



Preparation of glycosylated zein and retarding effect on lipid oxidation of ground pork



Xiao-Jie Wang, Xi-Qun Zheng, Xiao-Lan Liu ^{*}, Narasimha-Kumar Kopparapu, Wan-Suo Cong, Yong-Ping Deng

Heilongjiang Provincial Key University Laboratory of Processing Agricultural Products, College of Food and Bioengineering, Qiqihar University, Qiqihar 161006, PR China

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ABSTRACT

The focus of the present work was to investigate the glycosylation of zein, partial properties of the glycosylated zein (GZ) and its retarding effect on lipid oxidation of ground pork. Zein was glycosylated with chitosan (MW 1500 Da) by microbial transglutaminase, the reaction was verified by FT-IR. Under the optimized conditions, 97.48 mg of glucosamine was covalently conjugated to 1 g of zein, determined by HPLC. The solubility and the surface hydrophobicity of GZ were significantly improved. *In vitro* studies of GZ showed a dose-dependent scavenging activity against free radicals of DPPH, superoxide and hydroxyl radical, and the EC₅₀ value for DPPH radical was 1.99 µg TE/mg protein. In addition, reducing power and Fe²⁺-chelating capacity of it were 16.60 and 12.96 µg TE/mg protein, respectively. GZ resulted in low levels of thiobarbituric acid-reactive substances and peroxide value of ground pork. These results suggest that GZ is a potential natural antioxidant.

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1. Introduction

Corn gluten meal (CGM), a major co-product of corn wet-milling process, contains 67–71% proteins consisting of approximately 65% zein and 30% glutelin (Gioia, Cuq, & Guilbert, 1998). Zein, the predominant food grade protein in corn, is a mixture of highly hydrophobic proteins rich in proline, glutamine, leucine, and alanine (Pomes, 1971). Zein is insoluble in aqueous solutions but is readily soluble in organic solvents such as 60–70% isopropanol and 70–90% ethanol. Corn glutelin has a large number of inter- and intra-molecular disulfide bonds that create a closed compact structure, therefore it dissolves only in dilute acid or alkali solutions (Paraman, Hettiarachchy, Schaefer, & Beck, 2006). These characteristics make CGM less applicable in food industry, and it is mostly utilized as animal feed. If the protein from CGM is modified for utilizing in food industry, its value and presence in market place would be increased.

Protein glycosylation is regarded as one of the major protein post-translational modifications in cells. Glycosylation in body, an enzyme-driven process, is a crucial step in altering the biological activity of cells as it regulates the interaction between the cellular molecules (Hong, Gottardi, Ndagijimana, & Betti, 2014).

Glycosylation reaction can also occur in the food system. Compared to the native protein form, incorporation of carbohydrate into protein has shown promising improvements in several aspects of functionality of glycosylated protein. The Maillard reaction, also referred to as non-enzymatic glycosylation, has been studied extensively as it can significantly improve functional characteristics of native protein, such as solubility, emulsibility, foamability, gelling and antioxidant activities (Li et al., 2013; Xue, Li, Zhu, Wang, & Pan, 2013). Unfortunately, potential adverse effects of the Maillard-type glycosylation cannot be ignored, such as the formation of toxic and mutagenic compounds (Brands, Alink, Van-Boekel, & Jongen, 2000), undesired browning and flavour products (Guerra-Hernandez, Gomez, Garcia-Villanova, Sanchez, & Gomez, 2002) as well as difficulty to control the degree of glycosylation. Enzymatic glycosylation of protein catalyzed by transglutaminase (TGase, EC 2.3.2.13) is an alternative protocol because it increases the safety and occurs under mild reaction conditions.

TGase, i.e. protein-glutamine γ -glutamyltransferase, is an enzyme involved in post-translational modification of proteins (Trespalacios & Pla, 2007). The TGase derived from eukaryotic and prokaryotic sources, which does not require calcium ions, is popular in dairy and meat processing sectors in food industry. TGase catalyses the formation of an isopeptide bond between the group of γ -carboxamides of glutamine residues, as acyl donors, with the first order ϵ -amine groups of different compounds, as acyl acceptors. If lysine is an acceptor of acyl, the transfer of acyl onto a

^{*} Corresponding author at: College of Food and Bioengineering, 42 Wenhua Street, Qiqihar, Heilongjiang Province 161006, PR China.

E-mail address: liuxiaolan001@126.com (X.-L. Liu).

lysine residue bound in the polypeptide chain induces formation of inter- and intra-molecular cross-linkages (Kieliszek & Misiewicz, 2014). Moreover, reactive lysine may be substituted by several compounds containing primary amino groups in cross-linking reaction of proteins or peptides. Thus, saccharides containing primary amino groups or aminated saccharides could serve as an amine donor that allows TGase to bind its ϵ -amino group to a glutamine-containing peptide/protein to modify the functionality and bioactivity of some food proteins. Jiang and Zhao (2010–2012) studied glycosylation between glucosamine and soybean protein isolates (SPI) as well as with casein catalyzed by TGase and found that the modified product exhibited lower surface hydrophobicity, better interfacial properties, markedly increased apparent viscosity, and higher enzymatic digestibility *in vitro*. Song and Zhao (2013) and Song and Zhao (2014) reported that oligochitosan was incorporated into caseinate and SPI by TGase, resulting in a modified product with higher apparent viscosity. On the other hand, TGase was also applied to modify fish gelatin peptides and wheat gluten peptides at mild temperatures (25 °C or 37 °C), resulting in the synthesis of glycopeptides with higher antioxidant and antimicrobial properties (Gottardi, Hong, Ndagijimana, & Betti, 2014; Hong et al., 2014).

Since corn protein contains high proportion of glutamine (21.4%) and lacks lysine, the enzymatic glycosylation reaction can occur mainly between corn protein and saccharides with reactive primary amino group with low probability of intra-molecular cross-linkage between lysine and glutamine. Therefore, corn protein could be a good substrate in enzymatic glycosylation for preparation of glycoprotein. In the literature available, the conjugation of saccharides with primary amines to zein by TGase has not been reported so far. In the present study, zein was modified by chitosan using microbial TGase as biocatalyst. Partial functional properties of the modified products were evaluated, including solubility, surface hydrophobicity, and antioxidant activities, and effect of the glycosylated zein on inhibiting lipid oxidation of ground pork was also investigated.

2. Materials and methods

2.1. Material and chemicals

CGM was purchased from Longfeng Corn Development Co., Ltd. (Heilongjiang, China), with a total protein content of 61.25%. Microbial TGase (1000 U/g) was purchased from Yiming Biological Products Co., Ltd. (Taixing, Jiangsu, China). Chitosan, with a declared deacetylation degree of about 90% and an average molecular weight of 1500 Da by the supplier, was purchased from Shandong Bozhihuili Biochemical Co. (Qingdao, China). D-(+)-glucosamine hydrochloride of >99% purity was purchased from Sangon Biological Engineering Co., Ltd. (Shanghai, China). 1,1-diphenyl-2-picrylhydrazyl (DPPH), 8-anilino-1-naphthalene sulfonic acid (ANS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 1,1,3,3-Tetraethoxypropane (TEP) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All reagents in the HPLC analysis were HPLC grade. Other chemicals and reagents used were of analytical grade.

2.2. Preparation of zein

Zein was extracted according to the method described by Zheng et al. (2014) with some modifications. Extrusion and starch removal of CGM were used as pretreatment procedures before extraction. Briefly, the processed CGM powder was mixed with acetone (acetone: CGM = 10:1, v/w) for 30 min, and the mixture was centrifuged at 4000g for 15 min and the supernatant containing

most of the pigment in CGM was discarded. The sediment was collected and dispersed with 70% ethanol (ethanol: CGM = 10:1, v/w) at 60 °C for 2 h, followed by centrifugation at 4000g for 15 min. The supernatant was collected and the sediment was further processed once using the same method as above. Then, the supernatants were pooled, and concentrated to allow precipitation under rotary evaporation at 60 °C. Finally, zein powder was obtained by freeze-drying.

2.3. Glycosylation of zein

Glycosylation of zein was performed in a water bath with constant agitation. Briefly, zein was suspended in distilled water, and chitosan was added and mixed well, heated to appropriate temperature and adjusted to appropriate pH. Then, TGase was added to initiate glycosylation reaction. The following parameters were varied in the glycosylation: pH at 0.2–0.3 intervals from 7.0 to 8.0, temperature at 3 °C intervals from 31 to 43 °C, zein concentration at 1% intervals from 1% to 5%, molar ratio of acyl donor and acyl acceptor (i.e. zein and chitosan) of 1:1, 1:2, 1:3, 1:4 and 1:5, the E/S ratio at 10 U/g zein intervals from 40 to 80 U/g zein, and reaction time at 1 h intervals from 6 to 10 h. After glycosylation, the TGase in the samples was inactivated immediately by heat treatment at 85 °C for 5 min. Finally, excess of chitosan was removed by dialysis using 2000 Da cut off membrane against distilled water for two days at 4 °C. The control of cross-linked zein was prepared without chitosan. All prepared samples, i.e. the glycosylated zein and cross-linked zein, were lyophilized and stored in a desiccator for further study.

2.4. HPLC analysis of the glycosylated zein

Analysis of chitosan conjugated to zein was measured according to the method described by Jiang and Zhao (2010) with some modifications. Briefly, a RP-HPLC method using pre-column derivatization with anthranilic acid (AA) was applied to analyze the chitosan content, which was expressed on the basis of glucosamine. The analysis was performed on a liquid chromatograph 1200 series (Agilent Technologies Inc., Wilmington, DE, USA) with a C18-reversed phase column (Waters ODS 4.6 mm \times 250 mm i.d. 5 μ m, Waters Corporation, Milford, MA, US) with a flow rate of 1.0 mL/min. Solvent A consisted of 0.4% *n*-butylamine, 0.5% phosphoric acid and 1.0% tetrahydrofuran in water, and solvent B consisted of 50% solvent A and 50% acetonitrile. After sample application, the column was eluted with 96% solvent A and 4% solvent B for 30 min. Absorbance was monitored at 230 nm.

Hydrolysis and derivatization of the glycosylated zein products and standard glucosamine were conducted by the method of Jiang and Zhao (2010) with some modifications. 20 mg of the glycosylated zein was hydrolyzed with 5 mL of 6 mol/L HCl at 100 °C for 8.5 h, then 100 μ L of the obtained hydrolysates were dried using pressure blowing concentrator at 40 °C. A methanol-acetate-borate solution was prepared by dissolving 4.0 g sodium acetate and 2.0 g boric acid in 100 mL methanol. The AA-reagent was prepared by dissolving 30 mg of anthranilic acid and 20 mg of sodium cyanoborohydride in 1 mL of methanol-acetate-borate solution. 100 μ L of freshly prepared sodium acetate solution (1%, w/v) and 100 μ L of AA-reagent were added to the dried hydrolysates or 100 μ L of standard glucosamine solutions followed by derivatizing at 80 °C for 1 h, cooled to ambient temperature, diluted to 1 mL with HPLC solvent A, and then filtered through 0.22 μ m membranes. 10 μ L of the supernatant was loaded onto C18 column for analysis. The chitosan content conjugated to zein was expressed as mg of glucosamine per g of zein.

2.5. Fourier transform infrared spectroscopy analysis of the glycosylated zein

The glycosylated zein and original zein as well as cross-linked zein were characterized by Fourier transform infrared spectroscopy (FT-IR). FT-IR spectra of the samples were conducted in the form of KBr pellets by using a Spectrum One FT-IR instrument (Perkin Elmer Inc., Norwalk, CT, USA) at a frequency range of 400–4000 cm^{-1} with a resolution of 1 cm^{-1} .

2.6. Measurement of properties of the glycosylated zein

2.6.1. Measurement of solubility of the glycosylated zein

Solubility of the glycosylated zein was determined by the method of Babiker (2000) with slight modifications. Freeze-dried samples (original zein, cross-linked zein and the glycosylated zein) were dispersed in buffer solutions with various pHs: 0.05 mol/L citrate buffer at pH 3; 0.05 mol/L acetate buffer at pH 4–5; 0.05 mol/L phosphate buffer at pH 6–8; 0.05 mol/L carbonate buffer at pH 9–11. If necessary, the pH was adjusted by using 1 mol/L HCl or 1 mol/L NaOH. The samples were dispersed in the buffer and mixed with a vortex mixer for 30 s, and kept overnight at 4 °C to facilitate hydration. After centrifugation (9000g for 20 min, 4 °C), the protein content in the supernatant was determined according to Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). The total protein content of samples was measured by Kjeldhal method. Protein solubility (percent) was defined as the protein concentration in the supernatant divided by the total protein content of sample and then multiplied by 100. All measurements were performed in triplicate.

2.6.2. Determination of surface hydrophobicity of the glycosylated zein

Surface hydrophobicity of the glycosylated zein was determined according to the method of Kato and Nakai (1980) with some modifications. The samples (30 mg) were dispersed in 10 mmol/L phosphate buffer at pH 7.0 and incubated at room temperature for 24 h to facilitate hydration, then centrifuged at 10000g for 10 min and the supernatant was diluted further in phosphate buffer to obtain various concentrations ranging from 0.01 mg/mL to 0.2 mg/mL. The diluted protein solution (5 mL) was thoroughly mixed with 25 μL of 8 mmol/L ANS in 10 mmol/L phosphate buffer (pH 7.0) and incubated in dark at room temperature for 15 min. Fluorescence intensity (FI) was measured at an excitation wavelength of 390 nm and an emission wavelength of 470 nm at a constant slit of 5 nm using a Versafluor Fluorometer System (RF-5301PC model, Hitachi Co., Tokyo, Japan). The initial slope of the relative FI versus protein concentration (mg/mL) plot represented an index of the surface hydrophobicity of the protein sample evaluated. All measurements were performed in triplicate.

2.6.3. In vitro antioxidant activities of the glycosylated zein

The antioxidant activities including DPPH, superoxide, hydroxyl radical scavenging assays and reducing power assay as well as metal ions chelating capacity of the glycosylated zein were investigated *in vitro*. The antioxidant activities were determined according to our previously published methods (Wang et al., 2014). The copper ion-chelating capacity was determined as described by Kim (2013). In order to eliminate the physical blend of zein and chitosan, chitosan was used as negative control, since chitosan could contribute to antioxidant activity. Meanwhile, six standard Trolox calibration curves were prepared in a concentration range of 0–1100 μg . The antioxidant activities of the samples were calculated from Trolox standard curves and expressed as μg Trolox equivalent per mg of the sample ($\mu\text{g TE/mg sample}$).

All measurements were performed in triplicate.

2.7. Effect of the glycosylated zein on retarding lipid oxidation of ground pork

2.7.1. Preparation of ground pork

Fresh pork was purchased from a local market in Qiqihar, China. Lean pork and fat from pork back were separated manually using a butcher knife, and the connective tissues were discarded. The lean pork and fat were cut into uniform blocks and then grounded using a food processor at a ratio of 4:1 (w/w) to make ground pork. The ground pork and salt (2.0%) were homogenized in a B15 mechanical stirrer for 5 min, the sample was then divided into four groups, and the glycosylated zeins at levels of 0.00, 0.01, 0.03, and 0.05 g/100 g ground pork were added to the groups, respectively, by blending with a mixer. Aliquots (50 g) of the samples were vacuum-packed and stored in refrigerated incubators at 4 °C. Three random aliquots were taken for analysis from each group at 0, 3, 6, 9, 12, 15, 18, 21 and 24 days.

2.7.2. Determination of thiobarbituric acid-reactive substances

The thiobarbituric acid-reactive substances (TBARS) of samples were determined according to the method of Lee, Hendricks, and Cornforth (1999) with some modifications. The method is based on accumulation of lipid hydroperoxide decomposition products during storage of meat products, especially malondialdehyde (MDA). These products, after a thermo-activated reaction with thiobarbituric acid (TBA), produce a pink TBA-MDA adduct with a maximum absorbance at 532 nm. To 1 g of sample, 2.5 mL of TBA (stock solution) and 20 μL of 0.2% butylated hydroxytoluene (BHT) were added and placed in boiling water bath for 15 min, then cooled on ice, and centrifuged at 5500g for 20 min. The absorbance of the supernatant was measured at 532 nm against a reagent blank. TBARS values were calculated from a standard curve of MDA, which was a breakdown product of 1, 1, 3, 3-Tetraethoxypropane (TEP). TBARS values were expressed as mg of MDA per kg of sample. TBA stock solution was composed of 15g TCA, 0.375g TBA, 2.1 mL hydrochloric acid and 100 mL deionized water. The tests were performed in triplicate.

2.7.3. Determination of peroxide value (PV)

PV of samples was measured according to the method of Shi, Cui, Yin, Luo, and Zhou (2014) with some modifications. Ground pork (20 g) was homogenized in 50 mL water for 2 min. 2 mL of homogenate was mixed with 3 mL of isooctane/2-propanol (3:1, v/v) solution and centrifuged at 4000g for 5 min to obtain the organic solvent phase. An aliquot (400 μL) of this phase was mixed with 3 mL of methanol/1-butanol (2:1, v/v) solution, to this 30 mL of 30% ammonium thiocyanate and 30 mL of the ferrous chloride solution were added, and placed at room temperature for 20 min. Absorbance of the solution was measured at 500 nm. Results were expressed as milliequivalent peroxide per kg of meat sample (meq/kg).

2.8. Statistical analysis

All experiments were performed in at least three independent trials, and the results were reported as means \pm standard deviations. Results were subjected to the analysis of variance using SPSS, and significant differences were determined by Duncan's multiple range test and accepted at $P < 0.05$.

3. Results and discussion

3.1. Preparation of chitosan-conjugated zein by TGase

Chitosan is a deacetylated product of native chitin, a non toxic, biodegradable and biocompatible carbohydrate (Batista, Pinto,

Gomes, & Gomes, 2006). Free amine groups in chitosan provide an opportunity for its incorporation into food proteins to form glycosylated proteins by TGase. Zein was modified by TGase in the presence of chitosan to prepare the glycosylated zein. To obtain maximal content of chitosan conjugation, the reaction conditions were investigated. HPLC was used to analyze the amount of chitosan conjugated to zein, which was expressed on the basis of glucosamine. HPLC profiles of glucosamine, original zein and the glycosylated zein are shown in Fig. 1. When the glucosamine standard solution was analyzed, two peaks (peak 1 was AA-glucosamine, peak 2 was the epimer of AA-glucosamine, AA-mannosamine) were eluted at retention time of about 10 and 11 min (Fig. 1A), this phenomenon was also found in the glycosylated zein but not in the original one (Fig. 1B and C). The HPLC profiles confirmed that some chitosan was covalently conjugated to the zein. The suitable reaction conditions for the glycosylation were pH of 7.7, temperature of 37 °C, zein concentration of 3%, molar ratio of acyl donor and acyl acceptor of 1:3, TGase addition of 60 U/g zein and reaction time of 8 h, and under the optimized conditions, the content of chitosan conjugated to zein was 97.48 mg of glucosamine per 1 g of zein. In a previous study, Jiang and Zhao reportedly incorporated 3.3 mol of glucosamine to 1 mol of SPI (equals to 2.62 mg/g SPI), and 1.15 mol of glucosamine to

1 mol of casein (equals to 10.30 mg/g casein) by TGase (Jiang & Zhao, 2010, 2011). In a recent report by Song and Zhao, TGase was applied to catalyze glycosylation and cross-linking of oligochitosan (with a declared deacetylation degree of about 75% and an average molecular weight of 1 kDa) with caseinate and soybean protein, and the amount of glucosamine conjugated was 4.74 g/kg protein and 12.1 g/kg protein in the modified products, respectively (Song & Zhao, 2013, 2014). The conjugated saccharide content in the present study is higher than those reported earlier. It was estimated that the glycosylation sites of zein by TGase were more than those of soybean protein and casein since it contains high proportion of glutamine (21.4%), while subunits of soybean proteins (7S and 11S) and casein contain about 11.23–14.61% and 8.77–11.69% glutamine, respectively. Moreover, in our present work, chitosan (with a declared deacetylation degree of about 90% and an average molecular weight of 1500 Da by the supplier) contains more glucosamine compared to that used by earlier researchers (Song & Zhao, 2013, 2014).

3.2. FT-IR analysis of the glycosylated zein

FT-IR was used to confirm the successful glycosylation between chitosan and zein. FT-IR spectra of three samples are shown in Fig. 2. It indicated that in the range between 1140 and 1023 cm^{-1} the glycosylated zein were different apparently from original one or the cross-linked zein, while original zein and the cross-linked zein showed a similar model in this region. The peaks near 1080 to 1025 cm^{-1} were attributed to C–O stretching vibration ($\nu_{\text{C-O}}$) of the ring COH, COC and CH_2OH (Paluszkiwicz, Stodolak, Hasik, & Blazewicz, 2011). The $\nu_{\text{C-O}}$ and –OH deforming vibration will result in a typical absorbance at 1050–1150 cm^{-1} (Song & Zhao, 2014). The enhanced absorbance at 1075.74 cm^{-1} suggested that the glycosylated zein contained more C–OH than original one and the cross-linked zein. This indicates that the some chitosan covalently conjugated to the zein during the enzymatic reaction catalyzed by TGase, which is in agreement with the result of the HPLC analysis.

3.3. The impacts of conjugation of chitosan to zein on its partial physicochemical properties

The glycosylated zein was prepared under the optimal conditions as described above and its partial physicochemical properties

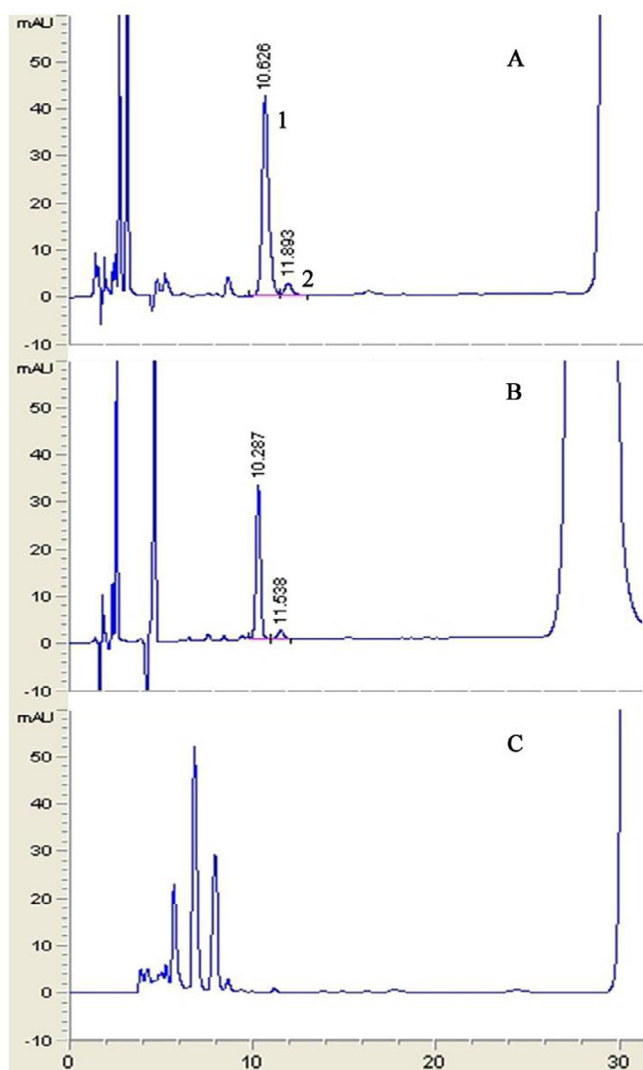


Fig. 1. HPLC profiles. (A) standard glucosamine; (B) glycosylated zein; (C) original zein. Peak 1 and 2 are AA-glucosamine peak and its epimer AA-mannosamine peak, respectively.

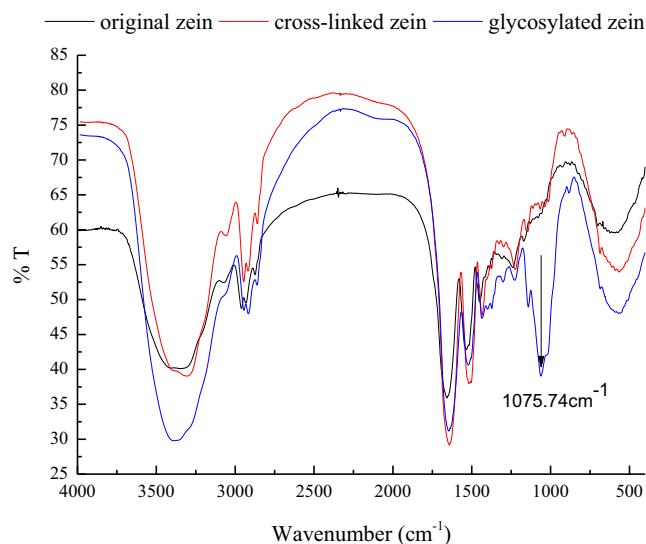


Fig. 2. FT-IR spectra of original zein, cross-linked zein and the glycosylated zein

were evaluated. At the same time, original zein and cross-linked zein were used as two controls.

Undesirable solubility of zein restricts its application in food industry. Therefore, the improvement of solubility of zein is a prerequisite for the performance of its other functional properties. Solubilities of the glycosylated zein, original zein and cross-linked zein in pH range of 3–11 are shown in Fig. 3. The original zein showed limited solubility at all tested pH levels which increased gradually up to 2.58% as the pH was raised to 11. The values in the alkaline pH range (8–11) were higher than those in the acidic pH range, which could be due to the predominance of $-COOH$ ionisable groups over $-NH_2$. It is noteworthy that the isoelectric point of the original zein was not detected in the tested pH range, because of zein's low polar and high non-polar amino acid contents (>50%), Casella and Whitaker also noticed this phenomenon (1990). Cross-linked zein had lower solubility than original zein. It could be speculated that when zein was modified by TGase in the absence of chitosan, cross-linking of the zein resulted in the formation of zein's polymers, which caused a decrease in its solubility. The solubility of the glycosylated zein was much higher than that of the control zein over a wide pH range (pH 3–11). The improvement in solubility was primarily due to an increased number of hydrophilic groups of the glycosylated zein by chitosan conjugation. Moreover, the carbohydrate moiety of the glycosylated zein might have imposed a steric hindrance to prevent its aggregation, giving rise to the improvement of the zein solubility. Similar phenomena were also observed by Jiang and Zhao (2011), Song and Zhao (2013), and Hrynets, Ndagijimana, and Betti (2014). Compared to original zein, the glycosylated zein showed excellent solubility at alkaline pHs (9–11) and extreme acidic pHs (3–4), and good solubility at neutral pHs (6.0–8.0), the values increased remarkably by 16.57%, 17.39%, 13.31%, 15.31% and 17.75% at pH 3, 4, 9, 10 and pH 11 respectively. The solubility of the glycosylated zein exhibited a typical profile of a protein with isoelectric point. This indicates that the glycosylation of zein added net charge on its surface.

Surface hydrophobicity has important influences on macromolecule structural stability, surface property and fat-binding ability of protein (Haskard & Li-Chan, 1998). Fig. 4 shows the surface hydrophobicities of three zein samples. Compared to that of original zein, the surface hydrophobicity of the glycosylated zein was decreased by 282.51, while the value of cross-linked zein was increased by 82.35. It could be speculated that the decreased

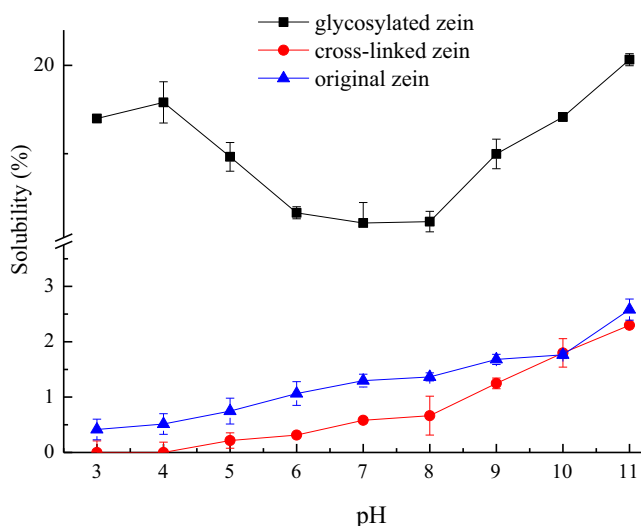


Fig. 3. Solubility profile of original zein, cross-linked zein and the glycosylated zein in pH range of 3–11.

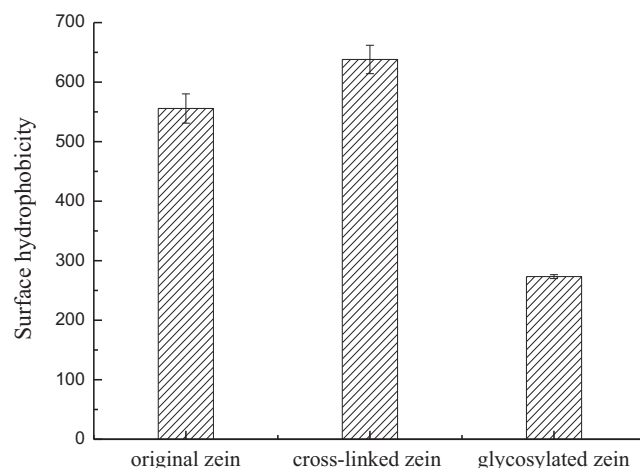


Fig. 4. Surface hydrophobicity of original zein, cross-linked zein and the glycosylated zein measured by ANS method.

surface hydrophobicity of the glycosylated zein was a result of an increased number of hydrophilic groups in the side chains of the zein by chitosan conjugation, while the increased value of the cross-linked zein by TGase was due to an unfolding of the zein structure. This resulted in an exposure of hydrophobic amino acid residues buried within the tertiary structure of original zein molecules. Similar results were observed in glucosamine-casein conjugated product (Jiang & Zhao, 2011) and oligochitosan-caseinate conjugated product (Song & Zhao, 2014).

3.4. The impacts of conjugation of chitosan to zein on its antioxidant properties

To evaluate the antioxidant activities of the glycosylated zein, its superoxide/hydroxyl /DPPH radical scavenging capacities, reducing power, and metal ions chelating activity were investigated, and original zein and cross-linked zein were used as controls (Table 1). As shown in Table 1, the original zein showed weak scavenging activity against DPPH free radical, reducing power and ferrous ion-chelating activity. Although it did not exhibit hydroxyl and superoxide radicals scavenging activities, it possessed notable copper ion-chelating activity. The antioxidant values of cross-linked zein did not display significant differences compared to that of the original zein, except in ferrous ion-chelating activity and reducing power. It was probable that less cross-linkage occurred between the γ -carboxamides in glutamine and ϵ -amino group in lysine residues of the zein, because it lacks lysine. The glycosylation reaction improved all the antioxidant values of zein significantly except Cu^{2+} -chelating capacity, and the antioxidant efficiency of the glycosylated zein was concentration dependent. The DPPH radical scavenging activity of the glycosylated zein was $4.72 \mu\text{g TE/mg protein}$, with an EC_{50} value of $1.99 \mu\text{g TE/mg protein}$. The reducing power, Fe^{2+} -chelating capacity, superoxide and hydroxyl radicals scavenging activities of the glycosylated zein were 16.60, 12.96, 1.42 and $9.70 \mu\text{g TE/mg protein}$, respectively. These activities might depend mainly on the oligosaccharides moiety of the glycosylated zein, which could discontinue the free radical chain reaction by donating more protons to react with free radicals and (or) limit the radicals productions by binding metals such as iron and copper as metal chelators to stabilize them in an inactive or insoluble form. In the literature available, antioxidant activity of the glycosylated protein by TGase has not been reported so far.

The chitosan exhibited $0.11 \mu\text{g TE/0.1 mg chitosan}$ scavenging effect on DPPH radical, and reducing power was $5.44 \mu\text{g TE/0.1 mg}$

Table 1
Antioxidant activities of the glycosylated zein, cross-linked zein, original zein and chitosan

Sample	DPPH radical scavenging activity	Hydroxyl radical scavenging activity	Fe ²⁺ chelating capacity	Cu ²⁺ chelating capacity	Reducing power	Superoxide radical scavenging activity
The glycosylated zein	4.72 ± 0.07 ^A	9.70 ± 1.23	12.96 ± 0.49 ^A	267.29 ± 32.78 ^A	16.60 ± 0.33 ^A	1.42 ± 0.49
Cross-linked zein	0.09 ± 0.00 ^B	–	10.50 ± 0.16 ^B	285.09 ± 32.41 ^A	3.84 ± 0.02 ^C	–
Original zein	0.08 ± 0.00 ^B	–	9.52 ± 0.49 ^C	273.32 ± 52.41 ^A	3.03 ± 0.17 ^D	–
Chitosan	0.11 ± 0.34 ^B	–	–	–	5.44 ± 1.83 ^B	–

Each value was carried out in triplicate (n = 3). Values with the same letters denote no significant difference ($P < 0.05$).

Note: Data of the glycosylated zein, cross-linked zein and original zein were expressed as μg Trolox equivalent per mg of protein, data of chitosan expressed as μg Trolox equivalent per 0.1 mg of chitosan just because the glycosylated zein of 1 mg contained chitosan of 0.1 mg about.

chitosan. Younes et al. (2014) reported that the chitosan exhibited antioxidant activity against DPPH and reducing power at concentrations of 1, 2, 3, 4 and 5 mg/mL, which is in agreement with our result. While some results of other researchers clearly showed that native chitosan displayed low or no antioxidant activity (Schreiber, Bozell, Hayes, & Zivanovic, 2013). The glycosylated zein of 1 mg contained about chitosan of 0.1 mg. The antioxidant efficiency of the glycosylated zein of 1 mg was significantly higher than that of chitosan of 0.1 mg and the original zein of 1 mg in our experimental range. These results suggest that the glycosylated zein possesses fine antioxidant activity, which could be due to the some chitosan covalently conjugated to the zein.

3.5. Effect of chitosan conjugation of zein on TBARS and PV of ground pork

Lipids are widely distributed in both intra- and extra-cellular space of meat as triacylglycerides, phospholipids and sterols. However, lipids are chemically unstable, easily prone to oxidation, especially during post-mortem handling and storage (Falowo, Fayemi, & Muchenje, 2014). A current protocol of preventing lipid oxidation is the usage of antioxidants. Synthetic antioxidants, such as EDTA, BHT, butylated hydroxyl anisole and propyl gallate, are available to prevent lipid oxidation of meats, but their potential health risk exists (Qin et al., 2013; Shi et al., 2014). The search and development for natural antioxidants with heightened safety is being continued.

The inhibition effect of the glycosylated zein on lipid oxidation of ground pork occurring during the storage for 24 d was evaluated by measuring TBARS and PV (Figs. 5 and 6). The TBARS assay expresses the content of MDA produced by secondary products from lipid oxidation (Qi, Huang, Huang, Wang, & Wei, 2015). In general, the increase of TBARS values is inevitable during the storage of meat without any antioxidant. It was found that the sample containing the glycosylated zein of 0.05% exhibited the lowest TBARS values during the experimental storage period (Fig. 5), and at the end of the period, the TBARS value of the control was 1.91 mg MDA/kg sample, whereas the TBARS values of the samples containing 0.03% and 0.05% of the glycosylated zein were 1.74 and 0.85 mg MDA/kg, respectively, which meant that the effect of the glycosylated zein on TBARS was dose-dependent in the range of 0.03–0.05%. Since TBARS reflects the formation of secondary lipid oxidation products, the results indicated that the glycosylated zein in the ground pork substantially inhibited lipid oxidation. It was proved that the glycosylated zein inhibited lipid oxidation by blocking radical chain reaction in the oxidation process because it had DPPH/hydroxyl/superoxide radicals scavenging activities and reducing power. Additionally, heme iron and non-heme iron catalyze lipid oxidation in meat products (Flaczyk, Rudzińska, Wasowicz, Korczak, & Amarowicz, 2006), the glycosylated zein possessed a ferrous ion-chelating activity which might prevent or reduce the negative effects of the Fe²⁺-catalyzed lipid oxidation. However, it is not clear why the ground pork containing the

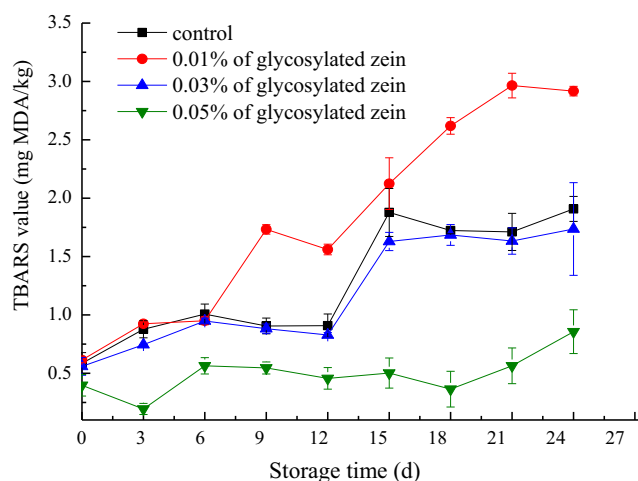


Fig. 5. Effect of the glycosylated zein on the thiobarbituric acid reactive substances values (mg malonaldehyde/kg sample) of ground pork during storage at 4 ± 1 °C.

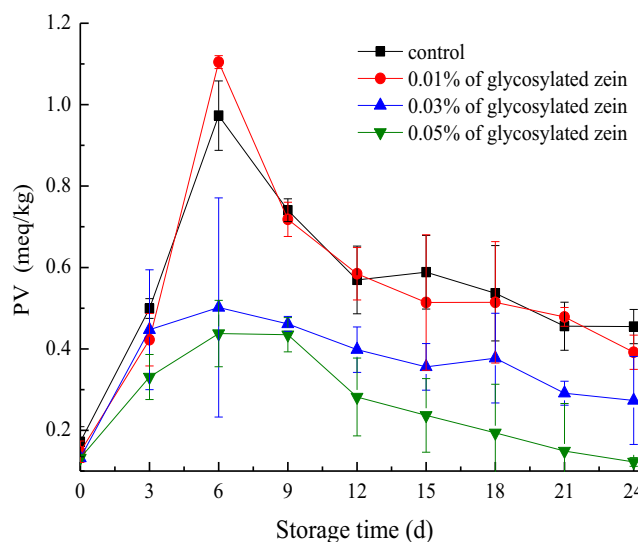


Fig. 6. Effect of the glycosylated zein on the peroxide value (PV) of ground pork during storage at 4 ± 1 °C.

glycosylated zein of 0.01% showed higher TBARS values compared with the control during the storage period of 24 d.

PV is an indicator of the initial stage of autoxidation or oxidative rancidity. The PV of the ground meat was 0.96 meq/kg on day 0, and all samples contained similar PV at the beginning of the test (Fig. 6). The PV of all samples showed peaks at 6 days and decreased thereafter. The PV profiles of the samples showed an increase at first and thereafter a decrease during the storage. The

increase of PV value with time is because hydroperoxide formation occurred at a faster rate than that of hydroperoxide degradation, and the decrease of PV observed with extended storage time is presumed to be due to the decomposition of hydroperoxides to form low molecular weight secondary oxidation compounds e.g., aldehydes and ketones. Other researchers also observed similar results (Qin et al., 2013; Shi et al., 2014; Wongwichian, Klomkiao, Panpipat, Benjakul, & Chaijan, 2015). In addition, there were significant differences among the treated samples during the storage period ($P < 0.05$), the control and the sample containing the glycosylated zein of 0.01% had similar PVs, while the PV in sample containing the zein of 0.05% was lowest at all sampling times. These results indicate that the glycosylated zein retarded the initial autooxidation in a dose-dependent manner.

4. Conclusions

Glycosylated zein was prepared by using TGase and chitosan. The content of conjugated chitosan was 97.48 mg of glucosamine per 1 g of zein. Solubility and *in vitro* antioxidant activities of the glycosylated zein were significantly enhanced, while its surface hydrophobicity decreased markedly, compared to original zein and TGase-induced cross linked zein. It retarded lipid oxidation of ground pork significantly. The results indicate that enzymatic glycosylation might be a new approach to modify partial functional properties of zein, and the glycosylated zein could be used as natural antioxidant to minimize lipid oxidation and to extend shelf life of meat.

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