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ORIGINAL PAPER

Transglutaminase-induced cross-linking and glucosamine conjugation in soybean protein isolates and its impacts on some functional properties of the products

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Abstract In the presented work, we exploited microbial transglutaminase as a biocatalyst and glucosamine as an acyl acceptor to modify soybean protein isolates (SPI) by cross-linking and glucosamine conjugation and evaluated some functional properties of the modified product prepared. Electrophoretic studies revealed that transglutaminase-induced cross-linking and glucosamine conjugation occurred simultaneously during modification reaction, and some polymers of glycoproteins with higher molecular weights were formed in the modified product. HPLC analysis demonstrated that about 3.3 mol of glucosamine could be conjugated to 1 mol of SPI, under the preparation conditions as following: SPI concentration of 3% (w/v), acyl donor in SPI/glucosamine acceptor molar ratio of 1:3, transglutaminase addition level of 10 U g^{-1} proteins, reaction temperature of 37 °C, and reaction time of 6 h. Compared to SPI and transglutaminase-induced cross-linked SPI, the modified product with glucosamine conjugation about 3.3 mol mol^{-1} SPI clearly exhibited lower surface hydrophobicity, better interfacial properties (especially in emulsion and foaming stability), markedly increased apparent viscosity in the prepared dispersion, and higher enzymatic digestibility in vitro. Our results showed that this modification technique might have the potential as an effective approach to improve the functional properties of SPI.

Keywords Transglutaminase · Soybean protein isolates · Glucosamine · Cross-linking · Functional property

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Introduction

Modification of food proteins has developed as an essential tool to meet continuously rising requirements of technologists, nutritionists, and consumers toward techno-functional [7, 14], tropho-functional [9], and sensory attributes [8] of food products. It has been well established that the functional properties of food proteins can be improved by some enzymatic or chemical modifications. Enzymatic protein modification may entail partial hydrolysis of the proteins [18], incorporation of cross-links within the protein molecules [20], or attachment of specific functional groups to the side residues of the proteins [49], while chemical modification can be achieved by acetylation [25], succinylation [53], esterification [35], amidation [36], phosphorylation [26], thiolation, and glycosylation [1] of the side residues of the proteins. The hydrophilic/hydrophobic balance or net charge at protein surface might be modified by glycosylation, leading to a modified isoelectric point and/or conformation, and finally functional behaviors of the food proteins. Specific functional characteristics that could be affected by glycosylation included solubility, interfacial properties, degree of hydration, tendency for gelation, and thermal stability of the proteins [22, 29].

The current strategies related to glycosylation of food proteins are mainly limited to chemical methods. Maillard

reaction recently is of growing scientific interest to modify functional properties of many food proteins [11, 21, 26, 32, 44], including solubility, emulsifying properties, foam-forming properties, gel-forming properties, and antioxidant properties. This modification is greatly accelerated by heating, and an extraneous compound (reducing sugar) is required [20]. However, Maillard reaction could result in formation of some mutagenic compounds and produce adverse browning, consequently affects sensory attributes of final products badly or gives rise to safety issue [4, 16, 45]. Therefore, there exists a need to study other modification methods to prepare glycosylated food proteins, because such modification might be also served as an alternative to modify the functional properties of common food proteins.

Transglutaminase (TGase, EC 2.3.2.13) can catalyze the formation of ε-(γ-glutamyl)-lysine cross-linking in food proteins via an acyl transfer reaction [27] and widely used in the research of food protein. The γ-carboxyamide groups of glutamine residues serve as the acyl donor, and the ε-amino groups of lysine residues serve as the acyl acceptor [13]. Moreover, reactive lysine may be substituted by several compounds containing primary amino groups, giving rise to a variety of derivatives. For example, TGase was previously and successfully applied to modify biological activities of some peptides [10, 28] and proteins [49–51] by covalently linking amine compounds (spermine, aminated dextran or aminated β-cyclodextrin) to their reactive endo-glutamine residues. Moreover, TGase was also employed for grafting of gelatin or ovalbumin with chitosan to prepare functional biomaterials [5, 39]. As the structural element of chitosan is glucosamine, 2-amino-2-deoxy-D-glucose, an amino monosaccharide with reactive primary amine, Ramezani et al. [41] had successfully conjugated glucosamine into lysozyme and casein using a chemical cross-linking agent, water-soluble carbodiimide and improved some functional properties of the modified proteins. However, conjugation of glucosamine into food proteins by an enzymatic approach, such as the catalysis of transglutaminase, is not reported in the literatures yet.

In the presented work, we used soybean protein isolates (SPI) as the acyl donor, glucosamine as the acyl acceptor, and commercial microbial transglutaminase as a biocatalyst to modify SPI and to prepare cross-linked and glucosamine-conjugated SPI. Some functional properties of the modified products were evaluated and compared to those of SPI and cross-linked SPI by transglutaminase, including emulsifying properties, foaming capacity, hardness of heat-induced gel, rheological properties, and enzymatic digestibility in vitro, to investigate the impacts of cross-linking and glucosamine conjugation by transglutaminase on these important properties of SPI.

Materials and methods

Materials and chemicals

Defatted soybean flour applied to prepare soybean protein isolates in this work was purchased from Harbin Hi-tech Soybean Food Co., Ltd. (Harbin, Heilongjiang, China). Microbial transglutaminase was donated by Jiangsu Yi-Ming Fine Chemical Industry Co., Ltd. (Qinxing, Jiangsu, China) with a declared activity of 100 units g^{-1}. Horseradish peroxidase (EC 1.11.1.7) was purchased from Shanghai Guoyuan Biotech Inc (Shanghai, China). D-(+)-Glucosamine hydrochloride (the purity >99%) was purchased from Sigma–Aldrich Co. (St. Louis, MO, US). All chemical reagents used in HPLC analysis were HPLC grade. Other chemicals were analytical grade. Highly purified water prepared with Milli-Q PLUS (Millipore Corporation, New York, NY, US) was used for the preparation of all buffers and solutions.

Preparation of the soybean protein isolates

Soybean protein isolates (SPI) were prepared with the method of Petruccelli and Anon [38]. An aqueous alkaline extraction from the defatted soybean flour (pH 8.0), followed by an isoelectric precipitation (pH 4.5) was carried out. The precipitate was resuspended in water, stirred at ambient temperature for 1 h, and centrifuged at 4000 × *g* for 20 min to remove acid residues. The isoelectric precipitate was dispersed in distilled water and adjusted to pH 7.0 with 2 mol L^{-1} NaOH. The dispersion obtained was lyophilized and ground to yield soybean protein isolates powder. Crude protein content of SPI determined by Kjeldhal method was 96.7% w/w (dry basis) (N × 6.25).

Modification of soybean protein isolates

The SPI of 3 g (on protein basis) and 1.94 g of glucosamine (giving approximately acyl donor in SPI/glucosamine acceptor molar ratio of 1:3) were added to 100 mL of distilled water, and the pH was adjusted to 7.5 by addition of 2 mol L^{-1} NaOH. The reaction was started by addition of 0.3 g of TGase (giving approximately E/S ratio of 10 U g^{-1} proteins) to the reaction system and mixed well. The reaction was carried out at 37 °C with continuous agitation. Aliquots were removed form reaction system at time intervals of 0.5, 2, 4, and 6 h. The TGase in the samples was inactivated immediately by heat treatment at 85 °C for 5 min. All separated samples were cooled to ambient temperature and dialyzed against distilled water overnight at 4 °C to remove unreacted glucosamine from the modified products. A control sample (cross-linked SPI) was treated as earlier, except that no glucosamine was

added. All prepared samples, the modified products and cross-linked SPI, were lyophilized and stored at −20 °C for further study.

SDS–PAGE analysis of the modified product

The cross-linking and glucosamine conjugation of SPI were confirmed by SDS–PAGE under reducing conditions using separating and stacking gels containing 12 and 3% acrylamide, as described by Laemmli [24]. The sample solutions (3 mg mL^{-1}) were prepared in a buffer containing 50 mmol L^{-1} Tris–HCl (pH 6.8), 2% (w/v) sodium dodecyl sulfate, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, and 0.1% (w/v) bromophenol blue, and then immersed in boiling water for 5 min to dissociate the proteins completely into individual polypeptide chains. Ten microliters of sample solution was applied in SDS–PAGE analysis in each lane. The stacking gels were run at 80 V, and the separating gels were run at 120 V in a SDS–Tris-glycine buffer system. For protein visualization, the gels were stained with 2.5% (w/v) Coomassie Brilliant Blue R250. For detection of the glycoprotein conjugates, the gels were stained by periodic acid-Schiff's reagent. SPI and horseradish peroxidase (HRP, a glycoprotein) were included as the negative and positive control, respectively. In the glycoprotein-specific staining, periodic acid oxidizes carbohydrates to aldehydes, which react with Schiff's reagent (a mixture of pararosaniline and sodium metabisulfite), releasing a pararosaniline adduct and staining the glyco-containing proteins pink [54].

HPLC analysis of glucosamine in the modified product

A RP–HPLC method using precolumn derivatization with anthranilic acid (AA) in methanol–acetate–borate reaction medium and fluorescence detection was applied to analyze the glucosamine conjugated into the modified product quantitatively. The analysis was performed on a liquid chromatograph 2695 series (Waters Corporation, Milford, MA, US) with a fluorescence detector, a C_{18}-reversed phase column (Hypersil ODS 250 mm × 4.6 mm i.d. 5 μm, Elite Technologies, Dalian, Liaoning, China) at ambient temperature with a flow rate of 1.0 mL min^{-1}. Solvent A consisted of 0.4% *n*-butylamine, 0.5% phosphoric acid, and 1.0% tetrahydrofuran in water. Solvent B consisted of 50% solvent A and 50% acetonitrile. The HPLC separation was performed at 6% B for 30 min. After each run, the column was washed with mobile phase B for 15 min and equilibrated with the initial mobile phase for 10 min. Fluorescence detection was carried out at an excitation wavelength of 230 nm and an emission wavelength of 425 nm.

Hydrolysis and derivatization of standard glucosamine solutions and the modified product followed the method of Raćaitytė et al. [40]. One hundred milligrams of the modified product or 10 μL standard glucosamine solution (0.25 ∼ 100 μg mL^{-1}) was hydrolyzed by 5 mL 20% trifluoroacetic acid at 100 °C for 8 h, the hydrolysates were dried in a vacuum centrifuge evaporator (Thermo Fisher Scientific Inc., Waltham, US). A methanol–acetate–borate solution was prepared by dissolving 2.4 g sodium acetate and 2.0 g boric acid in 100 mL methanol. The AA-derivatizing reagent was prepared by dissolving 30 mg anthranilic acid (AA) and 20 mg sodium cyanoborohydride in 1 mL of the methanol–acetate–borate solution. The dried hydrolysates were reconstituted in 10 mL of 1% (w/v) freshly prepared sodium acetate solution. One hundred microliters of the hydrolysates was mixed with 100 μL AA-derivatizing reagent, heated at 80 °C for 1 h, cooled to ambient temperature, diluted to 1 mL with HPLC solvent A, and then filtrated through 0.45-μm microporous filter membranes. Ten microliters of the supernatant was injected into the HPLC column for separation and analysis.

The number of moles of glucosamine conjugated into 1 mol of proteins was then calculated taking into account the molecular weights (MW) of glucosamine (215 Da) and SPI (about 270 kDa). It was noted that the molecular weight of SPI was estimated on the basis of SPI preparation consisted of 50% (w/w) 7S fraction (conglycinin, ca. MW 180 kDa) and 50% (w/w) 11S fraction (glycinin, ca. MW 360 kDa) [23], without regard to other protein fractions.

Characterizations of some functional properties

SPI, cross-linked SPI, or the modified product were dissolved in water or buffer solution with stirring at ambient temperature, and then kept at 4 °C overnight for complete rehydration. Prior to measurement, the sample solution was allowed to equilibrate at ambient temperature for at least 2 h and stirred gently to form homogeneous solution. Determinations of the functional properties were performed at least in triplicate.

Variation in surface hydrophobicity

Variation in surface hydrophobicity of the modified product was assessed according to intrinsic emission fluorescence spectroscopy. Intrinsic emission fluorescence spectra of the samples were obtained using a Hitachi F-4500 fluorophotometer (Hitachi Co., Kyoto, Japan). Protein dispersion of 5 mg mL^{-1} (on protein basis) was prepared in 50 mmol L^{-1} phosphate buffer (pH 7.0). The analysis samples were excited at 280 nm, and emission spectra were collected from 290 to 420 nm at a constant slit of 5 nm.

Emulsifying property

Emulsifying properties of the proteins were assessed by a turbidimetric method of Pearce and Kinsella [37]. To prepare emulsion, 25.0 mL of refined soybean oil and 75.0 mL of the protein solution (0.1% w/v) in 0.1 mol L^{-1} sodium phosphate buffer (pH 7.0) were shaken together in a plastic tube and homogenized by a high-speed homogenizer (BME 100L, Qidong Changjiang Mechanical and Electrical Equipment Co., Ltd. Jiangsu, China) at speed setting 12,000 r min^{-1} for 1 min. The emulsion was immediately transferred into a 250-mL capacity glass beaker. Aliquots of freshly prepared emulsion (50 μL) were taken 0.5 cm from the bottom of the beaker and dispersed into 5 mL of 0.1% (w/w) SDS solution as analysis sample. The absorbance of the sample was measured at 500 nm against 0.1% (w/w) SDS solution blank in a UV-2401 PC spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The emulsion was kept undisturbed for 10 min, and then 50 μL aliquots were taken 0.5 cm from the bottom of the beaker and dispersed into 5 mL of 0.1% (w/w) SDS solution as another analysis sample. The absorbance of the sample was also measured at 500 nm as described earlier. Emulsifying activity index (EAI, m^2 g^{-1}) and emulsion stability index (ESI, %) were calculated by using Eqs. 1 and 2. Each EAI and ESI evaluation was carried out triplicate.

$$\mathrm{EAI}\left(\mathrm{m^2\ g^{-1}}\right) = \frac{2 \times 2.303 \times \mathrm{A_{500}} \times \mathrm{dilution}}{C \times (1 - \Phi) \times 10^4} \quad (1)$$

$$\mathrm{ESI}(\%) = \frac{\mathrm{A_{10}}}{\mathrm{A_0}} \times 100 \quad (2)$$

where, A_{500} represents the absorbance at time zero at 500 nm, C is protein concentration (g mL^{-1}) before emulsification, Φ is the oil volume fraction (v/v) of the emulsion (Φ = 0.25 here), dilution = 100, while, A_{10} and A_0 represent the absorbance after 10 min and at time zero, respectively, at 500 nm.

Foaming property

Foaming capacity and foam stability of the proteins were evaluated by the method of Motoi et al. [33]. The analysis sample solution (0.1% w/v) was prepared in 50 mmol L^{-1} sodium phosphate buffer (pH 7.0). Then 20 mL sample was placed in a 200-mL glass cup and agitated at 10,000 r min^{-1} for 1 min with a blade type mixer (DS-1 Waring Blender, Shanghai Jingke Industrial Co. Ltd., Shanghai, China). The foams were carefully transferred into a measuring cylinder, and the whole volume was measured immediately. The initial foam volume, along with the foam volume after 30 min, was measured.

Foaming capacity was evaluated by relative overrun, and foam stability was determined by comparing the foam volume after 30 min with the initial foam volume (0 min) [17] and calculated with Eqs. 3 and 4.

$$\text{Relative overrun} = V_0/V_\mathrm{i} \quad (3)$$

$$\text{Foam stability} = V_{30}/V_0 \quad (4)$$

where, V_0 is foam volume at 0 min, V_{30} is foam volume at 30 min, and V_i is initial liquid volume before foaming.

Hardness of the heat-induced gel

The hardness of the heat-induced gel of the proteins was determined as described by Batista et al. [2]. The sample suspension (13% w/v, pH 7.0) was heated to 90 °C and lasted for 30 min in order to assure protein unfolding. The suspension was poured into 6-cm-diameter cylindrical containers, filled up to 3.5 cm height. The gels were allowed to set at a temperature of 4 °C in a refrigerator. The measurement was carried out 24 h after preparation of the gels. Before performing any measurement, the gels were allowed to equilibrate at ambient temperature for approximately 3 h.

The hardness was determined using a TA-XT2 texture analyzer (Stable Micro Systems, UK) with operating software Texture Expert. Penetration test was performed by using a 5-mm-diameter cylindrical probe (*p*/0.5) in gels contained in cylindrical glass flasks of 60 mm diameter and 45 mm height (0.02 N preload force, 10-mm penetration, 5-s waiting time, and 2 mm s^{-1} crosshead speed).

Apparent viscosity

Apparent viscosity of the protein dispersion prepared (10% w/v in water, pH 7.0) was measured using a Bohlin Gemini II Rheometer (Malvern Instruments Limited, Worcestershire, UK). A parallel plate measuring geometry with diameter of 20 mm and a gap width of 1 mm was used. The samples were loaded onto the rheometer and allowed to equilibrate to measuring temperature (25 °C) for 5 min. Apparent viscosity of the protein dispersion was obtained over different shear rates ranging from 0.1 to 100 s^{-1}, and the data were collected with Bohlin Software (Malvern Instruments Limited, Worcestershire, UK).

Digestibility in vitro

The digestibility of the protein samples in vitro was evaluated by the methods of Marciniak-Darmochwall and Kostyra [30] and Yin et al. [53]. For one-step hydrolysis, the starting solution was prepared as follows: 10 mL of protein dispersion (1%, w/v) of distilled water at pH 2.0

(by addition of 1 mol L^{-1} HCl), followed by addition of 2 mg pepsin (P-7000, Sigma, US). The hydrolysis was carried out at 37 °C for 2 h and stopped by adding an equal volume of 20% (w/v) trichloroacetic acid (TCA). Protein precipitates obtained were removed by centrifugation at 10,000 × *g* for 20 min. For two-step hydrolysis, the starting solution was prepared and treated as for pepsin hydrolysis. After 1-h incubation at 37 °C, the hydrolysis was stopped by heating at 90 °C for 5 min and cooling to 4 °C. Thereafter, the sample was lyophilized and reconstituted in 10 mL 0.2 mol L^{-1} phosphate buffer (pH 8.0), followed by addition of 6 mg trypsin (T-7409, Sigma, US). The incubation was carried out at 37 °C for 1 h, and the hydrolysis was stopped by adding an equal volume of 20% (w/v) TCA. Protein precipitates were removed by centrifugation at 10,000 × *g* for 20 min. The TCA-soluble nitrogen in the supernatants, released during the enzymatic digestion, was measured and compared by using the absorbance at 280 nm.

Statistical analysis

All data were expressed as mean ± SD (standard deviation) from at least three independent trials. The differences between the mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) with Duncan's multiple range tests. ANOVA data with $p < 0.05$ were classified as statistically significant. SPSS 13.0 software (SPSS Inc., Chicago, IL, US) and MS Excel 2003 (Microsoft Corporation, Redmond, WA, US) were used to analyze and report the data.

Results

Cross-linking and glucosamine conjugation of SPI by transglutaminase

The SPI prepared in our work was subjected to transglutaminase-catalyzed cross-linking reaction in the presence of glucosamine to prepare modified SPI product (the modified product), or in the absence of glucosamine to prepare cross-linked SPI. The peptide profiles from SDS–PAGE of SPI and the modified products, together with a control glycoprotein horseradish peroxidase, were stained for peptides by Coomassie brilliant blue R-250 or glycoproteins by periodic acid-Schiff's reagent and are shown in Fig. 1. Under reducing gel conditions applied, the main components of SPI, i.e., 7S fraction (conglycinin) and 11S fraction (glycinin), were dissociated into subunits, and the monomer of SPI were typically observed in lane 1 and 2 in Fig. 1a. Compared to that in SPI, the band color of most subunits in the modified products became weaker clearly,

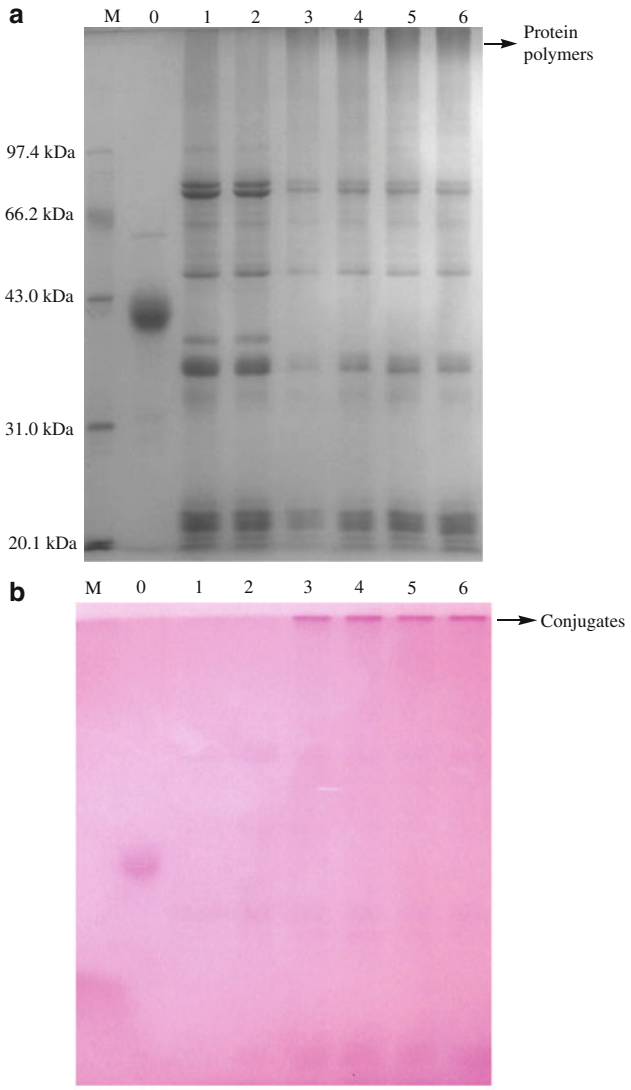

Fig. 1 SDS–PAGE profiles stained for the proteins with Coomassie brilliant blue R-250 (**a**) and stained for carbohydrate with periodic acid-Schiff's reagent (**b**). *Lane M* standard protein markers, *Lane 0* horseradish peroxidase, *Lane 1 and 2* SPI, *Lane 3–6* the modified products prepared at 3% (w/v) SPI solution, excessive glucosamine addition, transglutaminase addition 10 U g^{-1} proteins, pH 7.5, 37 °C with reaction times of 0.5, 2, 4, and 6 h, respectively

meanwhile some new peptide bands having lower mobility (i.e., higher molecular weights) appeared on the top of separating gel (see the top of lane 3–6 in Fig. 1a), which indicates that there existed some peptide polymers in the modified products. These peptide polymers had much higher molecular weights and were the products of cross-linking of SPI by transglutaminase. Also, it was revealed from the colored band in Fig. 1b that some saccharides were attached to the peptide polymers (see top of lane 3–6 in Fig. 1b) and implied that some glucosamine was conjugated into the peptide polymers after modification reaction of SPI. The SDS–PAGE analysis confirmed that

cross-linking and glucosamine conjugation in SPI during modification reaction occurred simultaneously.

Conjugated glucosamine in the modified product

HPLC analysis was employed to confirm and determine the glucosamine conjugated into the peptide polymers in the modified product. When the modified product prepared was hydrolyzed with trifluoroacetic acid, the released glucosamine and its epimer mannosamine reacted with AA-derivatizing reagent to form fluorescent derivatives, and later analyzed in HPLC to give two peaks (peak 1 and 2) with retention time in the range of 12–14 min as standard glucosamine solution did (see Fig. 2). Peak 2 was the epimer peak of AA-glucosamine (AA-mannosamine peak), as published literature stated [40], and totally included in glucosamine determination. Table 1 gives the amount of glucosamine conjugated into four modified products. After 6 h of reaction, the amount of glucosamine conjugation in SPI achieved the maximum (about 3.3 mol mol^{-1} SPI). Reaction time of 6 h was selected so as to achieve maximum reaction extent both in cross-linking and glucosamine conjugation, and the corresponding product was evaluated for some functional properties.

Some functional properties of the modified product

Some functional properties of the modified product, cross-linked SPI, or SPI were evaluated. The intrinsic emission fluorescence spectra of the dispersions prepared with SPI, the modified product, and cross-linked SPI were first measured with wavelength from 290 to 420 nm and are shown in Fig. 3. Compared to that of SPI, the dispersion of cross-linked SPI exhibited the increased maximum fluorescence intensity of emission fluorescence profile, but the dispersion of the modified product gave the decreased maximum fluorescence intensity of emission fluorescence profile. The result revealed that the modified product had lower surface hydrophobicity (or higher hydrophilicity) than SPI, and cross-linked SPI had higher surface hydrophobicity than SPI.

Interfacial properties of the modified product and cross-linked SPI were also evaluated and compared with those of SPI. The results showed that the emulsifying activity index, emulsion stability, overrun, and foam stability of the modified product were 92.6 m^2 g^{-1}, 87.2, 76.6, and 92.0%, respectively, and those indexes of cross-linked SPI or SPI were 59.4 m^2 g^{-1}, 55.2, 62.1, and 63.3%, or 77.6 m^2 g^{-1}, 64.8, 67.5, and 71.1%, respectively. Cross-linked SPI displayed the impaired emulsifying and forming properties than SPI did, because its emulsifying activity index and emulsion stability had a decrease of 23 and 15%, at same time its overrun and foam stability had a decrease of 8 and 11%, respectively. On the contrary, the modified product performed improved emulsifying and forming properties than original SPI did, as its emulsifying activity index and emulsion stability had an increase of 19 and 35%, at same time its overrun and foam stability had an increase of 14 and 29%, respectively. All these results are surmised and shown in Fig. 4.

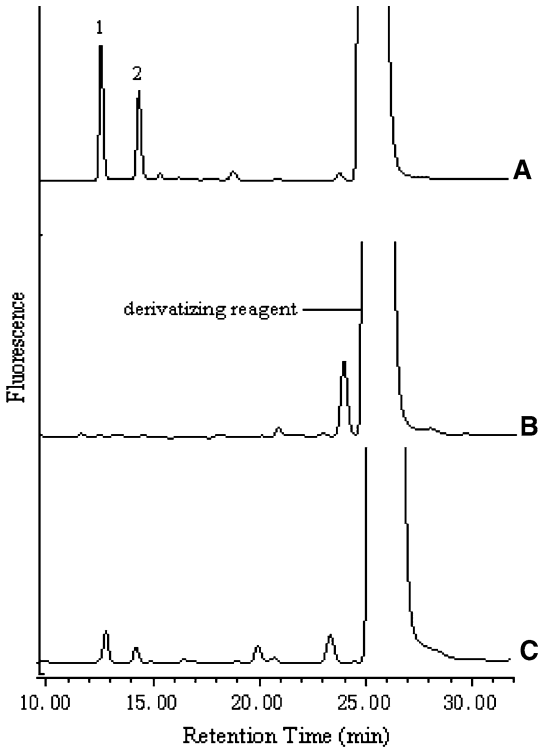

Fig. 2 HPLC–fluorescence profiles of anthranilic acid (AA)-derivatized glucosamine from a standard solution (**a**), SPI (**b**), and the modified product (**c**). *Peak 1 and 2* are AA-glucosamine peak and its epimer AA-mannosamine peak, respectively

After the suspensions (13% w/v on protein basis) of SPI (as control), cross-linked SPI and the modified product underwent heat-induced denaturation to form the gels, the hardness of the gels were measured and compared. The results are also given in Fig. 4. Compared to SPI, cross-linked SPI formed a harder gel with increased gel hardness of 30%, whereas the modified product formed a softer gel with decreased gel hardness of 22%.

Flow behaviors of the dispersions (10% w/v) prepared with SPI, cross-linked SPI, and the modified product were shown in Fig. 5. All the dispersions exhibited thixotropic behaviors (shear thinning), and the dispersion of the modified product gave the highest apparent viscosity, followed by the dispersions of cross-linked SPI and SPI. It was noticeable that the modified product had the ability to form a highly viscous solution. The evaluation result declared that the cross-linking and glucosamine conjugation of SPI

Table 1 Amount of glucosamine conjugation in soybean protein isolates (SPI) (mol glucosamine mol^{-1} SPI)

Reaction time (h)	0.5	2	4	6
Conjugated glucosamine in modified product	0.87 ± 0.02^{a}	3.21 ± 0.02^{b}	3.24 ± 0.03^{bc}	3.28 ± 0.03^{c}

Reaction conditions were SPI concentration of 3% (w/v), acyl donor in SPI/glucosamine acceptor molar ratio of 1:3, E/S ratio of 10 U g^{-1} proteins, 37 °C, and pH 7.5. The value is expressed as the mean ± standard deviation of three replicates. Different alphabets as superscripts after the values indicate the data differ significantly ($p < 0.05$)

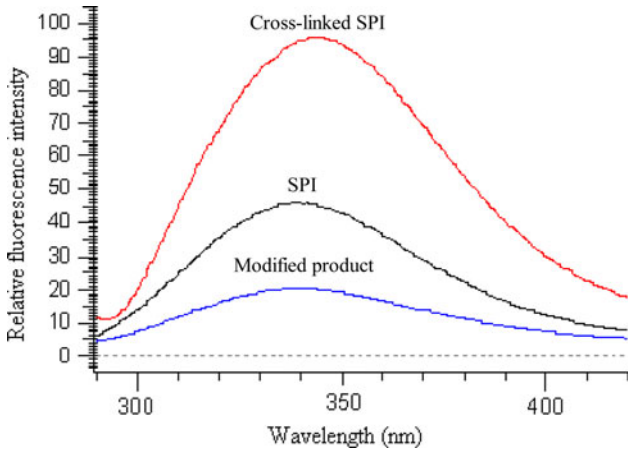

Fig. 3 Intrinsic emission fluorescence spectra of SPI, the modified product, and the cross-linked SPI. The protein solutions of 5 mg mL^{-1} were excited at 280 nm, and emission spectra were collected at a constant slit of 5 nm

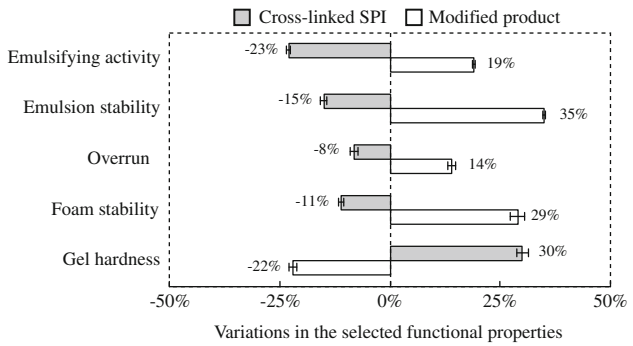

Fig. 4 Changes in some functional properties of cross-linked SPI and the modified product compared to that of SPI. Each value is the mean variation (%) ± standard deviation of three replicates

had helpful impact on the rheological property of the dispersion prepared.

Figure 6 shows the enzymatic digestibility of SPI, cross-linked SPI and modified product in vitro by pepsin or pepsin–trypsin hydrolysis, which was reflected by the absorbance at 280 nm of the TCA-soluble nitrogen released during the enzymatic digestion. Comparison with SPI, the modified product was more susceptive to enzymatic hydrolysis, and more peptides about 23% (in pepsin hydrolysis) or 14% (in pepsin-trypsin hydrolysis) released. The result indicated that modification of SPI by

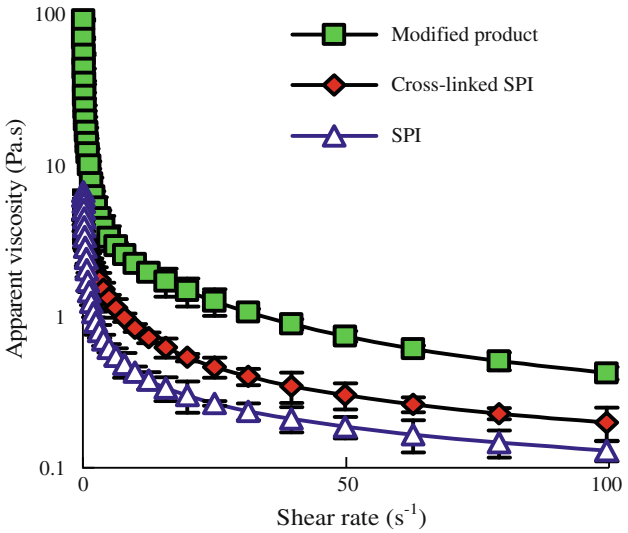

Fig. 5 Flow behaviors (apparent viscosity vs. shear rate) of the suspension of SPI, cross-linked SPI, and modified product at protein concentration of 10% (w/v) after aging for 24 h

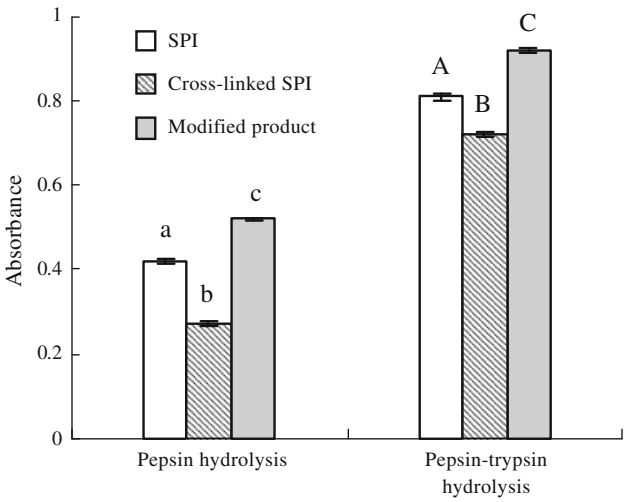

Fig. 6 The effects of cross-linking and glucosamine conjugation on digestibility of the modified SPI product evaluated by pepsin hydrolysis and pepsin–trypsin hydrolysis in vitro, respectively. Different letters (*a–c*, or *A–C*) above the columns for pepsin or pepsin–trypsin hydrolysis indicate significant difference ($p < 0.05$)

transglutaminase in the presence of glucosamine had no adverse impact on the in vitro bioavailability of the modified product. On the other hand, compared to SPI, cross-

linked SPI were more resistant to enzymatic hydrolysis, and the amount of the peptides released decreased about 36% (in pepsin hydrolysis) or 11% (in pepsin–trypsin hydrolysis).

Discussion

Cross-linking and glucosamine conjugation of SPI by transglutaminase

It is well known that transglutaminase has the ability to catalyze the formation of ε-(γ-glutamyl)-lysine cross-linking between the molecules of food proteins (Reaction A in Fig. 7), or to incorporate small primary amines into the protein substrate via an acyl transfer reaction (Reaction B in Fig. 7) [27]. Modification of food proteins by small molecular weight saccharides with primary amines is indeed an important reaction for transglutaminase considering that saccharide moiety is important to the functional properties of glycoproteins. Therefore, it is possible to prepare protein–saccharide conjugates with improved functional properties by transglutaminase-catalyzed glycosylation of the proteins with aminated saccharides or saccharides containing primary amines. Yan and Wold [52] had applied transglutaminase-catalyzed reaction to incorporate 8 mol of maltotriose into 1 mol of succinylated β-casein. Villalonga et al. [50] had exploited transglutaminase to catalyze synthesis of trypsin–cyclodextrin conjugates with improved stability properties. Valdivia et al. [49] also used transglutaminase to catalyze site-specific glycosylation of catalase with aminated dextran and improved the stability properties of catalase. Chen et al. [5] employed transglutaminase for grafting of gelatin with chitosan, and Pierro et al. [39] applied transglutaminase to prepare chitosan-ovalbumin films with good properties.

In our work, we exploited microbial transglutaminase to catalyze modification of SPI by two approach, cross-linking and glucosamine incorporation. Because of easier diffusion of small molecular weight amines, it was expected that the amines would be more reactive than the peptides' N-terminal amines or the ε-amines of lysine residues, which gave us opportunity to incorporate glucosamine into SPI. Electrophoretic analysis confirmed that transglutaminase was surely to induce cross-linking and glucosamine conjugation of SPI simultaneously during our preparation, as declared by Lorand and Conrad [27]. To stop modification reaction, heat inactivation of transglutaminase was used in our work as these studies [3, 42].

To obtain maximal glucosamine conjugation, some reaction conditions were studied in our work to reveal their influences on the amount of glucosamine conjugated into SPI, including E/S ratio (5, 10, 20, and 40 U g^{-1} proteins), reaction temperature (25, 30, 37, 42, and 50 °C), and pH of reaction mixture (6.0, 6.5, 7.0, 7.5, and 8.0). The final results showed that suitable reaction conditions for glucosamine conjugation were to be E/S ratio of 10 U g^{-1}proteins, reaction temperature of 37 °C, and pH 7.5, and reaction time of 6 h was selected to insure maximum reaction extent of cross-linking and glucosamine conjugation. Tang et al. [46] had treated glycinin-rich and β-conglycinin-rich SPI with microbial transglutaminase and studied the properties of the gels formed. The protein samples evaluated in their work were prepared at 37 °C and pH 7.5, with substrate concentration of 2 or 7% (w/v), an enzyme level of 20 U g^{-1} SPI, and reaction time of 4 or 6 h. Our selected reaction conditions shared similarity to Tang's, beside lower enzyme addition.

Under these reaction conditions, about 3.3 mol of glucosamine was conjugated into 1 mol of SPI, which is higher than that in the study of Ramezani et al. [41] who conjugated about 2 or 0.11 mol of glucosamine into 1 mol of casein or lysozyme, respectively, by using a water-soluble chemical, carbodiimide. We thus prepared the modified product with the amount of glucosamine conjugation about 3.3 mol mol^{-1} SPI. Some functional properties of the modified product were evaluated with SPI and cross-linked SPI as control, which might reflect the impacts of cross-linking and glucosamine conjugation on these properties of SPI.

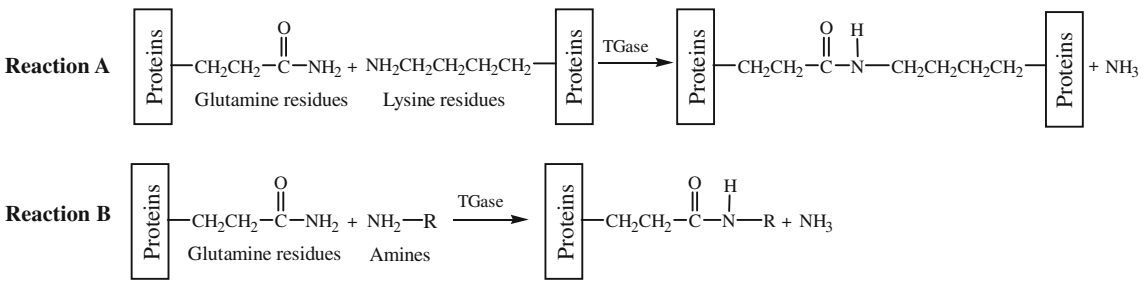

Fig. 7 Two typical reactions catalyzed by transglutaminase. *TGase* transglutaminase

The impacts of cross-linking and glucosamine conjugation of SPI on some functional properties

Our study results showed that after SPI was subjected to transglutaminase-catalyzed cross-linking reaction in the absence of glucosamine addition, some functional properties of cross-linked SPI changed significantly, characterized by the increased surface hydrophobicity, hardness of heat-induced gel as well as apparent viscosity. Meanwhile, interfacial properties and enzymatic digestibility in vitro of cross-linked SPI were impaired in relation to SPI, as those shown in Figs. 3, 4, 5, and 6. In case of preparation of the modified product by transglutaminase in the presence of glucosamine addition, the modified product with amount of glucosamine conjugation of 3.3 mol mol^{-1} SPI exhibited a decreased surface hydrophobicity as well as hardness of heat-induced gel, but better interfacial properties, increased apparent viscosity and higher digestibility in vitro in relation to original SPI, also shown in Figs. 3, 4, 5, and 6. Totally, the functional properties of the modified product evaluated in the presented work were different from those of cross-linked SPI, especially in interfacial and rheological properties. Thus, it can be speculated here that it is glucosamine conjugation in SPI that confers the modified product better functional properties.

When SPI was catalyzed by transglutaminase in the absence of glucosamine addition, only the self cross-linking reaction of the proteins occurred, and some protein polymers with higher molecular weights were formed, which accounted for the changes in some functional properties of cross-linked SPI. It was found the oligomerization of milk proteins unfolded the structure of the proteins, leading to exposure of hydrophobic amino acid residues buried within the tertiary structure of original protein molecules [20], which accounts for the increase in surface hydrophobicity of the cross-linked proteins. At the same time, the highly increased molar weights as well as lower activity toward interface of cross-linked proteins make their foam-forming capacity and emulsifying activity impaired [12, 47]. Also, highly cross-linked proteins sterically shield the peptide bonds against proteolysis, thus decreased enzymatic digestibility in vitro [43], which supported our results that cross-linked SPI had lower enzymatic digestibility in vitro than SPI. Protein polymers have larger molecular volume than the proteins in aqueous solution, which leads to the corresponding dispersion a higher apparent viscosity [48]. So the dispersion of cross-linked SPI had a higher apparent viscosity than that of SPI did.

If SPI was catalyzed by transglutaminase in the presence of glucosamine addition, besides the cross-linking, glucosamine was conjugated into SPI. Some functional properties of the modified product were altered by both cross-linking and glucosamine conjugation, for example, surface hydrophobicity of the modified product might be increased by cross-linking while surface hydrophilicity of the modified product might be also increased by glucosamine conjugation. The final result was that surface hydrophobicity (hydrophilicity) of the modified product decreased (increased), because the maximum fluorescence intensity of emission fluorescence profile of the modified product decreased (see Fig. 3). The evaluation data presented in Figs. 4, 5, and 6 declared that some functional properties of the modified product were totally better than those of cross-linked SPI, which revealed the helpful effect of glucosamine conjugation in SPI. It had been reported that the hydrophobic residues of proteins might be anchored to the surface of oil/gas droplets in an emulsion or foam and the hydrophilic residues oriented to the water phase covering the oil/gas droplets [19]. A more stable emulsion or foam might be formed in the presence of protein–saccharide conjugates, because the attached saccharides in the side chains of the proteins would increase the surface hydrophilicity of the proteins and enhance hydration or water-binding capacity of the molecules, which was beneficial to the stability of the emulsion or foam or to the apparent viscosity of the dispersions. Also, as indicated by Miller and Gerrard [31] that binding of saccharide molecules to globular proteins might facilitate their proteolysis, so the modified product prepared in our work performed an improved enzymatic digestibility in vitro.

Recently, Maillard reaction is of growing scientific and practical interest to physico-chemically modify food proteins to improve their functional properties. It was reported that Maillard products of β-lactoglobulin and lactose exhibited superior foam-forming properties [6], while that of casein and dextran performed better emulsifying activity [11]. It was also found that Maillard products of sodium caseinate and glucose could form viscous solutions [34], and that of SPI and glucose had decreased surface hydrophobicity [15], and that of pea protein and glucosamine were more susceptive to enzymatic hydrolysis [30]. These results indicated that glycosylation of food proteins by Maillard reaction improved the functional properties of the product. Our study results shared similarity to these studies. Unfortunately, it has been reported that glycosylation by Maillard reaction can result in formation of some mutagenic compounds [4]. Hence, any food ingredient created by the Maillard reaction might cause safety issue. As an enzymatic reaction, the modification approach applied in our work might have some advantages over Maillard reaction and could be used as an effective approach or alternative to improve the functional properties of food proteins.

Conclusions

SPI were prepared from defatted soybean flour and subjected to transglutaminase-catalyzed reaction in the presence of glucosamine addition to prepare modified SPI product. Electrophoretic studies revealed that transglutaminase could induce cross-linking and glucosamine conjugation of SPI simultaneously. HPLC analysis demonstrated that about 3.3 mol of glucosamine could be conjugated into 1 mol SPI, under the preparation conditions as following: SPI concentration of 3% (w/v), acyl donor in SPI/glucosamine acceptor molar ratio of 1:3, transglutaminase addition level of 10 U g^{-1} proteins, reaction temperature of 37 °C, and reaction time of 6 h. Some functional properties of the modified product prepared were evaluated and compared to those of SPI and cross-linked SPI prepared in the absence of glucosamine addition. The analysis results showed that the modified product with amount of glucosamine conjugation of 3.3 mol mol^{-1} SPI had improved interfacial properties and enhanced apparent viscosity in dispersions, but gave a softer gel; meanwhile, the modified product displayed unimpaired digestibility in vitro. The results indicated that this modification might be served as an effective approach or alternative of Maillard reaction to improve some functional properties of SPI.

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