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Effects of saccharide on the structure and antigenicity of β-conglycinin in soybean protein isolate by glycation

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Abstract Soybean is a high-quality plant protein resource and also a major food allergen. Glycation is widely used to modify protein allergens. In the current report, the influences of different saccharides on soybean protein structure and antigenicity through glycation were investigated. Soybean protein isolate (SPI) and saccharides (glucose, galactose, maltose, lactose, and dextran), at 1:1 weight ratio, were dry-heated at 60 °C and 79 % relative humidity for different times. The content of free amino group in glycated products was decreased by trinitrobenzene sulfonic acid method. In addition, high-molecular aggregates were generated in the glycated SPI, indicating that glycation reaction occurred in SPI–saccharide conjugates. Moreover, the structure of SPI in conjugates changed with exposure to aromatic side chains. Of all the SPI–saccharide conjugates, with time increased from 0 to 72 h, the antigenicity inhibition rate of β-conglycinin in SPI–glucose complexes declined from 83.55 (0 h) to 29.80 % (48 h), suggesting that introducing saccharides in SPI is an effective method to reduce the antigenicity of β-conglycinin.

Introduction

As the occurrence of food allergies has risen rapidly, food allergy has become a major worldwide public health problem. The eight common food allergens are soybeans, milk, eggs, fish, crustacean, peanuts, tree nuts, and wheat [1].

In a number of epidemiological studies, allergy due to soybean protein allergens impacts a significant amount of people, especially infants, young children, and many animals that are fed with soybean products. About 1–6 % of infants are influenced by allergies to soybean products [2], and the incidence in adults is higher because of more consumption of soy products [3]. Several processing methods have been researched in for removing soybean protein allergenicity, including heat treatment, enzymatic hydrolysis, fermentation, and glycation [4–7]. Two major soybean allergens are β-conglycinin and glycinin with account for 30 and 40 % of the total storage proteins, respectively [8]. β-conglycinin is aggregated into trimer that contains three subunits, namely α (~67 kDa), α′ (~71 kDa), and β (~50 kDa) [9]. Glycinin is composed of five different subunits, G1 (A1aB1b, 53.6 kDa), G2 (A2B1a, 52.4 kDa), G3 (A1bB2, 52.2 kDa), G4 (A5A4B3, 61.2 kDa), and G5 (A3B4, 55.4 kDa) [10].

Among the trials of reducing soybean protein allergenicity, conjugation with reducing sugars through Maillard reaction is a promising approach for shielding food protein allergenicity because of its safety. Besides, it also seemed to be an effective means to improve the functional properties of proteins [11]. Liu et al. [12] revealed that the emulsifying and foaming abilities of peanut protein

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isolate (PPI) were enhanced significantly by conjugation with dextran through Maillard reaction. Hattori et al. [13] also demonstrated that β-LG-acidic oligosaccharide conjugates exhibited reduced immunogenicity by shielding the B cell epitopes. Furthermore, Hiller and Lorenzen [14] indicated the heat stability, pancreatic in vitro digestibility, and emulsifying activity of milk protein were improved by Maillard reaction with glucose, lactose, pectin, and dextran.

The glycation reaction, called non-enzymatic glycosylation between the ε-amino group in lysine residues and carbonyl groups of reducing saccharides with a reaction cascade of rearrangements, is a promising protein modification method used in food industry. Various studies have investigated the effect of glycation on allergenicity and other functional properties of soybean proteins. For example, Xue et al. [15] examined the effect of soybean protein isolate (SPI)–maltodextrin and SPI–gum acacia conjugate using Maillard reaction, and found that after dry-heated treatment at 60 °C and 79 % relative humidity for three days, the solubility and the emulsifying properties of SPI were increased remarkably. When SPI was added with fructose or fructooligosaccharides by Maillard reaction, the electrophoretic behavior of allergenic β-conglycinin and glycinin was modified with the antigenicity of the glycated product reduced up to 20 % [16]. Furthermore, non-enzymatic glycosylation caused the pea globulin 7S to have a lowered affinity to antibodies and stimulate the maturing of Th0 cells to Th2 cells. The role of glycation in the process of food allergy was identified and conformed as well [17]. For example, the influence of glycation on the structural properties of soy protein was investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), high-performance size exclusion chromatography (HPSEC), dynamic light scattering (DLS), atomic force microscopy (AFM), and circular dichroism spectroscopy (CD) [18]. Glycation was found to inhibit the thermal aggregate of protein, and the tertiary structure of conjugate protein changed with increased number of aromatic side chains exposed. And covalent attachment of fenugreek gum to soy whey protein isolate through Maillard reaction improved the emulsion stability of protein [19].

Previous studies mainly focused on the effect of glycation on the antigenicity of soybean protein, the mechanism of functional properties as well as attention in stability, emulsibility, and solubility accompanied by changes in structural properties. The relationship between the alteration of antigenicity by β-conglycinin due to Maillard reaction with different saccharides and the change of protein structure has not been understood yet.

The aim of the current study was to investigate the structure via SDS-PAGE, UV, and FTIR approaches and the antigenicity change of β-conglycinin in soybean protein isolate by glycation with different saccharides (glucose, galactose, maltose, lactose, and dextran, respectively). The antigenicity could be depicted in the ability of the specific binding between an antigen and an antibody corresponding to the antigen and indicated by the inhibition rate. The antigen inhibition rate in this study was measured by the indirect competitive ELISA.

Materials and methods

Materials

Glucose and galactose were obtained from Tianjin Kermel Chemical Reagent Co. Ltd (Tianjin, China). Maltose, lactose, and dextran (molecular weight 20,000–40,000) were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Soybean protein isolate (SPI, containing 92.46 % protein) was purchased from Gushen Biological Technology Group Co., Ltd HRP-labeled goat anti-rabbit IgG (A6154) and β-conglycinin (C5868) were obtained from Sigma Chemical Co. Ltd (St. Louis, MO, USA). TMB chromogenic single-component liquid was obtained from Beijing Solarbio Science and Technology Co. Ltd (Beijing, China). The anti-β-conglycinin serum from rabbit was generated by our own laboratory. All other reagents were of analytical grade.

Preparation of soybean protein isolate (SPI)–saccharides conjugates

Freeze-dried SPI and dextran (20,000), dextran (40,000), maltose, lactose, glucose or galactose mixtures (weight ratio 1:1) were incubated at 60 °C and 79 % relative humidity controlled by saturated solution of KBr at different time (sampled at an interval of 12 h) [20]. The resulted samples were stored at −20 °C prior to analysis.

Determination of free amino groups

The amount of free amino groups was determined by the trinitrobenzene sulfonic acid (TNBS) method [21]. The sample was dissolved with 1 % (w/v) sodium dodecyl sulfate (SDS) to ensure the final protein concentration of 5 mg/mL. The TNBS reagent was freshly prepared each time. To 0.25 mL sample solution containing 2 mL 0.2 mol/L phosphate buffer (pH 8.2), 2 mL 0.1 % TNBS reagent was added. After incubated at 50 °C for 60 min, 4 mL of 0.1 mol/L HCl was added to end the reaction. The absorbance was read at 420 nm after 30 min incubation. A calibration curve was obtained using L-leucine as a standard ranging from 0 to 3.5 mmol/L.

Table 1 Immunization procedure of New Zealand rabbits

Sequence	Intervals (days)	Antigen sensitization	Dose (μg protein/kg weight)	Treatment
1	1	Antigen/Freud complete adjuvant	300	Crural intramuscular
2	14	Antigen/Freud incomplete adjuvant	200	Dorsal subcutaneous
3	24	Antigen/Freud incomplete adjuvant	200	Dorsal subcutaneous
4	34	Antigen/Freud incomplete adjuvant	200	Dorsal subcutaneous
5	44	Antigen/Freud incomplete adjuvant	200	Dorsal subcutaneous
6	54	Antigen	400	Otic plexus

Measurement of glycated products molecular weight

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was conducted on a discontinuous buffered system according to the method of Laemmli [22], using 12 % separating gel and 4 % stacking gel. Five microliter of sample buffer (containing 0.5 M Tris–HCl buffer, 10 % SDS, 2 % β-mercaptoethanol, 20 % glycerol, and 0.5 % bromophenol blue) was added to 5 μL protein sample (protein concentration: 2 mg/mL) and heated for 4 min in boiling water before electrophoresis. After electrophoresis, gels were stained with Coomassie brilliant blue R-250, and destained with 10 % acetic acid containing 25 % ethanol.

Structural characteristics of glycated products

Ultraviolet spectrum

UV absorption spectra analysis was performed by TU-1901 double beam UV–Vis spectrophotometer (Beijing Purkinje General Instrument Co. Ltd, Beijing, China). The 0 h sample concentration was 1 mg/mL. The UV absorption spectra were scanned from 230 to 420 nm [23].

Fourier transform infrared spectrum

A WQF-510 Fourier transform infrared spectrum equipped with a computer (Beijing Ruili Analytical Instruments Co. Ltd, Beijing, China) was used for FTIR analysis. The glycated products were mixed with potassium bromide (KBr) (1:100, w/w), ground with a mortar to form homogeneous powder, pressed into a sheet and scanned from 4,000 to 400 cm^{-1} within 32 scans using the Fourier transform infrared spectrophotometer [24].

Antigenicity of glycated products

Immunization

For the sensitization experiments, three young male New Zealand rabbits, average weighing 2.5 kg, from the Experimental Animal Center, Zhengzhou University (Zhengzhou, China) were used. Rabbits were sensitized with β-conglycinin by parenteral administration as shown in Table 1. The titers of antibodies generated were controlled by indirect ELISA method with blood drawn from the rabbit marginal vein 3 days before the subsequent immunization. Four days after the sixth sensitization, blood samples were collected from the rabbit hearts.

Blood samples were incubated at room temperature for 1 h and left at 4 °C overnight. Supernatant (serum) was obtained after the blood was centrifuged for 20 min at 3,000 r/min. After clot formation and stored at −20 °C, the antigenicity of β-conglycinin was measured by indirect competitive ELISA.

Analysis of β-conglycinin antigenicity by indirect competitive ELISA

The antigenicity of the glycated products was determined by indirect competitive ELISA [16]. Microtiter plates with 96-well (Costar 3590 High Binding, Corning, NY, USA) were coated with 100 μL/well of β-conglycinin antigen diluted in 50 mmol/L carbonate buffer (pH 9.8) with the concentration determined earlier in indirect ELISA method (0.3 μg/mL β-conglycinin) and incubated at 4 °C overnight. Solutions of glycated products and β-conglycinin antigens with an equivalent volume of rabbit anti-β-conglycinin serum (1:3,200) diluted in 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4) containing 1 % (w/v) BSA and 0.1 % Tween-20 (PBST-BSA) were incubated, respectively, in test tubes overnight at 4 °C. Next day, the wells were washed four times (250 μL/well, 3 min/time) with PBST buffer (0.01 mol/L PBS, pH 7.4, 0.05 % Tween-20). The washing procedure was repeated after each analytical step. After washing, the plate was filled with 100 μL/well of PBST-BSA to block residual free binding sites and incubated at 37 °C for 1 h. The plate was washed, added the reactive mixtures of glycated samples and polyclonal rabbit antibodies (100 μL/well), and incubated for 1 h at 37 °C. After plate washing, 100 μL/well of HRP-labeled goats anti-rabbit IgG (A6154, Sigma-Aldrich, St. Louis, USA) was added to each well and the plate was incubated at 37 °C for 1 h. The plate was washed again, added the

substrate 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) (100 μL/well), and incubated at 37 °C for 10 min. The reaction was stopped by adding to 50 μL/well of 2 mol/L H_2SO_4. The optical density (OD) was detected at the dual wavelengths of 450 and 620 nm, respectively, by using a microplate reader (Thermo Fisher Scientific Instrument Co. Ltd, New York, USA). OD was obtained by subtracting OD_{620} from OD_{450}.

The antigenicity of glycated products in SPI was represented by inhibition rate of β-conglycinin by glycation reaction, which was calculated as follows:

$$\text{Inhibition rate (\%)} = \frac{B_0 - B}{B_0} \times 100$$

where B is the OD of glycated samples and B_0 is the OD of native β-conglycinin.

Statistical analysis

Three independent experiments were performed for each parameter. One-way analysis of variance (ANOVA) was performed using the SPSS 16.0 statistical analysis system. Secondary structure of proteins from FTIR was analyzed using the Peak Fit Version4.12 software.

Results and discussion

Changes in free amino groups

The free amino groups after dry-heated treatment for different time in SPI–monosaccharide complexes, SPI–disaccharide complexes, and SPI–polysaccharide complexes are shown in Fig. 1. With reaction time increased, the content of free amino group in glycated products gradually reduced. The greatest reduction of free amino group content in SPI–monosaccharide complex and SPI–disaccharide complexes was found during the first 24 and 60 h, respectively (Fig. 1a, b). For SPI–polysaccharide complexes, the free amino groups contained in glycated products decreased gradually as well with reaction time increased (Fig. 1c).

The decrease in the concentration of free amino group indicated that Maillard reaction between free amino groups in protein and saccharides occurred [25]. The more decrease of free amino groups in SPI–monosaccharide than SPI–disaccharide and SPI–polysaccharide conjugates indicated that the size of saccharides may influence the rate and degree of glycation [26]. At the beginning of glycosylation reaction, free amino groups exposed at the protein molecules surface were more prone to start reactions than
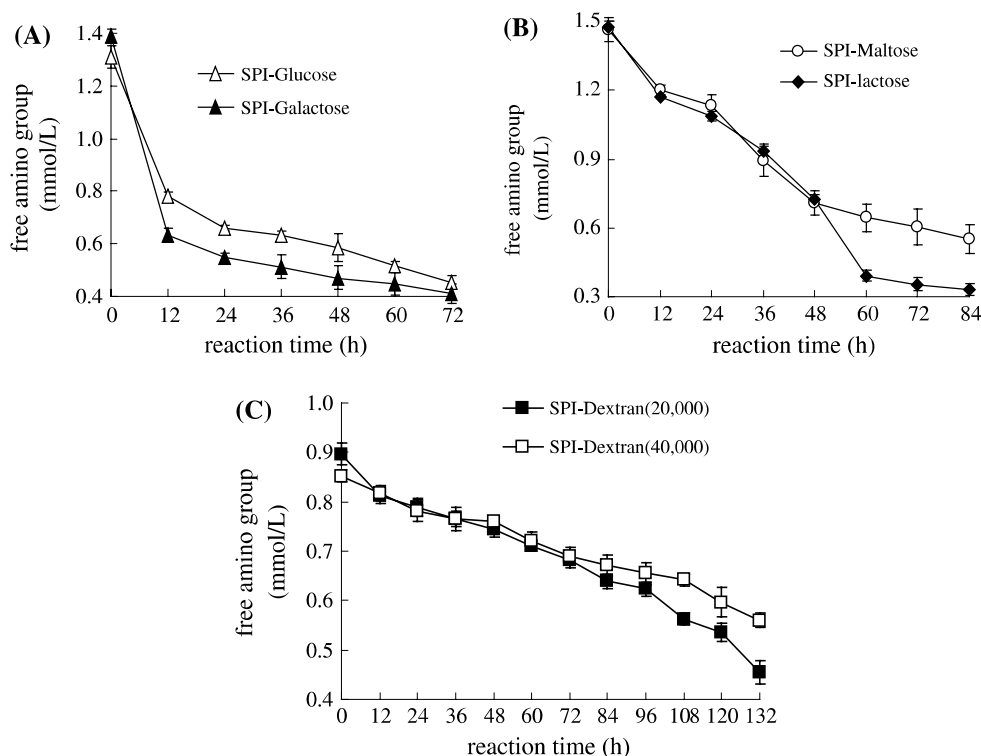

Fig. 1 Concentration of free amino groups in glycated conjugates after incubation at 60 °C and 79 % relative humidity for different time. **a** SPI–glucose (1:1, w/w) and SPI–galactose (1:1, w/w); **b** SPI–maltose (1:1, w/w) and SPI–lactose (1:1, w/w); **c** SPI–dextran (20,000) (1:1, w/w) and SPI–dextran (40,000) (1:1, w/w). *Data points represent mean values ± standard deviations (SD) of three independent experiments*
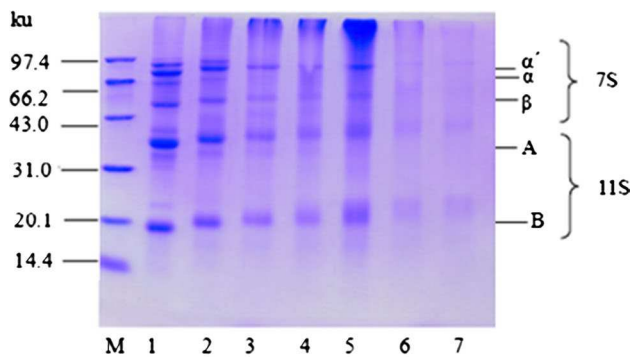
Fig. 2 SDS-PAGE of SPI–glucose conjugates (1:1, w/w) after incubation at 60 °C and 79 % relative humidity for different time. *M* molecular weight markers; *lanes 1–7* SPI–glucose conjugates incubated for 0, 12, 24, 36, 48, 60, and 72 h, respectively
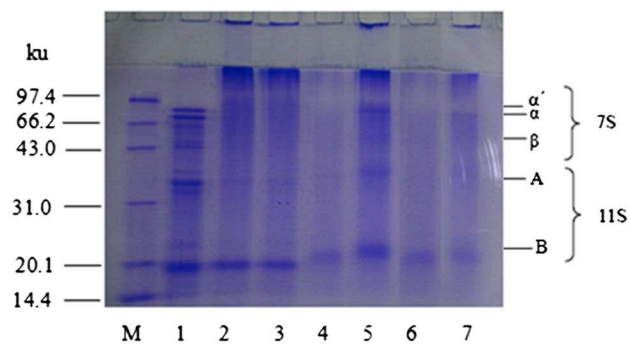

Fig. 3 SDS-PAGE of unglycated and glycated SPI conjugates (1:1, w/w) after incubation at 60 °C and 79 % relative humidity for different time. *M* molecular weight markers; *lanes 1–7* native SPI, SPI–glucose incubated for 48 h, SPI–galactose incubated for 60 h, SPI–maltose incubated for 72 h, SPI–lactose incubated for 72 h, SPI–dextran (20,000) incubated for 132 h, SPI–dextran (40,000) incubated for 132 h

that hidden inside proteins. Reactions occurring in the free amino groups inside the protein molecules may need to wait until the protein structure changed.

SDS-PAGE profiles of the conjugates

Molecular weight of individual components in the glycated products is shown in Figs. 2 and 3. As shown in Fig. 2, SPI–glucose conjugates incubated for different time had a similar band pattern. When reaction time increased, the molecular weight of conjugates also increased, and the band color of most subunits reduced clearly. Meanwhile, the migration rate of main soy protein slowed down and the high molecular mass aggregates increased (the top of electrophoresis gel). These results suggested that glucose bound to SPI during Maillard reaction and high molecular weight products formed [20, 27].

Compared with native SPI, glycated SPI components decreased in their mobility rate with simultaneous appearance of a large number of high molecular mass aggregates (Fig. 3, lanes 2–7). The differences in the extent of mass aggregates might depend on the size of the saccharides. For instance, the degree of SPI–dextran conjugate reduced more than SPI–glucose as the mobility rate of the protein molecule decreased (Fig. 3, lanes 2, 6, 7). Similar results were found in other reports [26, 28]. The reason may be explained that during glycation with monosaccharide, polymerization occurred because of covalent bonds other than the disulfide bonds and the sulfydryl-disulfide interchange reaction caused the polymerization between proteins and polysaccharides. Covalent bonds were suggested corresponding to the degree of saccharide polymerization [28]. With the formation of numerous polymers, the antigenicity of glycated products might be altered since these generated adducts may mask IgE binding epitopes [29, 30].

Ultraviolet spectrum analysis

The UV spectrum pattern of glycated products is shown in Fig. 4. In the vicinity of 280 nm, both native SPI and glycated products exhibited maximum absorption ability, while the SPI–saccharides had lower peak values than native SPI. Compared with SPI, the absorption of glycated products at 260–280 nm was weaker and shifted to shorter wavelength, a phenomenon called blueshift.

Protein molecules containing aromatic amino acids such as tryptophan, tyrosine, and phenylalanine are capable of absorbing ultraviolet light in the vicinity of 280 nm. The current results indicated that the amino acid residues in the SPI–conjugates were surrounded more by hydrophobic environment than those in SPI [12, 31]. The sugar chain made protein easy to expand protein peptide chain so that the interior amino acids exposed. When the auxochromes were conjugated with carbonyl group in sugar chains, the electron orbit energy level was increased and the energy required by electronic transition increased as well, leading to blue shift of the absorption bands.

Fourier transforms infrared spectrum analysis

Fourier transform infrared spectroscopy was utilized to analyze protein structure and is able to reflect the changes in the structure of the peptide chain. The characteristic absorption band of protein amino I in the 1,600–1,700 cm^{-1} range reflects the protein secondary structure. The following parameters were used in the current manuscript based on literature review: β-sheet, 1,600–1,640 cm^{-1}; disordered structure, 1,640–1,650 cm^{-1}; α-helix structure, 1,650–1,660 cm^{-1}; and β-turn structure, 1,660–1,700 cm^{-1} [32].

Fig. 4 Ultraviolet spectra of unglycated and glycated SPI conjugates (1:1, w/w) after incubation at 60 °C and 79 % relative humidity for different time. *1–7* native SPI, SPI–glucose incubated for 48 h, SPI–dextran (20,000) incubated for 132 h, SPI–maltose incubated for 72 h, SPI–lactose incubated for 72 h, SPI–dextran (40,000) incubation for 132 h, and SPI–galactose incubated for 60 h, respectively
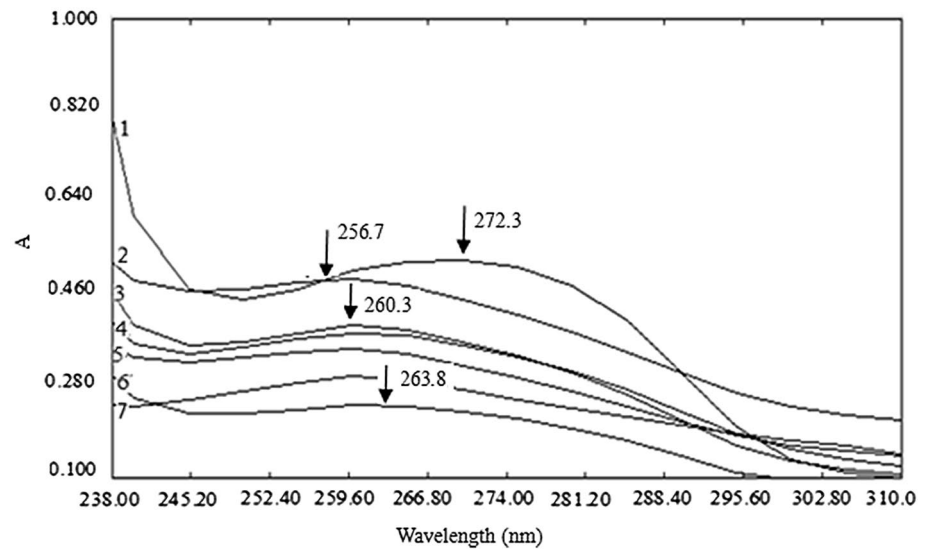

Table 2 Secondary structure of the glycated SPI measured by Fourier transform infrared spectrum and analyzed by peakfit V4.12

Samples	Content of secondary structure (%)			
	α-Helix (1,650–1,660 cm^{-1})	β-Sheet (1,600–1,640 cm^{-1})	β-Turns (1,660–1,700 cm^{-1})	Random coil (1,640–1,650 cm^{-1})
SPI	12.22	41.42	33.69	12.67
SPI–glucose (w:w = 1:1, 48 h)	11.54	42.71	34.73	12.02
SPI–galactose (w:w = 1:1, 60 h)	10.33	43.08	34.29	12.31
SPI–maltose (w:w = 1:1, 72 h)	11.60	41.89	34.40	12.11
SPI–lactose (w:w = 1:1, 72 h)	11.58	40.61	35.92	11.88
SPI–dextran (20,000) (w:w = 1:1, 132 h)	12.35	38.70	37.26	11.69
SPI–dextran (40,000) (w:w = 1:1, 132 h)	12.33	35.84	40.10	11.73

The changes in protein secondary structure were analyzed by the Peak Fit version 4.12 software (Table 2). The SPI–monosaccharide complexes and SPI–disaccharide complexes lost α-helix and random coil structure with a concomitant increase in β-turns and β-sheet. In the SPI–polysaccharide complexes, while there was an observed increase in α-helix and β-turn, there was a decrease in β-fold and random coil. These results implied that the secondary structures of SPI might be changed by Maillard reaction. The interaction of biopolymers and heat denaturation during Maillard reaction between protein and saccharide could affect the secondary structure of proteins [12]. The α-helix and β-sheet of proteins are usually buried in the interior of polypeptide chains. The cross-linking of saccharide to protein led to an unfolding of protein molecules and changes in spatial structure [15].

It was reported that the higher the helical structure, the lower the protein hydrophobic sites [33]. Therefore, the SPI–polysaccharide had a lower extent of exposuring their hydrophobic sites than SPI–monosaccharide and SPI–disaccharide. This speculation was supported by the results in SDS-PAGE (Fig. 3). This type of information may further the understanding of protein and saccharide cross-linking.

Antigenicity of the glycated products

The changes of β-conglycinin antigenicity in glycated products with reaction time are shown in Fig. 5. Maillard reaction in SPI and monosaccharide or SPI and disaccharide or SPI and polysaccharide reduced the antigenicity of β-conglycinin. Although the antigenicity of all glycated products was decreased, the antigenicity of β-conglycinin in SPI–monosaccharide significantly declined compared with the SPI–disaccharide complexes and SPI–polysaccharide complexes. In the glycated products, the initial antigenicity inhibition rate of β-conglycinin in SPI–glucose complexes was 83.55 %. After 48 h later, the antigenicity inhibition rate of β-conglycinin decreased to 29.80 %, that is, a reduction up to 50 %. The SPI–glucose complex incubated for 48 h had less antigenicity (Fig. 5) with smaller free amino group lost than other glycated products (Fig. 1).
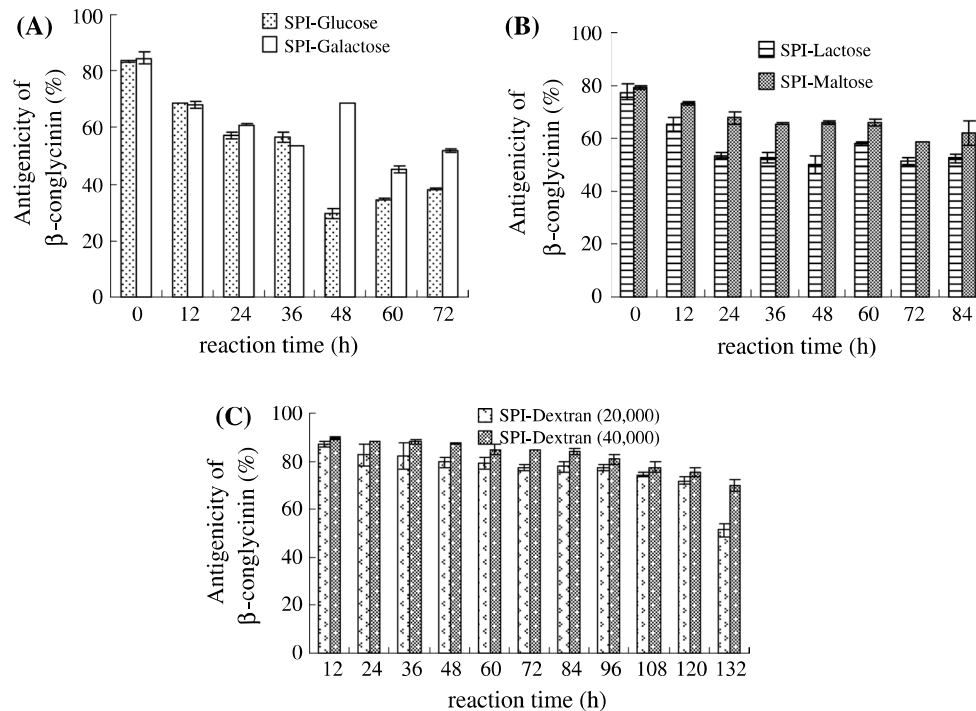
Fig. 5 Effect of incubation at 60 °C and 79 % relative humidity for different time on the antigenicity of β-conglycinin in glycated conjugates. **a** SPI–glucose (1:1, w/w) conjugates; **b** SPI–dissaccharide (1:1, w/w) conjugates; **c** SPI–polysaccharide (1:1, w/w) conjugates

So SPI and glucose incubated for 48 h seems a good Maillard reaction condition to reducing antigenicity.

The antigenicity of allergenic protein could decrease by Maillard reaction, which is in agreement with the studies of Lagemaat et al. [16] and Bu et al. [25]. With the increased incubation time, the antigenicity of glycated products reduced, suggesting that the extent of glycation might be a factor leading to decreased antigenicity. In addition, the changes of antigenicity might also depend on the difference in the binding specificity of saccharide to the antigenic sites of protein [26]. The secondary structure (α-helix, β-structure) of SPI through Maillard reaction was unfolded, which could be observed in the SDS-PAGE pattern and FTIR pattern. This change presumably caused partial shield of the linear antigenic epitopes of SPI resulting in antigen and antibody binding specificity, thereby the antigenicity was reduced.

The antigenicity of protein may reduce after glycation treatment. Lagemaat et al. [16] reported that the antigenicity of wet-heated SPI in the absence of fructooligosaccharides or fructose reduced up to 20 %, and the electrophoretic behavior of allergenic β-conglycinin and glycinin was modified. High temperature for longtime may make protein denature and expose the buried sulfhydryl groups, exchange sulfhydryl–disulfide in molecular, and then the antigenic epitopes hidden inside exposed owing to the protein unfolding of conformational structure [29, 34].

In fact, different saccharides have the similar characteristic, which could cross-link protein through Maillard reaction to reduce the allergenicity of protein, and could also improve the functional properties such as the solubility, the emulsifying abilities, and the foaming abilities [12, 15], while, in the same conditions, the reactivity between different saccharides and protein may be different, such as reaction time, the degree of browning, and the free amino group contents [35, 36]. In addition, Li et al. [36] investigated the glycation of rice proteins with different reducing sugars (glucose, lactose, maltdextrin, and dextrin) in order to improve their functional properties, and found that glucose could provide the most improvement of the solubility and the emulsifying capacity.

Conclusions

SPI–saccharide conjugates were prepared by dry-heated Maillard reaction. The reduction of free amino group content in protein proved that Maillard reaction was occurred. And Maillard reaction could induce conjugation between saccharide and SPI. Adding sugar chains to the protein by Maillard reaction changed the native protein spatial structure. In the glycated products mentioned above, the antigenicity inhibition rate of β-conglycinin in SPI–glucose was reduced to a level of 29.80 % at 48 h, which was a

reduction up to 50 % compared with native SPI. These results suggest that the extent of glycation might be an important factor leading to decreased antigenicity. The structural modification of protein through glycation might alter protein's antigenicity due to the mask of allergen epitopes. Although the antigenicity of β-conglycinin was not completely eliminated, it is concluded that the glycation of SPI with saccharide is a promising way of lowering β-conglycinin antigenicity. In the future, the relationship between the mechanism of reducing antigenicity and glycate effective sites needs to be further exploration.

Conflict of Interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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