorbance at 294 nm is often representative of the intermediate products (i.e. fructosazine and deoxyfructosazine), whereas the pre-melanoidins and melanoidins formed in the final stage can be estimated at 420 nm [28]. Non-enzymatic browning reaction of GlcN and GlcN-ZC at 294 and 420 nm is presented in Fig. 3. As shown in the graphs, the rate of

Fig. 1 FTIR spectra of GlcN (A), GlcN-ZC (B, 240 min) and GlcN-ZC (C, 300 min)

Fig. 2 X-ray diffraction of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, GlcN and GlcN-ZC

accumulation of GlcN and GlcN-ZC condensation products incubated at 100 °C increases during the course of the reaction time. Figure 3(A) shows that browning of GlcN results in a higher intensity at 294 nm at all incubation times as compared to GlcN-ZC. This indicates a faster GlcN condensation rate in the absence of Zn(II). Furthermore, GlcN-ZC shows an increased UV absorbance at 294 nm during 180 min, then keep invariant in the time range of 180–300 min. Figure 3(B) demonstrated the progress on the formation of pre-melanoidins and melanoidins at 420 nm. GlcN produced higher absorbance intensity than GlcN-ZC. However, pre-melanoidin formations of GlcN-ZC reached to a reaction plateau at 180 min. Correspondingly, it was colourless for the GlcN and GlcN-ZC browning solutions in the initial stage. During the

intermediate stage, the colour of the solutions gradually became yellow, and with the time prolonged, a dark brown colour was observed in the solutions.

Based on the above results, intervention of Zn(II) plays an important role in the non-enzymatic browning reaction of GlcN. In the absence of Zn(II), the rate of browning in GlcN solution increases during the complete heating period, especially during the final stage. Addition of Zn(II) leads to a significant decrease intermediate of products of browning reaction during the complete reaction period. Therefore, Zn(II) may inhibit interaction between intermediate products, or even catalyze the degradation of high molecular weight melanoidins formed during interactions. This result is in accordance with the conclusion of the literature data [29].

Fluorescence intensity

Fluorescence spectroscopy can be used to indicate the formation of advanced glycation end products (AGEs) through the Maillard reaction [30, 31]. AGEs are normally produced in the final stage of Maillard reaction in foods and biological systems. These products play an important role in vascular stiffening, atherosclerosis, osteoarthritis, inflammatory arthritis and cataracts. AGEs are highly colored compounds and have typical fluorescence properties at an excitation wavelength of 343 nm and at an emission wavelength of 415 nm [32, 33]. Fluorescent intensities of GlcN and GlcN-ZC over reaction time at excitation wavelength of 343 nm and emission wavelength of 415 nm are depicted in Fig. 4. Solutions of GlcN and GlcN-ZC showed a significant increase in fluorescence intensity that reached a maximum at 60 min, then decreased over the investigated reaction time, indicating a

Fig. 3 Non-enzymatic browning reaction of GlcN and GlcN-ZC at 294 (A) and 420 nm (B). Insert the gradient colour of GlcN(I) and GlcN-ZC(II) browning solutions (from left to right, 0, 60, 120, 180, 240, and 300 min, respectively)

Fig. 4 Fluorescent intensity of GlcN and GlcN-ZC over reaction time at an excitation wavelength of 343 nm and an emission wavelength of 415 nm

high production of AGEs during non-enzymatic browning reaction. This phenomena indicated that AGEs formed during early stage were the precursors of the brown pigments such as melanoidins.

Antioxidant properties

The scavenging of DPPH radicals is widely used to measure the capacity of antioxidants. DPPH is a purple-colored stable organic nitrogen centered free radical and shows a maximum absorbance at 517 nm. However, the radicals become colorless and the absorbance is reduced when it encounters a proton-donor substrate such as antioxidants. Productions of non-enzymatic browning is normally associated with an increase of antioxidant capacity of MRPs. MRPs directly react with DPPH radicals by donation of hydrogen to form a stable DPPH-H molecule. The colour changed from purple to yellow by acceptance of a

Fig. 5 DPPH radical scavenging activity (A) and reducing power (B) of GlcN and GlcN-ZC as a function of reaction time

Fig. 6 The diameter of the inhibition zone of GlcN and GlcN-ZC at different reaction time. (A) Against *E. coli*; (B) against *S. aureus*

hydrogen atom form MRPs. The DPPH radical scavenging activities of GlcN and GlcN-ZC at different reaction time are shown in Fig. 5(A). GlcN and GlcN-ZC caramel solutions produced at 100 °C show a progressive increase in radical scavenging activity with prolonged incubation duration, and reach maximum 61.5 and 79.2% at 180 min, but decrease thereafter. This suggests that the radical scavenging activity did not entirely due to the melanoidins and the formation of the UV-absorbing intermediates may also have contributed to this activity [14]. The DPPH scavenging activity of GlcN-ZC is dramatically enhanced in comparison with GlcN. At the same reaction time, the DPPH radical scavenging activities of GlcN-ZC are higher than that of GlcN, which indicate Zn(II) can significantly improve DPPH radical scavenging activities of MRPs.

The reducing power is an important antioxidant assay for determining the capacities of antioxidant compounds. Generally, the reducing power of MRPs can be attributed to the Amadori products and intermediate reactants generated during the heating process, which can donate electrons to reduce the ferric chloride ferricyanide complex to ferrous form [34, 35]. Consequently, the contents of intermediate MRPs are greatly correlated with the reducing power. The reducing power of GlcN and GlcN-ZC as a function of reaction time is showed in Fig. 5(B). GlcN-ZC exhibits significantly higher reducing power when compared with GlcN browning solution. The reducing power of GlcN and GlcN-ZC was dramatically enhanced along with increased the reaction time, and reached a maximum at 60 min. A trend for reducing power was similar with the DPPH scavenging activity. Thus, the extent of the reducing power of GlcN-ZC could be explained not only to the formation of intermediate MRPs, but also to the distinct role of Zn(II) in the complex of reducing conditions.

Antibacterial evaluation

The inhibition of antimicrobial activity of GlcN and GlcN-ZC browning solutions produced at different times against *E. coli* and *S. aureus* is depicted in Fig. 6 by the method of antibacterial zone diameter. As shown in Fig. 6(A), the antibacterial activity of GlcN against *E. coli* is relatively weak during all the incubated time, whereas GlcN-ZC shows a time-dependent inhibition profile over *E. coli*. The diameter of the inhibition zone of GlcN-ZC at reaction time 300 min is around 19.8 mm as compared to the control of 6.0 mm against *E. coli*. The results indicate that melanoidins derived from GlcN could significantly inhibit the growth and survival of pathogenic bacteria by chelating Zn(II). In contrast, the antibacterial activities of GlcN and GlcN-ZC against *S. aureus* gradually decreases with prolonged heating time [Fig. 6(B)]. However, GlcN-ZC is more active against *S. aureus* than GlcN caramel solutions.

It is worth noting that GlcN-ZC without heating has intensive antibacterial activity against *S. aureus* with the diameter of the inhibition zone 23.1 mm.

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