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Preparation, characterization and antioxidant activity analysis of three Maillard glycosylated bone collagen hydrolysates from chicken, porcine and bovine

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ABSTRACT

Bone collagen hydrolysates (peptides) derived from byproduct of animal product processing have been used to produce commercially valuable products due to their potential antioxidant activity. Maillard glycosylated reaction is considered as a promising method to enhance the antioxidant activity of peptides. Hence, this research aims at investigating the Maillard glycosylation activity and antioxidant activity of bone collagen hydrolysates from different sources. In this study, 3 glycosylated bone collagen hydrolysates were prepared and characterized, and cytotoxicity and antioxidant activity were analyzed and evaluated. The free amino groups loss, browning intensity, and fluorescence intensity of G-Cbcp (glycosylated chicken bone collagen hydrolysates (peptides)) were the heaviest, followed by G-Pbcp (glycosylated porcine bone collagen hydrolysates (peptides)) and G-Bbcp (glycosylated bovine bone collagen hydrolysates (peptides)). The results of amino acid analysis showed that amino acid composition of different bone collagen hydrolysates was significantly different and the amino acid decreased to different degrees after Maillard glycosylated reaction, which may lead to differences in Maillard glycosylated reaction activity. Furthermore, the 3 glycosylated hydrolysates showed no significant cytotoxicity. The results showed that glycosylation process significantly increased the antioxidant activity of bone collagen hydrolysates, and G-Cbcp showed the strongest antioxidant activity, followed by G-Pbcp and G-Bbcp. Therefore, compared with the bone collagen hydrolysates, 3 glycosylated hydrolysates showed significant characteristic and structural changes, and higher antioxidant activity.

1. Introduction

About 130 million tons of bone by-products are generated annually in the word, whereas the deep processing rate of livestock and poultry bone (such as chicken, pig, and bovine bone) is below 1%[1]. Collagen is the major structural protein of livestock and poultry bone (approximately 30% of the total protein)[1-2]. Furthermore, bone collagen is one of the most abundant food-source of collagen in the world, which has a high nutritional value and can promote physiological health[3-4]. Therefore, it is of great significance to make high value utilization of bone collagen and its hydrolysates from livestock and poultry bone by-products under the current global resource shortage. At present, bone collagen has been extensively used in tissue engineering, food packaging, cosmetics, and other fields[5]. With the constant study of enhancing the functional properties of bone collagen, the biological peptides from bone collagen have attracted extensive attention[6]. Studies have shown that the biological peptides with certain antioxidant activity can be released by the enzymatic hydrolysis of bone collagen[7-8]. For example, He et al.[9] reported that collagen peptides can prevent oxidation in various food systems as a natural antioxidant. Nonetheless, compared with some antioxidants, the limited antioxidant activity of bone collagen hydrolysates does not

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meet the market demand and limits its application. Therefore, how to obtain the bone collagen hydrolysates with higher antioxidant activity by chemical modification has become a research hotspot.

The Maillard reaction, referred to the "nonenzymatic browning reaction", is a covalent grafting reaction between the amino groups (including amino acids, peptides, proteins), and the carbonyl groups (including aldehydes, ketones, and reducing sugars)[10-11]. Many studies have shown that Maillard reaction can improve the functional properties of polypeptides such as its antioxidation[11], emulsifying[12-13] and anti-allergenicity[14]. The Maillard reaction can be fallen into 3 phases, and its glycosylation products formed at the end of the first phase or at the beginning of the second Maillard reaction usually have optimal antioxidant activity[15]. In recent years, Maillard glycosylated reaction is also considered as a hopeful method to improve the antioxidant activity of hydrolysates (polypeptides)[16], which has attracted more and more attention and been widely applied in food industry[17-18]. For example, Yang et al.[19] enhanced the antioxidant activity of fish protein hydrolysates through Maillard glycosylation reaction explored its protective effect on liver. Habinshuti et al.[20] explored the effect of Maillard glycosylation reaction on the antioxidant activity of sweet potato protein hydrolysates obtained from ultrasonic assisted enzyme treatment. However, there are few studies focusing on the effect of Maillard glycosylated reaction on the antioxidant activity and safety of collagen hydrolysates from livestock and poultry bones (chicken, pig, bovine). Therefore, it is necessary to systematically explore the antioxidation activity and potential toxicity of Maillard glycosylated bone collagen hydrolysates.

In this study, 3 glycosylated bone collagen hydrolysates were successfully prepared and characterized, and their antioxidant activity were analyzed and evaluated before and after Maillard reaction. In addition, the safety of glycosylated bone collagen hydrolysates was also explored by cells experiments. This study will lay a necessary foundation for using Maillard glycosylation reaction in improving the antioxidant activity of different livestock and poultry bone collagen hydrolysates, and provide a theoretical reference for the high-value utilization of collagen hydrolysates from livestock and poultry bone.

2. Materials and methods

2.1 Materials and reagents

The bone collagen in the article is from our own laboratory. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), *D*-galactose, 2'-azino bis(3-ethylbenzothiazoline-6-sulphonicacid) (ABTS) were offered by Sinopharm Chemical Reagent Co., Ltd. 2',7'-Dichloro-fluorescin diacetate (DCFH-DA), Dulbecco's modified Eagle's medium (DMEM) with fetal bovine serum (FBS) were obtained from Solarbio (Beijing, China). All reagents are analytical grade unless otherwise specified.

2.2 Preparation of three glycosylated collagen hydrolysates

Deionized water was used to prepare the collagen solution to get a substrate concentration of 5%, next to additions of trypsin (5 000 U/g). The enzymatic hydrolysis conditions were pH 7.8 and 37 °C. The enzymatic reaction was performed constantly for 12 h and the solution was immediately treated at 95 °C for 10 min. After 15-min centrifugation at 10 000 r/min to eliminate precipitates, the supernatant collected was lyophilized at –40 °C for further experiments.

Glycosylated bone collagen hydrolysates were prepared on basis of the approach of Zhang et al.[21] with minor modifications. Three glycosylated collagen hydrolysates (chicken, porcine and bovine) were prepared by the same process and marked as G-Cbcp (glycosylated chicken bone collagen hydrolysates (peptides)), G-Pbcp (glycosylated porcine bone collagen hydrolysates (peptides)) and G-Bbcp (glycosylated bovine bone collagen hydrolysates (peptides)), respectively. The dissolution of bone collagen hydrolysates and *D*-galactose (1:1, *m*/*m*) was made in water with 30 mg/mL. The initial pH was changed to 9.0 with 1 mol/L sodium hydroxide (NaOH). The 5-h heating of solution was made in a water bath at 95 °C. Next, centrifugation of mixture was conducted at 8 000 × *g* for 20 min. The supernatant was lyophilized and then was kept at –20 °C before use.

2.3 Chemical composition analysis of glycosylated bone collagen hydrolysates

2.3.1 D-galactose content determination

The *D*-galactose in the different sample was determined by ion chromatography. Chromatography: ICS-5000 + DC ion chromatography; detector: pulse amperometric detector; chromatographic column: Dionex carbon Pac PA200 column (250 mm × 5 mm, 5.5 μm), Dionex carbon Pac PA200 protection column (50 mm × 50 mm, 5.5 μm); flow rate: 0.4 mL/min; the column temperature was 30 °C; injection volume: 25 μL. Pure water was used as mobile phase A, 0.25 mol/L sodium hydroxide as mobile phase B, and 0.25 mol/L sodium acetate as mobile phase C. Gradient elution was performed.

2.3.2 Total nitrogen content determination

The overall nitrogen content of different bone collagen hydrolysates and glycosylated hydrolysates was determined by automatic Kjeldahl nitrogen analyzer (Kjeltec8400).

2.3.3 Moisture content determination

The moisture content of different bone collagen hydrolysates and glycosylated hydrolysates was determined according to direct drying method in GB 5009.3−2016.

2.4 Fourier transform-infrared spectroscopy (FTIR) analysis

The measurement of FTIR of the sample was made based on the approach of Zhang et al.[22] with a minor adjustment. FTIR was recorded with an FTIR spectrometer (Tensor-27, Bruker Company, Germany). After pressing KBr together with dried sample (100:1) into a 1 mm pellet, the measurement was made in a frequency scope of 4 000–450 cm^{-1} at a resolution of 4 cm^{-1} in the transmission mode.

2.5 Determination of molecular weight distribution

The molecular weight of bone collagen hydrolysates and glycosylated hydrolysates was assessed by gel permeation chromatography based on the approach of Zhang et al.[22] with a minor adjustment. The dissolution of samples was made in the mobile phase at the final concentration of 1 mg/mL, which included 30% acetonitrile solution and 0.1% of trifluoroacetic acid. With injection volume of sample of 10 μL and the velocity of constant gradient elution of 0.5 mL/min, absorbance was monitored at 214 nm. The standards were cytochrome C (12 327 Da), aprotinin (6 511 Da), bacitracin (1 422 Da), Gly-Gly-Tyr-Arg (451 Da) and Gly-Sar (146 Da).

2.6 Determination of UV-absorbance and browning

The UV-absorption and browning intensity of the samples were determined based on the approach of Ajandouz et al.[23] with a slight change. Typically, the dissolution of sample was made in 0.1 mol/L phosphate buffer saline (PBS, pH 7.0) at the final concentration of 5 mg/mL. UV-600 spectrophotometer was adopted to measure absorbance. The generation of intermediate in the Maillard reaction was assessed with the absorbance at 294 nm, the generation of later stage in Maillard reaction was assessed with the absorbance at 420 nm. Glycosylation products mainly accumulated in the middle phase of Maillard reaction[24].

2.7 Determination of intrinsic fluorescence spectra

The emission spectra and intrinsic fluorescence excitation of the samples were determined with F-4500 fluorescence spectrophotometer. Typically, the dissolution of sample was made in 0.1 mol/L PBS (pH 7.0) at 0.3 mg/mL. While recording the emission spectrum of 370–570 nm, the fixation of excitation wavelength was made at 340 nm. While recording the excitation spectrum from 300 to 400 nm, the emission spectrum was fixed at 420 nm.

2.8 Determination of free amino group content

The content of free amino group in the sample was determined by *o*-phthalic aldehyde (OPA) method. OPA solution was used and prepared on the spot. Typically, the dissolution of sample was made in 0.01 mol/L PBS (pH 7.0) at 0.2 mg/mL. Briefly, sample solution (100 μL) was poured into OPA reagent (3 mL). The 5-min reaction was made in the dark at 25 °C. The measurement of absorbance of the sample was made at 340 nm. The calculation of content of free amino group was made based on the standard curve, which was established with *L*-leucine as the standard. The blank group was treated with ultrapure water instead of the sample.

2.9 Amino acid analysis

2.9.1 Total amino acid

Automatic amino acid analyzer (L-8900, Hitachi LTD, Japan) was adopted to determine the total amino acid content of the sample based on the approach of Shen et al.[25] with a minor adjustment. The dissolution of sample was made in 6 mol/L hydrochloric acid solution at a certain concentration and then transferred to an oven for hydrolysis at 110 °C for 24 h. After cooling at room temperature, 1 mL of hydrolysate was dried by nitrogen, and then redissolved with ultrapure water. The above prepared solution was filtered through 0.45 μm membrane and used for subsequent amino acid exploration.

2.9.2 Free amino acid

The same approach (Section 2.9.1) was used to determine the free amino acid content of samples, except that samples shall not be hydrolyzed.

2.10 Evaluation of potential cytotoxic

Caco-2 cells were cultured in the RPMI 1640 medium. The seeding of Caco-2 cells was made (1×10^5 cells/mL) in 96-well plates, followed by 24-h cultured at 37 °C in an atmosphere containing 5% CO_2. Next, the culture medium was eliminated and replaced with RPMI 1640 medium with G-Cbcp, G-Pbcp or G-Bbcp at 0.5, 1.0 and 2.0 mg/mL. The culture of Caco-2 cells was made in RPMI 1640 culture medium were the control group. The 48-h incubation of all groups of cells was made. Apoptosis, which was used to assess the potential cytotoxicity of glycosylated hydrolysates, was detected by flow cytometry.

2.11 Determination of antioxidant properties

2.11.1 DPPH radical scavenging activity

The DPPH radical scavenging activity of the sample was decided based on the approach of Li et al.[26] with a minor change. Typically, the dissolution of samples was made in 0.01 mol/L phosphate buffer (pH 7.0) at final concentrations of 3, 6, 9, 12 and 15 mg/mL. Sample solution (160 μL) was added to 640 μL of 0.01 mmol/L DPPH solution (the dissolution of DPPH in analytical grade ethanol). After mixing well, the above solution was put at room temperature and reacted in the dark for 30 min. Next, measurement of the absorbance (517 nm) of the above solution was made. The absorbance of the sample with DPPH in ethanol was used as the experimental group (A_{sample}). The absorbance of the dissolution of the sample with ethanol was used as the control group ($A_{control}$). The absorbance of ultrapure water containing DPPH dissolved in ethanol was adopted as blank group (A_{blank}). The DPPH radical scavenging activity was calculated with the formula below:

$$\text{DPPH radical scavenging activity (\%)} = \left(1 - \frac{A_{sample} - A_{control}}{A_{blank}}\right) \times 100 \quad (1)$$

2.11.2 ABTS cation radical scavenging activity

The ABTS cation radical scavenging activity of the sample was decided based on the approach of Tan et al.[27] with a minor change. Typically, the mixture of potassium persulfate solution of 2.45 mmol/L and ABTS solution of 7 mmol/L was made in equal volume and reacted for 12 h at 25 °C. The dilution of above solution was made with 5 mmol/L PBS (pH 7.0) and its absorbance at 734 nm was about

0.7. Samples (20 μL) with concentration of 3, 6, 9, 12 and 15 mg/mL were poured into diluted ABTS solution (2 mL), and the above mixed solution reacted for 6 min at room temperature. The measurement of absorbance of the above solution was made at 734 nm (A_s). Ultrapure water was adopted as blank group instead of sample (A_0). The ABTS cation radical scavenging activity was calulated with formula below:

$$\text{ABTS cation radical scavenging activity (\%)} = (1 - \frac{A_s}{A_0}) \times 100 \quad (2)$$

2.11.3 Hydroxyl radical scavenging ability

The hydroxyl radical scavenging ability of the samples was decided based on the approach reported by Camarena-Tello et al.[28] with minor modifications. Sample solutions (Cbcp, Pbcp, Bbcp, G-Cbcp, G-Pbcp and G-Bbcp) were prepared at final concentrations of 0.1, 0.5, 1, 2 and 4 mg/mL and $FeSO_4$ solution of 9 mmol/L and salicylic acid-ethanol (dissolved in ethanol) of 9 mmol/L were made. Then, 0.5 mL every of $FeSO_4$ solution and salicylic acid-ethanol were poured to all test tube and numbered 1–4. Deionized water (6 mL) and 0.5 mL of H_2O_2 solution (8.8 mmol/L) were poured to No. 1. To No. 2, 6.5 mL of deionized water was poured as blank. The sample solution of 5 mL, deionized water of 1 mL, and H_2O_2 solution of 0.5 mL were poured to No. 3. Sample solution (5 mL) and 1.5 mL of deionized water were poured to No. 4. The all tubes were kept in a water bath kept at 37 °C for 15 min and the absorbance was decided at 510 nm. The hydroxyl radical scavenging ability was decided with the formula below:

$$\text{Hydroxyl radical scavenging ability (\%)} = 1 - (\frac{A_2 - A_3}{A_1}) \times 100 \quad (3)$$

Where A_1 means the absorbance of No. 1, A_2 refers to the absorbance of No. 3 and A_3 stands for the absorbance of No. 4.

2.11.4 Ferrous reducing power

The ferrous reducing power of the samples was decided based on the approach of Zhang et al.[21] with minor modifications. The dilution of samples was made with 0.01 mol/L phosphate buffer (pH 7.0) to 3, 6, 9, 12 and 15 mg/mL. Every sample (0.5 mL) was poured to 2.5 mL of PBS (pH 6.6, 0.2 mol/L) followed by 1% potassium ferricyanide (2.5 mL). The 20-min incubation of well mixed reaction solution was made at 50 °C. Next, the solution was quickly cooled and centrifugal of the above mixture was made at 1 000 × *g* for 10 min after adding 2.5 mL of 10% trichloroacetic acid (TCA). Then, 2.5 mL of deionized water and 0.5 mL of 0.1% ferric chloride was mixed with 2.5 mL of the upper layer obtained. Absorbance was recorded at 700 nm.

2.12 Superoxide detection in Caco-2 cells

The culture of Caco-2 cells was made in DMEM with 10% FBS in 5% CO_2 incubator at 37 °C. Cell propagation adopted trypsin digestion approach. When the cells reach 70%–80% of confluence, trypsin was used for enzymatic hydrolysis to generate cell suspension for the subsequent cell experiments.

The inoculation of cells into 96-well plates was made at a cell concentration of 2.5×10^4 cells/mL for qualitative and quantitative detection of reactive oxygen species (ROS). The cell experimental model was fallen into 5 groups: 1) Control group: DMEM with 10% FBS; 2) Perhydrol treatment group: DMEM with 10% FBS and 0.3 mmol/L H_2O_2; 3) G-Cbcp treatment group: 10% FBS, 0.3 mmol/L H_2O_2 DMEM with different concentrations of G-Cbcp; 4) G-Pbcp treatment group: 10% FBS, 0.3 mmol/L H_2O_2 DMEM containing different concentrations of G-Pbcp; 5) G-Bbcp treatment group: 10% FBS, 0.3 mmol/L H_2O_2 DMEM containing different concentrations of G-Bbcp. Qualitative detection: after incubation for 24 h, the Caco-2 cells were incubated in DMEM containing various concentrations of glycosylated hydrolysates and/or H_2O_2 for 24 h. After treatment, the Caco-2 cells were washed with PBS, followed by 20 min incubation with DCFH-DA at 37 °C. The fluorescence intensity of cells in each group was observed by using fluorescence microscope. The fluorescence intensity of cells was measured quantitatively with ImageJ software.

2.13 Statistical analysis

The measurement of all data was made three times, followed by expression as mean ± standard deviation (SD). ANOVA statistical analyses were made on all data with SPSS 20.0, and data were tested for significant differences ($P < 0.05$) with Duncan's test.

3. Results and discussion

3.1 Composition of glycosylated hydrolysates

Table 1 showed the chemical compositions of bone collagen hydrolysates and glycosylated hydrolysates. There was no significant difference in the total nitrogen content of the 3 collagen hydrolysates. Furthermore, there was no significant difference in *D*-galactose content among the three collagen hydrolysates, the content of *D*-galactose in G-Bbcp, G-Pbcp and G-Cbcp were 15.168, 14.201, and 13.270 mg/100 mg, respectively. The galactose consumption of G-Cbcp was the most, next to that of G-Pbcp and G-Bbcp ($P < 0.05$). It indicated that Cbcp may have higher Maillard glycosylation activity than Pbcp and Bbcp. In addition, the moisture content of different samples was also not significantly different, which avoided the influence of moisture difference on the experimental results.

3.2 FTIR spectra

FTIR is an important analytical method, which is widely used to analyze the chemical components of complex compounds and the secondary structures of proteins and hydrolysates[29]. The FTIR spectra of bone collagen hydrolysates (Cbcp, Pbcp, Bbcp) and glycosylated hydrolysates (G-Cbcp, G-Pbcp, G-Bbcp) was shown in Fig. 1. The FTIR spectra of bone collagen hydrolysates showed a broad peak at 3 500–3 200 cm^{-1}. After Maillard reaction, the broad peak intensity of the infrared spectrum of glycosylated hydrolysates at 3 500–3 200 cm^{-1} became weaker. This may be due to the stretching vibration of free hydroxyl after the introduction of galactose. The absorption peaks of FTIR spectroscopy at 1 676 and 1 539 cm^{-1} were caused by N–H

Table 1
Chemical composition of bone collagen hydrolysates and glycosylated bone collagen hydrolysates (mg/100 mg).

Chemical composition	Bbcp	Pbcp	Cbcp	G-Bbcp	G-Pbcp	G-Cbcp
Total nitrogen	15.131 ± 0.129^{a}	15.100 ± 0.117^{a}	15.109 ± 0.108^{a}	7.566 ± 0.046^{b}	7.551 ± 0.039^{b}	7.554 ± 0.036^{b}
D-Galactose	0.013 ± 0.001^{d}	0.013 ± 0.001^{d}	0.012 ± 0.001^{d}	15.168 ± 0.002^{a}	14.201 ± 0.001^{b}	13.270 ± 0.001^{c}
Moisture	5.121 ± 0.421^{b}	5.292 ± 0.332^{b}	5.401 ± 0.371^{b}	5.534 ± 0.523^{a}	5.482 ± 0.514^{a}	5.447 ± 0.490^{a}

Note: Data are expressed as the means ± SD (n = 3); different letters in the same row denote significant difference (P < 0.05). The same below.

bending vibration (1 655–1 540 cm^{-1}) and C=O stretching vibration (1 680–1 630 cm^{-1}), which from amides I and II. By comparing with bone collagen hydrolysates, the intensity of glycosylated peptides decreased slightly, which may be due to the increased absorption caused by the production of Schiff base (C=N), pyrazines (C−N) and Amadori compound (C=O). Nevertheless, macromolecular compounds were formed by polymerizing amino and carbonyl groups during Maillard reaction, which resulted in a decline in absorption. Our results are consistent with previous studies[30-31]. The N−H changes and C−N stretching caused the amide III band (1 450–1 240 cm^{-1}). The absorption peak of glycosylated products at 1 393 cm^{-1} was weaker than that of bone collagen hydrolysates, which may be caused by O−H in-plane deformation vibration. Compared with bone collagen hydrolysates, the glycosylated products showed a stronger peak in the scope of 1 137–938 cm^{-1}, indicating that galactose was attached to the skeleton of bone collagen hydrolysates, leading to partial changes in the peptide structure. In addition, compared with bone collagen hydrolysates, the absorption of glycosylated products was weaker at 660.03 cm^{-1}, which meant that amino groups were consumed during Maillard reaction, resulting in the reduction of N−H bending vibration[32]. The above phenomena confirmed the structural changes caused by glycosylation reaction.

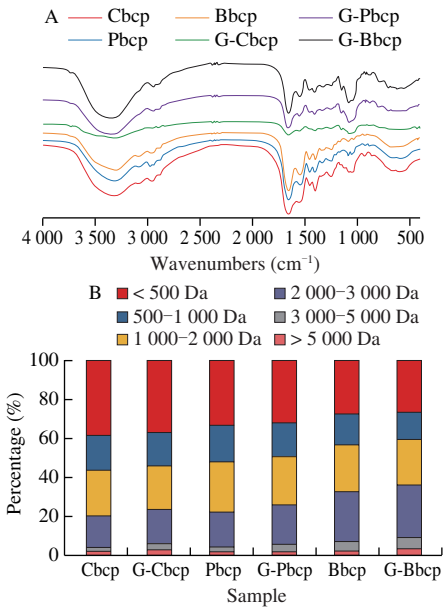

Fig. 1 (A) FTIR spectra of the bone collagen hydrolysates (Cbcp, Pbcp, Bbcp) and glycosylated hydrolysates (G-Cbcp, G-Pbcp, G-Bbcp) at 4 000–450 cm^{-1}; (B) Molecular weight distribution of the bone collagen hydrolysates (Cbcp, Pbcp, Bbcp) and glycosylated hydrolysates (G-Cbcp, G-Pbcp, G-Bbcp).

3.3 Molecular weight distribution

Fig. 1B shows the molecular weight distribution of bone collagen hydrolysates (Cbcp, Pbcp, Bbcp) and glycosylated hydrolysates. The results of molecular weight distribution showed that Cbcp has more low molecular weight collagen peptides, followed by Pbcp and Bbcp. Therefore, Bbcp may contain more complex or more secondary structures than Cbcp and Pbcp, which will affect the reaction activity of free amino groups by steric hindrance effect. Some studies have also shown smaller molecular weight could promote the glycosylation reaction activity, thus explaining that Cbcp showed highest glycosylation reaction activity among three hydrolysates[20]. Furthermore, after the glycosylation, the molecular weight of the glycosylated hydrolysates (G-Cbcp, G-Pbcp, G-Bbcp) was higher than that of the corresponding bone collagen hydrolysates (Cbcp, Pbcp, Bbcp). It might be contributed to the reasons that bone collagen hydrolysates were crosslinked with galactose to form conjugates during glycosylation reaction, which will greatly increase the molecular weight.

3.4 UV-absorbance and browning intensity

The UV-absorption and browning intensity of bone collagen hydrolysates (Cbcp, Pbcp, Bbcp) and glycosylated products (G-Cbcp, G-Pbcp, G-Bbcp) are displayed in Fig. 2A. The UV-absorption of the sample at 294 and 420 nm is the important index of the products in the middle stage and late stage of Maillard reaction, respectively[33]. Compared with collagen hydrolysates, the UV-absorption of glycosylated products increased significantly at 294 and 420 nm (P < 0.05). This may be due to the Maillard glycosylation reaction between bone collagen hydrolysates and *D*-galactose, resulting in the Maillard middle stage or later stage products, which mainly included various carbonyl compounds, such as hydroxymethylfurfural and reduced ketone. Moreover, the UV-absorption of glycosylated products at 294 nm was significantly higher than that at 420 nm (P < 0.05). This implied that three glycosylated bone collagen hydrolysates mainly contained intermediate products of the Maillard reaction rather than the late state products. This meant that the three glycosylated bone collagen hydrolysates may be safe. This is similar to the result of Zhang et al.[21]. In addition, G-Cbcp showed the strongest UV-absorption at 294 nm, followed by G-Pbcp and G-Bbcp. This is because under the same reaction conditions, the Cbcp produced the most Maillard glycosylated intermediate products, followed by the Pbcp and Bbcp. The results showed that of G-Cbcp may be more conducive to glycosylation than G-Pbcp and G-Bbcp. This may be due to the different amino acid composition, different

molecular weight distribution, or different spatial structure of the three bone collagen hydrolysates[20,34].

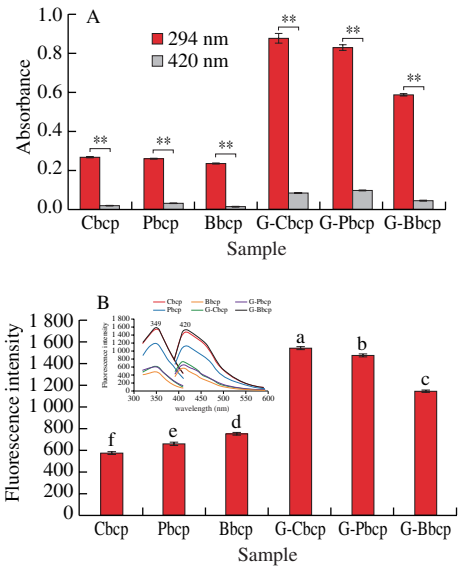

Fig. 2 (A) Changes in browning intensity and absorbance at 294 nm. (B) Fluorescence intensity. Error bars represent the mean ± SD of triplicate experiments. The insert map in Fig. 2B shows the fluorescence intensity, fluorescence excitation spectrum (left), and fluorescence emission spectrum (right) of the sample. ** Means $P < 0.01$. Different letters on top of the bars denote significant difference ($P < 0.05$).

3.5 Fluorescence intensity

In the process of glycosylation reaction, fluorescent groups appeared before the generation of the visible brown pigments[35]. Fluorescence spectroscopic analysis was used to characterize glycosylated products. The fluorescence intensity, fluorescence excitation spectrum (left), and fluorescence emission spectrum (right) of the sample are shown in Fig. 2B. From the fluorescence spectrum of the sample, the fluorescence excitation spectrum peak appears at about 349 nm, and the fluorescence emission spectrum peak appears at about 420 nm. This agrees with the research results of Zhang et al.[21]. The fluorescence intensity of glycosylated products was significantly higher than that of bone collagen hydrolysates ($P < 0.05$). This is similar to research of Chen et al.[36] on Maillard reaction of soybean protein hydrolysates, that is, the sample after Maillard reaction has higher fluorescence intensity in the scope of 400–600 nm. A resembling phenomenon was also observed by Liu et al.[37] that the fluorescence intensity enhanced with the growing time in the intermediate stage of the Maillard reaction in the fructose-histidine model system. Moreover, G-Cbcp displayed the highest fluorescence intensity, followed by G-Pbcp and G-Bbcp ($P < 0.05$). The results showed that a large number of fluorescent compounds were generated in the samples during glycosylation reaction, and the formation rate in G-Cbcp was the fastest, followed by G-Pbcp, and the slowest was G-Bbcp. These fluorescent compounds reached to the maximum before glycosylation products were transformed into brown pigments. In addition, fluorescent compounds obtained from Maillard reaction were revealed to generate hydrogen atoms to eliminate free radical, suggesting that fluorescent compounds derived from Maillard reaction might improve the antioxidant activities of Maillard reaction products[20]. Despite unclear structure of the fluorescent compounds, several researchers speculate that the chemical structure of the fluorescent compound is a Schiff base combined with an electron-donating group, a cyclic unsaturated carbonyl compound or a nitrogen-containing compound[38].

3.6 Change of free amino groups

The variation of free amino groups is a significant reference indicator in the process of glycosylation reaction[39]. The variation of free amino groups in the sample is shown in Fig. 3. Cbcp showed significantly higher content of free amino group than Bbcp. This may be due to the more complete enzymatic hydrolysis of Cbcp, resulting in the explosion of more free amino groups. Compared with collagen hydrolysates (Cbcp, Pbcp, Bbcp), the free amino groups of glycosylated products (G-Cbcp, G-Pbcp, G-Bbcp) decreased significantly ($P < 0.05$), which because α-NH_2 or ε-NH_2 groups in samples were covalently attached to galactose to generate glycosylated hydrolysates. This is similar to research of Zhang et al.[21] on Maillard glycosylation reaction of whey protein hydrolysates, that is, compared with that of whey protein hydrolysates, the free amino groups of glycosylation whey protein hydrolysates sharply declined ($P < 0.05$) since α-NH_2 or ε-NH_2 in whey protein hydrolysate were covalently attached to *D*-galactose to generate advanced glycation end products. A similar situation was also observed by Liu et al.[40] that in porcine plasma protein hydrolysate and galactose reaction system, free amino groups were remarkably decreased after Maillard glycosylation reaction. In addition, the free amino groups decreased most after glycosylation of Cbcp, followed by Pbcp and Bbcp. This indicated that Cbcp showed the highest glycosylation activity, followed by Pbcp and Bbcp. It might be contributed to the reason that Cbcp contains more α-NH_2 groups or ε-NH_2 groups than Pbcp and Bbcp. Because it has been shown that the free amino groups of peptides or amino acids are the sites of the Maillard glycosylation reaction with the sugar[41].

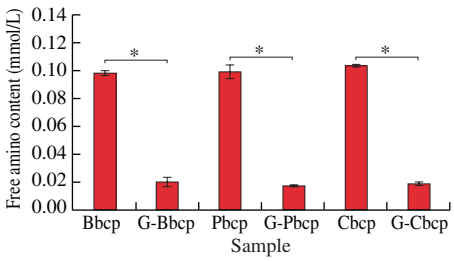

Fig. 3 Content of free amino groups before and after glycosylation of bone collagen peptide from chicken, porcine and bovine. Error bars represent the mean ± SD of triplicate experiments. * Denote significant difference ($P < 0.05$).

3.7 Amino acids

Proportion of different kinds of amino acids in free amino acids and total amino acids is shown in Fig. 4. Tryptophan was not measured due to its destruction during acid hydrolysis. The ratio of basic amino acids (Lys, Arg, His) in Cbcp was significantly higher than that in Pbcp and Bbcp ($P < 0.05$). The reactivity of basic amino acids in Maillard reaction was higher than that of other amino acids[34]. In particular, arginine and lysine in basic amino acids are key amino acids in Maillard reaction. Arginine and lysine are also

the cleavage sites of trypsin, which maybe lead to more complete enzymatic hydrolysis of bone collagen and produce more small molecular peptides and free amino groups. However, after Maillard glycosylation reaction, the proportion of basic amino acids (Lys, Arg, His) in the free amino acids of G-Cbcp was significantly lower than that of G-Pbcp and G-Bbcp. Furthermore, there was no significant difference in the proportion of basic amino acids in the total amino acids of G-Cbcp, G-Pbcp and G-Bbcp after Maillard glycosylation. The results showed that the digestion of basic amino acids in Cbcp was the most during the Maillard reaction, followed by the basic amino acids in Pbcp and Bbcp. Furthermore, Cbcp were significantly higher the contents of phenylalanine and tyrosine than Pbcp and Bbcp (Table 2). Studies have shown that heterocyclic components and rearranged peptides containing aromatic amino acids generated by Maillard reaction may contribute to enhanced antioxidant activity[34]. Phenylalanine, tryptophan (non-polar amino acid) and tyrosine are also the main cleavage sites of pepsin (the extraction of collagen involves pepsin), which maybe also lead to different degrees of enzymatic hydrolysis of collagen already occurring when it is extracted from bone. These above phenomena may lead to the different Maillard glycosylation reaction activity of the three bone collagen hydrolysates, which may lead to their different antioxidant activities. The content of total amino acids and free amino acids of bone collagen hydrolysates (Cbcp, Pbcp, Bbcp) and glycosylated hydrolysates (G-Cbcp, G-Pbcp, G-Bbcp) are shown in Tables 2 and 3. The content of each amino acid in glycosylated hydrolysates was significantly less than that in bone collagen hydrolysates ($P < 0.05$). It may be contributed to the reason that *D*-galactose was crosslinked with amino acids to form macromolecular compounds during glycosylation, resulting in a significant decrease in the content of amino acids.

3.8 Evaluation of potential cytotoxic effects

Flow cytometry was used to further quantitatively determine the potential cytotoxic of three glycosylated hydrolysates. Fig. 5 shows the results of the potential cytotoxic of three glycosylated hydrolysates. The normal living cells in the control group reached 89.89%.

Table 2
Total amino acid content of collagen hydrolysates and glycosylated hydrolysates (mg/g).

Amino acid	Cbcp	G-Cbcp	Pbcp	G-Pbcp	Bbcp	G-Bbcp
Asp	65.088 ± 5.171^{a}	25.099 ± 0.461^{c}	55.603 ± 0.912^{b}	26.557 ± 0.459^{c}	52.823 ± 4.792^{b}	23.367 ± 0.831^{c}
Thr	26.879 ± 1.919^{a}	9.623 ± 0.803^{cd}	16.211 ± 0.591^{b}	7.053 ± 0.059^{d}	15.341 ± 1.352^{bc}	6.635 ± 0.313^{d}
Ser	23.999 ± 0.619^{a}	7.794 ± 0.238^{b}	26.953 ± 1.913^{a}	11.285 ± 0.502^{b}	25.195 ± 3.049^{a}	9.224 ± 0.030^{b}
Glu	114.288 ± 5.774^{a}	56.302 ± 0.631^{d}	97.957 ± 5.848^{c}	52.827 ± 1.204^{d}	104.234 ± 0.300^{b}	53.830 ± 1.629^{d}
Gly	302.398 ± 9.072^{a}	64.516 ± 1.149^{d}	305.956 ± 8.353^{a}	97.541 ± 2.770^{c}	300.061 ± 6.873^{a}	109.116 ± 0.433^{b}
Ala	85.401 ± 3.924^{a}	30.122 ± 0.277^{d}	75.981 ± 5.532^{b}	39.746 ± 1.024^{c}	82.982 ± 1.868^{a}	45.042 ± 0.581^{c}
Cys	3.606 ± 0.723^{b}	1.737 ± 0.751^{bc}	6.488 ± 0.069^{a}	2.811 ± 3.380^{b}	1.297 ± 1.678^{bc}	0.436 ± 0.120^{c}
Val	21.964 ± 0.723^{b}	10.101 ± 1.014^{c}	29.834 ± 0.040^{a}	11.455 ± 1.318^{c}	27.034 ± 2.361^{a}	10.238 ± 0.567^{c}
Met	10.677 ± 1.382^{a}	3.692 ± 1.094^{c}	8.934 ± 0.821^{a}	1.661 ± 0.700^{c}	5.765 ± 0.886^{b}	2.031 ± 1.302^{c}
Ile	16.533 ± 0.332^{a}	8.834 ± 0.158^{c}	14.061 ± 0.010^{b}	5.724 ± 2.804^{d}	11.662 ± 2.314^{bc}	4.473 ± 2.081^{d}
Leu	37.371 ± 1.851^{a}	15.119 ± 0.229^{d}	31.537 ± 1.363^{b}	13.977 ± 0.031^{de}	27.918 ± 1.478^{c}	11.975 ± 0.569^{e}
Tyr	15.358 ± 0.011^{a}	5.811 ± 2.038^{c}	15.025 ± 5.042^{a}	11.975 ± 2.601^{b}	11.487 ± 0.010^{b}	2.556 ± 1.474^{d}
Phe	27.115 ± 1.390^{a}	12.105 ± 1.032^{b}	23.923 ± 3.918^{a}	14.343 ± 0.679^{b}	22.057 ± 2.291^{a}	10.578 ± 0.058^{b}
Lys	40.414 ± 2.104^{a}	9.028 ± 1.008^{c}	33.993 ± 1.087^{b}	11.031 ± 2.030^{c}	35.683 ± 0.261^{b}	10.657 ± 0.313^{c}
His	10.681 ± 0.647^{a}	4.047 ± 0.412^{c}	8.515 ± 1.212^{b}	3.507 ± 0.527^{cd}	6.694 ± 2.143^{b}	2.097 ± 0.901^{d}
Arg	70.704 ± 6.586^{a}	23.238 ± 0.478^{c}	66.505 ± 3.871^{a}	29.802 ± 0.963^{bc}	68.274 ± 1.528^{a}	32.196 ± 1.021^{b}

Note: the unit mg/g is based on the weight of the original hydrolysates.

Table 3
Free amino acid content of collagen hydrolysates and glycosylated hydrolysates (mg/g).

Amino acid	Cbcp	G-Cbcp	Pbcp	G-Pbcp	Bbcp	G-Bbcp
Asp	6.52 ± 0.21^{a}	1.27 ± 0.04^{d}	4.70 ± 0.03^{c}	0.89 ± 0.01^{e}	5.17 ± 0.27^{b}	1.26 ± 0.07^{d}
Thr	2.69 ± 0.06^{a}	0.52 ± 0.01^{d}	1.37 ± 0.08^{c}	0.26 ± 0.02^{e}	1.50 ± 0.07^{b}	0.37 ± 0.02^{e}
Ser	2.41 ± 0.10^{a}	0.47 ± 0.02^{b}	2.28 ± 0.11^{a}	0.43 ± 0.02^{b}	2.47 ± 0.20^{a}	0.60 ± 0.05^{b}
Glu	11.47 ± 0.10^{a}	2.23 ± 0.02^{d}	8.28 ± 0.31^{c}	1.56 ± 0.06^{e}	10.23 ± 0.37^{b}	2.50 ± 0.09^{d}
Gly	16.49 ± 0.03^{c}	3.21 ± 0.01^{e}	17.75 ± 0.30^{b}	3.35 ± 0.06^{e}	22.47 ± 0.21^{a}	5.49 ± 0.05^{d}
Ala	8.57 ± 0.13^{a}	1.67 ± 0.02^{cd}	6.42 ± 0.33^{b}	1.21 ± 0.06^{d}	8.15 ± 0.50^{a}	1.99 ± 0.12^{c}
Cys	0.23 ± 0.12^{b}	0.04 ± 0.02^{c}	0.55 ± 0.01^{a}	0.10 ± 0.00^{bc}	0.23 ± 0.04^{b}	0.06 ± 0.01^{c}
Val	2.21 ± 0.07^{b}	0.43 ± 0.01^{d}	2.52 ± 0.06^{a}	0.48 ± 0.01^{d}	2.65 ± 0.13^{a}	0.65 ± 0.03^{c}
Met	1.07 ± 0.11^{a}	0.21 ± 0.02^{d}	0.75 ± 0.05^{b}	0.14 ± 0.01^{d}	0.56 ± 0.07^{c}	0.14 ± 0.02^{d}
Ile	1.86 ± 0.10^{a}	0.36 ± 0.02^{c}	1.19 ± 0.03^{b}	0.22 ± 0.01^{c}	1.14 ± 0.18^{b}	0.28 ± 0.04^{c}
Leu	3.95 ± 0.05^{a}	0.77 ± 0.01^{c}	2.67 ± 0.06^{b}	0.50 ± 0.01^{c}	2.74 ± 0.04^{b}	0.67 ± 0.01^{d}
Tyr	0.83 ± 0.08^{b}	0.16 ± 0.02^{c}	1.45 ± 0.47^{a}	0.27 ± 0.09^{c}	1.05 ± 0.06^{ab}	0.26 ± 0.02^{c}
Phe	2.32 ± 0.01^{a}	0.45 ± 0.00^{b}	2.37 ± 0.39^{a}	0.45 ± 0.07^{b}	2.16 ± 0.14^{a}	0.53 ± 0.03^{b}
Lys	4.06 ± 0.03^{a}	0.79 ± 0.01^{d}	2.88 ± 0.16^{c}	0.54 ± 0.03^{e}	3.50 ± 0.11^{b}	0.85 ± 0.03^{d}
His	1.07 ± 0.01^{a}	0.21 ± 0.00^{c}	0.72 ± 0.12^{b}	0.14 ± 0.02^{c}	0.65 ± 0.18^{b}	0.16 ± 0.05^{c}
Arg	6.37 ± 0.42^{a}	1.24 ± 0.08^{cd}	5.62 ± 0.20^{b}	1.06 ± 0.04^{d}	6.69 ± 0.11^{a}	1.63 ± 0.03^{c}

Note: the unit mg/g is based on the weight of the original hydrolysates.

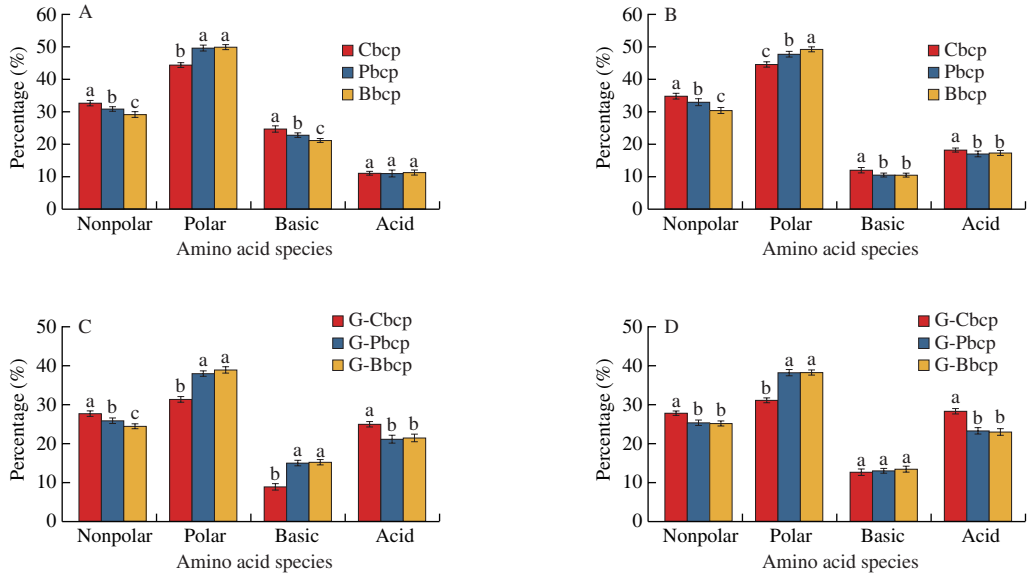

Fig. 4 (A) The proportion of different amino acids in the free amino acids of collagen hydrolysates. (B) The proportion of different amino acids in the total amino acids of collagen hydrolysates. (C) The proportion of different amino acids in the free amino acids of glycosylation hydrolysates. (D) The proportion of different amino acids in the total amino acids of glycosylation hydrolysates. Error bars represent the mean ± SD of triplicate experiments. Different letters on top of the bars in same amino acid specie denote significant difference ($P < 0.05$).

The normal living cells accounted for 89.73%, 90.27% and 90.48% (mean value) in the G-Cbcp treatment groups with a concentration of 0.5–2.0 mg/mL, respectively. The normal living cells accounted for 90.40%, 90.26% and 90.20% (mean value) in the G-Pbcp treatment groups with a concentration of 0.5–2.0 mg/mL, respectively. The normal living cells accounted for 89.58%, 89.66% and 90.39% (mean value) in the G-Bbcp treatment groups with a concentration of 0.5–2.0 mg/mL, respectively. By comparing with the control group, all 6 groups of samples did not significantly inhibit cell activity at 0.5–2.0 mg/mL at 48 h, indicating that 3 glycosylated hydrolysates showed no obvious toxicity. Therefore, the 3 glycosylated hydrolysates can be safely applied to food as natural antioxidants.

3.9 Antioxidant properties

DPPH, ABTS, hydroxyl radical scavenging activity and ferrous reducing power of bone collagen hydrolysates and glycosylated hydrolysates is shown in Figs. 6−9. The DPPH, ABTS cation, hydroxyl radical scavenging activity and ferrous reducing power of all 6 samples significantly increased when concentration ranged from 3 to 15 mg/mL (hydroxyl: 0.25−4.00 mg/mL) ($P < 0.05$). The DPPH, ABTS cation, hydroxyl radical scavenging activity and ferrous reducing power of glycosylated hydrolysates (G-Cbcp, G-Pbcp, G-Bbcp) was significantly higher than that of bone collagen hydrolysates (Cbcp, Pbcp, Bbcp). In addition, the DPPH, ABTS cation, hydroxyl radical scavenging activity and ferrous reducing power of G-Cbcp was the strongest, followed by G-Pbcp and G-Bbcp. DPPH radical scavenging activity stands for the hydrogen-donating capacity of an antioxidant. According to the report, the intermediate compounds (reductone) of Maillard reaction could provide hydrogen atoms and destroy the radical chain[42]. ABTS experiments detected compounds that play a role through radical quenching by hydrogen atom transfer (HAT) mechanism or direct reduction by electron transfer. This indicated that glycosylation products might play the role of hydrogen donor and displayed well ABTS cation radical scavenging activity. Therefore, compared with the bone collagen hydrolysates, the three glycosylated hydrolysates showed significant characteristic and structural changes and higher antioxidant activity.

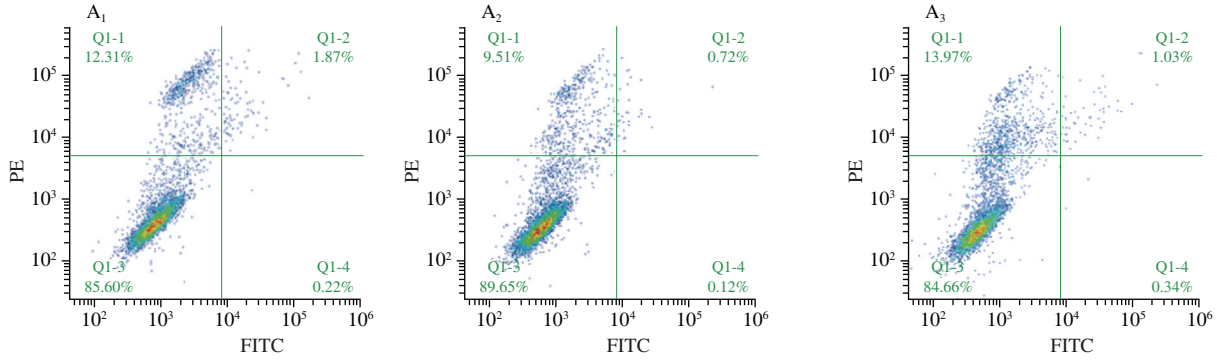

Fig. 5 (A) Apoptosis of Caco-2 cells in the experimental group was detected by flow cytometry. (A_1–A_3) G-Bbcp treatment groups with a concentration of 2.0, 1.0, 0.5 mg/mL, respectively; (A_4–A_6) G-Pbcp treatment groups with a concentration of 2.0, 1.0, 0.5 mg/mL, respectively; (A_7–A_9) G-Cbcp treatment groups with a concentration of 2.0, 1.0, 0.5 mg/mL, respectively. (B) Apoptosis of Caco-2 cells in the control group was detected by flow cytometry. (C) Quantitative histogram of apoptosis of Caco-2 cells. Cells in Q1-3 range are normal living cells. PE, phycoerythri; FITC, fluorescein isothiocyanate.

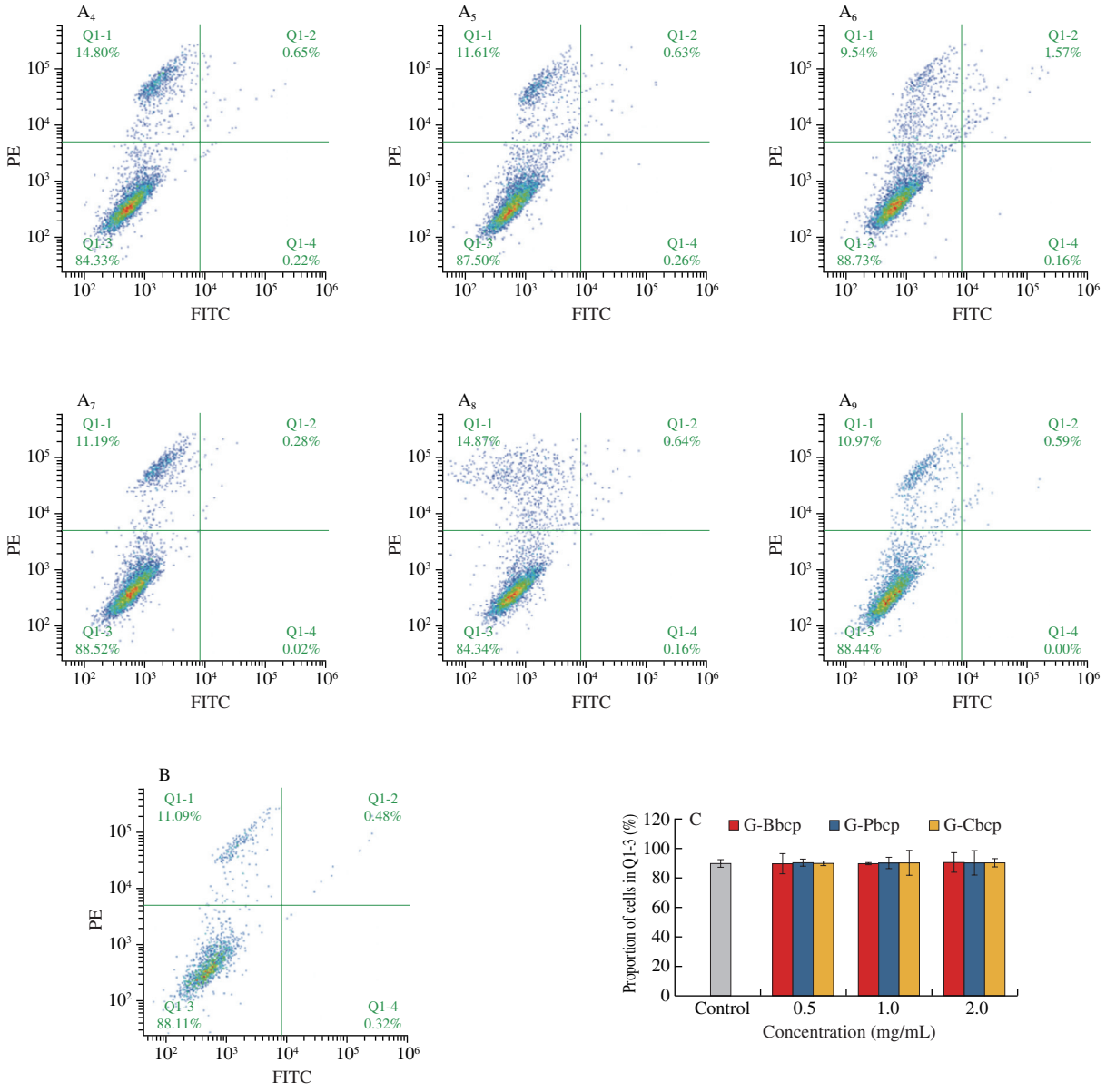

Fig. 5 (*Continued*)

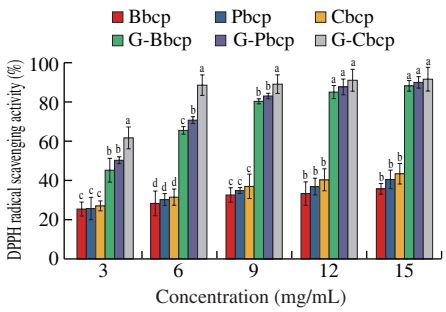

Fig. 6 DPPH radical scavenging activity of the bone collagen hydrolysates and glycosylated hydrolysates. Error bars represent the mean ± SD of triplicate experiments. Different letters on top of the bars in same concentration denote significant difference ($P < 0.05$).

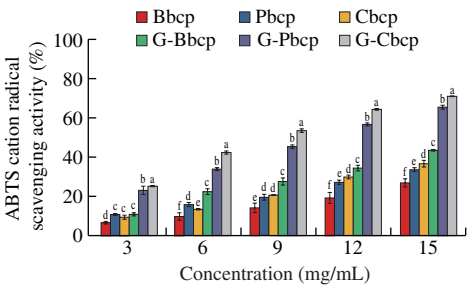

Fig. 7 ABTS cation radical scavenging activity of the bone collagen hydrolysates and glycosylated hydrolysates. Error bars represent the mean ± SD of triplicate experiments. Different letters on top of the bars in same concentration denote significant difference ($P < 0.05$).

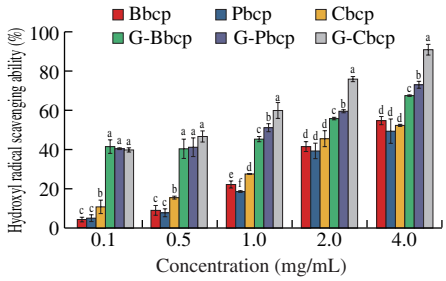

Fig. 8 Hydroxyl radical scavenging ability of the bone collagen hydrolysates and glycosylated hydrolysates. Error bars represent the mean ± SD of triplicate experiments. Different letters on top of the bars in same concentration denote significant difference ($P < 0.05$).

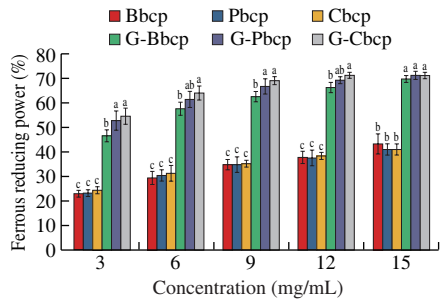

Fig. 9 Ferrous reducing power of the bone collagen hydrolysates and glycosylated hydrolysates. Error bars represent the mean ± SD of triplicate experiments. Different letters on top of the bars in same concentration denote significant difference ($P < 0.05$).

3.10 Antioxidant activity in Caco-2 cells

ROS is an unstable molecule with high activity and short half-life. Studies have shown that antioxidants can clear ROS directly or indirectly by activating antioxidant enzymes in cells[43-44]. ROS variations in every group were found qualitatively with a fluorescence microscope. According to Fig. 10, the cells in the control group displayed almost no green fluorescence, while the cells in the hydrogen peroxide treatment group displayed the strongest green fluorescence intensity, indicating that hydrogen peroxide induced excessive ROS production. In the experimental group with glycosylated hydrolysates, the green fluorescence intensity or ROS level decreased significantly with the increase of glycosylated hydrolysates concentration ($P < 0.05$). In addition, the experimental group containing G-Cbcp showed the weakest fluorescence intensity or the lowest ROS level, indicating that the ROS scavenging ability of G-Cbcp was the strongest, followed by G-Pbcp and G-Bbcp. This is consistent with the above experimental results of ABTS, DPPH, hydroxyl radical scavenging rate and ferrous reducing power.

4. Conclusion

In this study, the antioxidant activities of the three collagen hydrolysates (Cbcp, Pbcp, and Bbcp) were significantly enhanced after Maillard glycosylation reaction. Compared with Pbcp and Bbcp, Cbcp was more suitable for the preparation of high antioxidant activity peptides by Maillard glycosylation reaction. The difference of amino acid composition and the degree of hydrolysis may be the main reasons for the difference of Maillard glycosylation reaction activity and antioxidant activity of glycosylation products of the three bone collagen hydrolysates. The 3 glycosylated bone collagen hydrolysates showed no obvious cytotoxicity and can be safely applied to food as natural antioxidants. These results provide theoretical support for the application of the 3 glycosylated hydrolysates as antioxidants in food, and also provide theoretical reference for the high-value utilization of collagen hydrolysates from livestock and poultry bones.

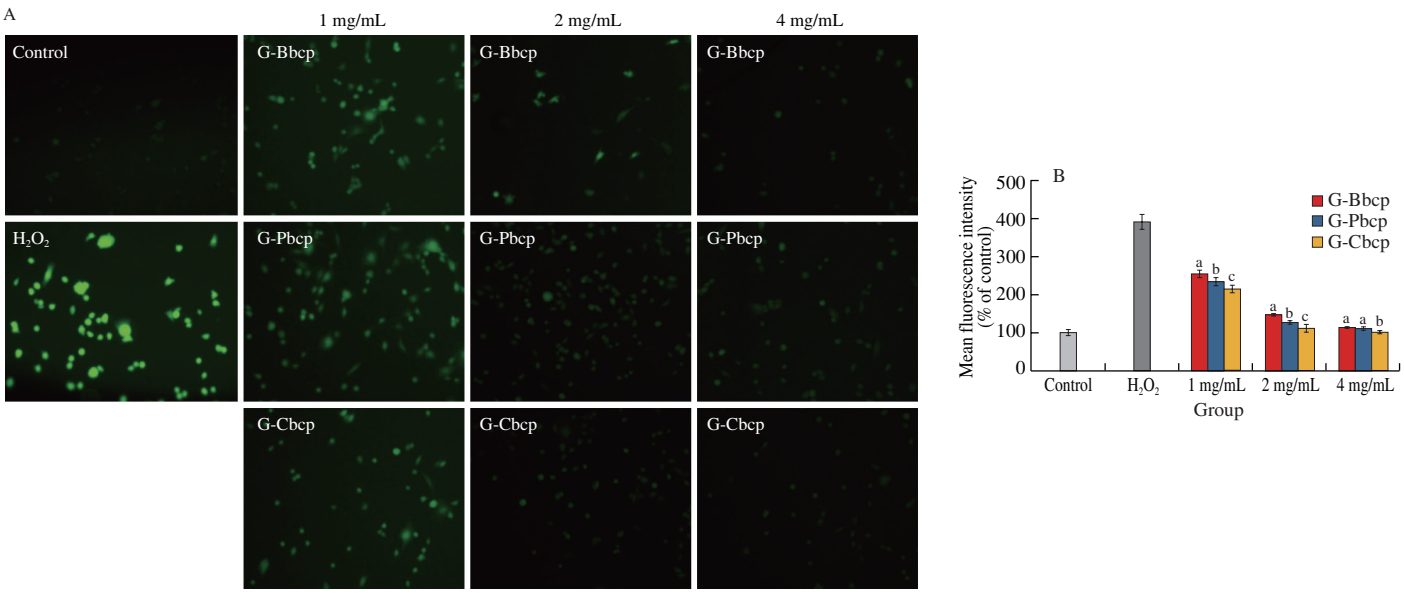

Fig. 10 Antioxidant activity of glycosylated hydrolysates. (A) ROS distribution observed under fluorescent microscope. (B) Quantitative histogram of fluorescence intensity. Error bars represent the mean ± SD of triplicate experiments. Different letters on top of the bars in same concentration denote significant difference ($P < 0.05$).

Declaration of competing interest

The authors declare that there is no conflict of interests.

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