



Characterization of soy β -conglycinin–dextran conjugate prepared by Maillard reaction in crowded liquid system

Xi Zhang^a, Jun-Ru Qi^{a,*}, Kang-Kang Li^a, Shou-Wei Yin^a, Jin-Mei Wang^a, Jian-Hua Zhu^b, Xiao-Quan Yang^a

^a College of Light Industry and Food Sciences, South China University of Technology, Guangzhou 510640, China

^b Department of YingDong Food Science and Technology, Shaoguan College, Shaoguan 512005, Guangdong, China

ARTICLE INFO

Article history:

Received 23 March 2012

Accepted 6 September 2012

Keywords:

Soy β -conglycinin

Dextran

Covalent conjugate

Macromolecular crowding

Maillard reaction

ABSTRACT

The conjugation reaction between soy β -conglycinin and dextran via Maillard reaction in crowded liquid system was studied. Apparent viscosity indicated that environment became more and more crowded with the increase of dextran concentration. The covalently linked conjugate was produced by incubating aqueous solution containing soy β -conglycinin and dextran at pH 7.0 and 95 °C for 6 h.

Free amino group content and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profile showed that soy β -conglycinin–dextran conjugate was successfully formed and macromolecular crowding enhanced the extent of glycation. The results of atomic force microscopy indicated that glycation inhibited the thermal aggregate of protein. Meanwhile, the tertiary structure of protein in conjugate changed with increasing number of aromatic side-chains exposed in heating environment. All data showed that the introduction of macromolecular crowding in soy β -conglycinin–dextran conjugate was an effective and promising method for linking polysaccharides to proteins.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Maillard-type protein–polysaccharide conjugates, the product of non-enzymatic browning reaction, are formed between the ϵ -lysyl amino groups of a protein and the reducing end carbonyl group of a polysaccharide through an Amadori-type linkage (Kato, Minaki, & Kobayashi, 1993). This is demonstrated to be a promising approach to improve functional properties, such as solubility, foaming, thermal stability, and emulsifying, and chemical properties, such as antioxidant activity (Akhtar & Dickinson, 2007; Fujiwara, Oosawa, & Saeki, 1998; Jafar, & Fadia, 2010; Nakamura, Kato, & Kobayashi, 1992; Zhu, Damodaran, & Lucey, 2010). This protein modification is generally considered to be suitable for application in the food industry, because it improves physicochemical properties without adding extraneous chemicals (Shepherd, Robertson, & Ofman, 2000).

Dry-heating has usually been adopted to prepare Maillard-type protein–polysaccharide conjugates under controlled temperature and relative humidity conditions (Akhtar & Dickinson, 2007; Diftis & Kiosseoglou, 2006; Xu & Yao, 2009). This process usually takes a long reaction time up to several days or weeks, and the reaction extent is uncontrollable, which may lead to excessive browning development. From an industrial viewpoint, this dry-heating processing is not attractive, and there is no commercially manufactured conjugate ingredient (Zhu, Damodaran, & Lucey, 2008). To avoid these problems, wet heating has been adopted to prepare protein–

saccharide conjugates (Jing & Kitts, 2002; Lertittikul, Benjakul, & Tanaka, 2007). Wet heating largely shortens the reaction time to only several hours at high temperature and short reaction time limits the Maillard reaction to the initial stage to provide better control of browning. However there are also some drawbacks of wet heating. At high temperature, protein aggregation will affect the Maillard reaction and result to low degree of glycation (Zhu et al., 2008).

To solve these problems, “Macromolecular Crowding Effect”, a new concept in life science was introduced to the present study. In recent years scientists have strongly suggested that much like pH, ionic strength, or solution composition, the degree of molecular crowding should be considered an important factor for describing the environmental conditions in a solution (Ellis, 2001). In the presence of high concentrations of biological macromolecules, the reaction follows excluded volume theory and crowded environment promotes the reaction (Zimmerman & Minton, 1993). In addition, the extent of protein aggregation could potentially be minimized in macromolecular crowding environment (Ellis, 2001; Zhu et al., 2008).

Soy β -conglycinin is an important component of soy protein and closely related to its functional properties. At present, although there are abundant researches about soybean protein glycation with different sugars (Diftis & Kiosseoglou, 2003, 2004; Xu & Yao, 2009), researches focused on soy β -conglycinin glycation are scant. Our studies have shown that the main component of soy involved in glycation reaction between soy protein and dextran is β -conglycinin (Qi, Liao, Yin, Zhu, & Yang, 2010). Dextran is a family of microbial 1 $\rightarrow$ 6- α -D-glucans derived from *Leuconostoc mesenteroides* with varying proportions of other linkage types (1 $\rightarrow$ 2-a-, 1 $\rightarrow$ 3-a-, or

* Corresponding author. Tel.: +86 20 87114262; fax: +86 20 87114263.
E-mail address: [jq@scut.edu.cn](mailto:jrq@scut.edu.cn) (J.-R. Qi).

1→4- α -branch linkage). Due to the high percentage of α (1→6) glucosidic linkage, dextran is a very flexible polysaccharide with a high solubility and low solution viscosity and is unable to form gels at high concentration. In addition, dextran is a good macromolecular crowding agent (Hall & Minton, 2003). Xu and co-workers reported the formation of soy β -conglycinin–dextran conjugates in dry-heating and the effect of β -conglycinin glycation on the thermal aggregation of protein. The results showed that the glycation of β -conglycinin inhibited its thermal aggregation at various pH or ionic strength values. Besides, they (Xu, Yang, et al., 2010; Xu, Yu, et al., 2010) further researched the emulsifying properties of β -conglycinin and dextran conjugates in a pressurized liquid system. However, protein polymerization will be very serious in this condition, and few studies of conjugates focused on liquid system.

Heat-induced aggregation of soy protein has poor properties in foods (Kinsella, 1979), like low solubility. We hope to introduce macromolecular crowding, which is carried out by a new reaction model, to solve these problems. β -Conglycinin has the efficacy of lowering serum triacylglycerol (Ohara et al., 2007), so it is promising in functional food and medicine market. Besides, glycated protein improves technological properties, especially emulsifying property (Diftis, Biliaderis, & Kiosseoglou, 2005). Glycated protein with reduced aggregation can be industrially applied in soluble protein additives for sports drinks, medicines, salad dressing emulsions, and so on.

This study aims to prepare β -conglycinin–dextran conjugate in aqueous solution, introduce “Macromolecular Crowding Effect” to generate protein–polysaccharide functional macromolecules, and study aggregation behavior and structure of protein.

2. Materials and methods

2.1. Materials

β -Conglycinin was purified from defatted soybean seed flour (commercially produced during soy bean oil production by low-temperature technology; provided by Shandong Yuwang Industrial and Commercial Co., Ltd., Shandong Province, China) using the method described by Nagano, Hirotsuka, Mori, Kohyama, and Nishinari (1992). The protein content of the prepared powder was 92.05% (dry weight, $N \times 5.71$) as determined by the Dumas combustion method (Elemental Analyzer rapid N cube, Hanau, Germany). Dextran (67 kDa), o-phthalaldehyde (OPA) and 8-anilino-1-naphthalene sulfonic acid (ANS) reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thyroglobulin from bovine thyroid (669 kDa), ferritin from horse spleen (440 kDa), aldolase from rabbit muscle (158 kDa), conalbumin from chicken egg white (75 kDa) and ovalbumin from hen egg (43 kDa) used as molecular weight markers were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, U.K.). All other reagents and chemicals were of analytical grade.

2.2. Preparation of β -conglycinin–dextran conjugate by heat treatment

Mixtures of β -conglycinin–dextran in various ratios (β -conglycinin:dextran = 2:1, 1:1, 1:2, 1:3, 1:4, by weight) were dissolved in deionized water. The protein concentration of dilute solution samples is 0.5%, and the protein concentration of all other solutions is 5%. The sample solutions were stirred on a magnetic stirrer at room temperature for 2 h to completely dissolve the mixture. The pH values of the solutions (pH 7.0) were adjusted by carefully adding 0.1 N HCl or 0.1 N NaOH. Solutions were gently stirred overnight at 5 °C to ensure the complete hydration of the macromolecules. Aliquots of the solutions were placed in a water bath heated at 95 °C for different times (from 0 h to 6 h), and then immediately cooled in an ice-water bath followed by centrifugation (himac CR 22 G High-Speed Refrigerated Centrifuge, Hitachi, Tokyo, Japan) at 10,000 g for 15 min, and almost no precipitate

was observed after centrifugation. The supernatant was then freeze-dried. Native β -conglycinin, heated β -conglycinin at 95 °C for 6 h and mixture (β -conglycinin:dextran = 1:3) without heating were used as controls.

2.3. Apparent viscosity measurement

Flow curves were evaluated on a stress-controlled HAAKE RS600 rheometer (HAAKE Co., Karlsruhe, Germany) with parallel plates ($d = 27.83$ mm), at 25 °C. The dispersions were placed between two parallel plates, and the gap between two plates was set to 1.0 mm. The temperature of protein samples was monitored through the lower plate. Apparent viscosity measurements were carried out with a linearly incremental shear rate from 0.01 to 500 s^{-1} in 3 min.

2.4. Measurement of degree of glycation (DG)

The content of free amino groups was determined by the OPA method (Guan, Qiu, Liu, Hua, & Ma, 2006). 40 mg OPA was dissolved in 1 ml of 95% ethanol, 2.5 ml of 20% (w/v) sodium dodecyl sulfate (SDS), and 25 ml of 0.1 M sodium tetraborate buffer solution (containing 100 μ l 2-ME) and then replenished to 50 ml with deionized water. The OPA reagent was prepared freshly and used within 2 h. To 200 μ l of sample solution (2.5 mg/ml β -conglycinin equivalent), 4 ml of OPA reagent was added. The absorption was measured at 340 nm immediately after being placed in a water bath at 35 °C for 2 min. The content of free amino groups was calculated by using the calibration curve of Lysine as a standard. DG was calculated based on the loss of amino groups compared to unreacted protein.

2.5. SDS-PAGE

Gel electrophoresis was conducted according to the method of Laemmli (1970) using 12% and 5% acrylamide separating and stacking gel, respectively. Samples were prepared in 0.125 M Tris–HCl buffer, containing 1% (w/v) SDS, 2% (v/v) 2-ME, 20% (v/v) glycerol and 0.025% (w/v) bromophenol blue, and heated for 5 min in boiling water before electrophoresis. After the electrophoresis, the gel sheets were stained for protein with Coomassie brilliant blue R-250 and stained for carbohydrate with periodate–Schiff solution (Zacharius, Zell, Morrison, & Woodlock, 1969), respectively. The protein stain was destained with 10% acetic acid (v/v) containing 10% methanol (v/v).

2.6. Turbidity measurements

Native β -conglycinin, β -conglycinin–dextran mixture and glycosylated β -conglycinin were dissolved in distilled water. The final protein concentration was 2.0 mg/ml (i.e., protein of the conjugate and mixture). The pH of the sample solutions was adjusted to the required value by adding 0.1 N NaOH or 0.1 N HCl dropwise with gentle stirring. The turbidity of each solution was estimated by measuring the absorbance of the solutions at 540 nm (Damodaran & Kinsella, 1982) as determined on a UV2300 spectrophotometer (Tianmei Ltd., Shanghai, China) in a 1 cm cuvette. Each sample was shaken fully to ensure a uniform suspension of particles before turbidity test.

2.7. High performance size exclusion chromatography

The HPSEC experiment was performed using a TSK-GEL G4000SWXL column (Tosoh, Tokyo, Japan), connected to a Waters high-performance liquid chromatography (HPLC) equipped with a Waters 1525 HPLC pump and a Waters 2487 UV–visible detector operating at 280 nm (Waters, Division of Millipore, Milford, MA, USA). The column was equilibrated and eluted with 50 mM phosphate buffer (pH 7.2) containing 50 mM

NaCl at an elution rate of 0.6 ml/min at room temperature. The protein solutions were suspended in elutes, stirred for 2 h and then stored overnight at 4 °C to ensure complete hydration. After centrifugation of the bulk protein solutions (10,000 g for 10 min at 25 °C), the supernatants were further filtered with 0.22 µm membrane (Whatman International Ltd., Maidstone, England). The protein concentration and the amount of injected samples were 5.0 mg/ml and 10 µl, respectively.

2.8. Dynamic light scattering

The samples were diluted to 1 mg/ml with distilled water (pH 7.0) filtered through a 0.22 µm filter (Fisher Scientific). After centrifugation of the protein solutions (10,000 g for 10 min), the supernatants were further filtered with 0.22 µm filter before measurement. The sample was placed in a 1 cm × 1 cm cuvette. DLS analysis was performed at a fixed angle of 173° using a Zetasizer Nano-ZS instrument (Malvern Instruments, Worcestershire, U.K.) equipped with a 4 mW He–Ne laser (633 nm wavelength) at 25 °C. The appropriate viscosity (dispersant, water 0.8872 cP) and refractive index (material, protein/polysaccharide 1.450; dispersant, water 1.330) parameters for each solution were set. The time-average (or “total”) intensity (kcps) of the scattered light at different points was collected to obtain qualitative information about the time required for the formation of aggregates using Dispersion Technology Software (DTS) (V4.20) (Malvern Instruments Ltd.).

2.9. Atomic force microscopy

AFM images were recorded at room temperature in tapping mode at a drive frequency of approximately 320 kHz, and the scan rate was 1.0 Hz using a MultiMode SPM microscope equipped with a Nanoscope IIIa Controller (Digital Instruments, Veeco, Santa Barbara, CA, USA). PointProbe NCHR silicon tips of 125 µm in length with a spring constant of 42 N/m were purchased from NanoWorld (Arrow NC cantilevers, Nanoworld, Switzerland). Typical resonant frequencies of these tips were about 290 kHz. Aliquots (2 µl) of dispersions (diluted to 10 µg/ml with distilled water filtered through a 0.22 µm filter) were placed on a freshly cleaved mica disk and air-dried for 10 min at ambient temperature. Control samples (freshly cleaved mica) were detected to exclude possible artifacts. Images were analyzed using Digital Nanoscope software (version 5.30r3, Digital Instruments, Veeco, Santa Barbara, CA, USA).

2.10. Surface hydrophobicity (H_0) measurement

H_0 was determined using ANS as a fluorescence probe according to the method of Haskard and Li-Chan (1998). Samples were dispersed into sodium phosphate buffer solution (0.01 M, pH 7.0) to achieve different concentrations (0.02–0.5 mg/ml). Aliquots of ANS solution (20 µl, 8 mM in the same phosphate buffer) were then added to 4 ml of each sample. Fluorescence intensity was measured using F7000 fluorescence spectrophotometer (Hitachi Co., Tokyo, Japan), at an excitation wavelength of 390 nm and an emission wavelength of 488 nm. The initial slope of fluorescence intensity vs. protein concentration (mg/ml) was calculated by linear regression analysis and used as the index of H_0 .

2.11. Circular dichroism spectroscopy

CD spectra were obtained using a MOS-450 spectropolarimeter (Bio-Logic Science Instruments, Grenoble, France). The near-UV CD spectrum measurements were performed in a quartz cuvette of 10 mm with protein concentration 1.0 mg/ml. The sample was scanned over a wavelength range from 250 to 350 nm. For the measurements, the spectrum was an average of six scans. The following parameters were used: step resolution, 0.5 nm; acquisition duration,

1 s; bandwidth, 0.5 nm; sensitivity, 100 mdeg. The cell was thermostated with a Peltier element at 25 °C. The spectrum of distilled water as a control was subtracted from the spectra of the samples. CD measurements were expressed as mean residue ellipticity in deg·cm²/dmol.

2.12. Statistical analysis

Unless specified otherwise, three independent trials were performed, each with a new batch of sample preparation. All of the tests were carried out in duplicate or triplicate, and an analysis of variance (ANOVA) of the data was performed using the SPSS 13.0 statistical analysis system. A least significant difference (LSD) test with a confidence interval of 95% was used to compare the means.

3. Results and discussion

3.1. Formation of β-conglycinin–dextran conjugate in crowded liquid system

3.1.1. Apparent viscosity

Apparent viscosity was performed to demonstrate the crowded degree of the system. The evolution of viscosity versus shear rate measured for different dextran concentrations and heating time is illustrated in Fig. 1. The protein concentration of all samples was 5% w/v. All the solutions exhibited shear-thinning behavior at shear rate in the range 0.1–100 s^{−1}. This phenomenon is attributed to that intermolecular interactions broke after the parallel plates rotating static solution. Solutions displayed a Newtonian shear behavior at shear rate from 100 to 500 s^{−1}. In Fig. 1A, as the dextran concentration increased, the viscosity increased, indicating the environment becomes more and more crowded. And there was a significant increase when the dextran concentration increased from 15% to 20%. Larissa et al. (2004) reported that too high viscosity may lead to a dramatic acceleration in the rate of protein aggregation under macromolecule crowding environment. And at too high concentration of crowding reagent the rate of glycation reaction may decrease due to high viscosity effect. Thus, 15% dextran was selected for the following study.

In Fig. 1B, 5% β-conglycinin and 15% dextran were used to further research the crowded situation of the system in heating process. There was no obvious change in the viscosities of β-conglycinin and dextran which equilibrated at 0.0035 Pa·s and 0.01 Pa·s, respectively, during heating at 95 °C for 6 h. The viscosities of mixture at 0 h were almost the same as dextran. However, the viscosity of mixture increased from 0.01 Pa·s to 0.038 Pa·s after heating 6 h, demonstrating that the environment became more and more crowded during heating process and indirectly confirmed the formation of covalent conjugate. The viscosity of mixture significantly increased while in separate heating it did not, which can be due to a new macromolecule that is gradually forming during heating.

3.1.2. Glycation degree

Glycation occurs by covalent attachment of carbonyl group in reducing sugars with free amino groups in proteins to form Schiff base (Singh, Barden, Mori, & Beilin, 2001) and DG is often used to evaluate the extent of Maillard reactions (Laroque et al., 2008). The DG of glycated products, as the concentration of dextran is increasing, is shown in Fig. 2. The dilute solutions as control aimed at exploring the effects of protein–dextran ratio on the glycation degree. The DG of concentrated solutions was much higher than the DG of dilute solutions, indicating that crowding prompted glycation. As dextran proportion increased, the DG almost didn't change in dilute solutions, while the DG enhanced in concentrated solutions. These results suggest that the reason of the raise of DG is macromolecule crowding

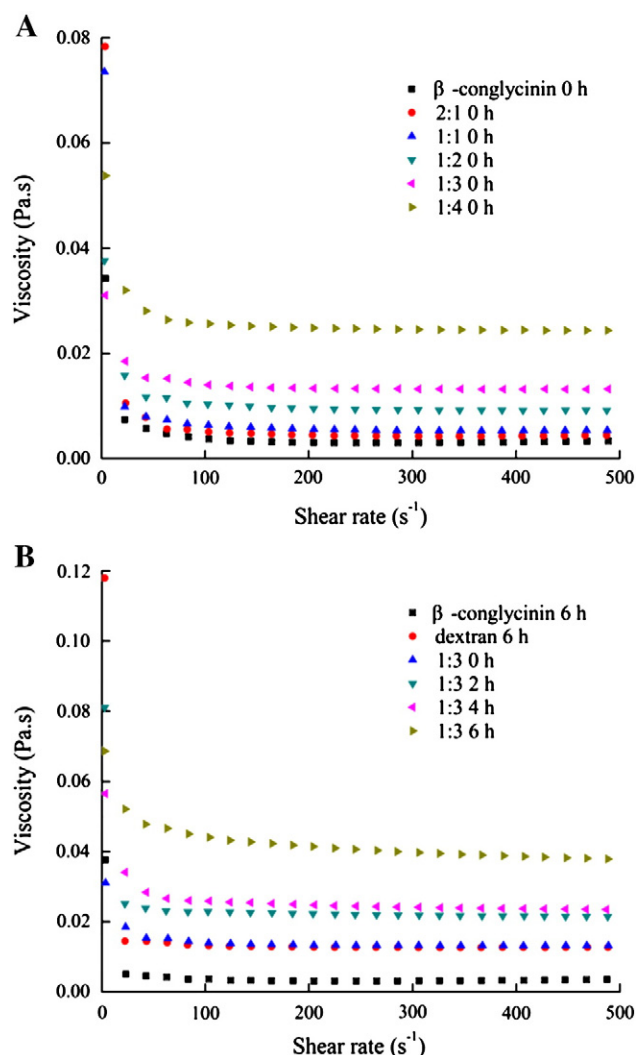


Fig. 1. The apparent viscosity versus shear rate curves for β -conglycinin/dextran dispersions prepared at various initial protein/polysaccharide ratios and different heating times.

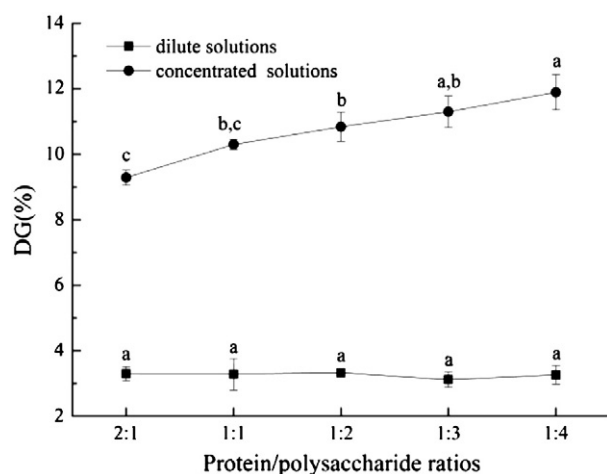


Fig. 2. The DG for glycosylated β -conglycinin. β -Conglycinin:dextran = 2:1, 1:1, 1:2, 1:3, 1:4, heated at pH 7.0 and 95 °C for 6 h. Dilute solutions: protein concentration 0.5%; concentrated solutions: protein concentration 5%. Different letters (a–c) on the top of the dot indicate significant differences ($p < 0.05$).

instead of protein–dextran ratio. Higher concentration of crowding agent dextran results in higher reaction degree.

3.1.3. SDS-PAGE analyses

To further confirm the covalent conjugating of dextran to β -conglycinin and the impact of crowded environment on covalent coupling, SDS-PAGE was performed, as shown in Fig. 3. On visualizing the gel, the characteristic protein bands (α' -sub, α -sub and β -sub) were visible under protein staining (Fig. 3A). On the top of the separating gel, dense polydispersed bands were observed for the β -conglycinin–dextran mixtures heated at 95 °C for 6 h (lanes 5–9). The same bands were also observed under carbohydrate staining (B), indicating the formation of high molecular weight products and that dextran was covalently bonded with β -conglycinin (Diftis & Kiosseoglou, 2006; Shepherd et al., 2000) because most noncovalent interactions are generally disrupted in SDS-PAGE and the uncharged dextran does not migrate in gel electrophoresis (lane 1). On increasing the dextran concentration from 2.5% (lane 5) to 20% (lane 7), the blue-staining protein and pink-staining carbohydrate components were increasingly retained, which confirms that crowded environment promotes the formation of glycation products. Such dense polydispersed bands on the top of the separating gel were not

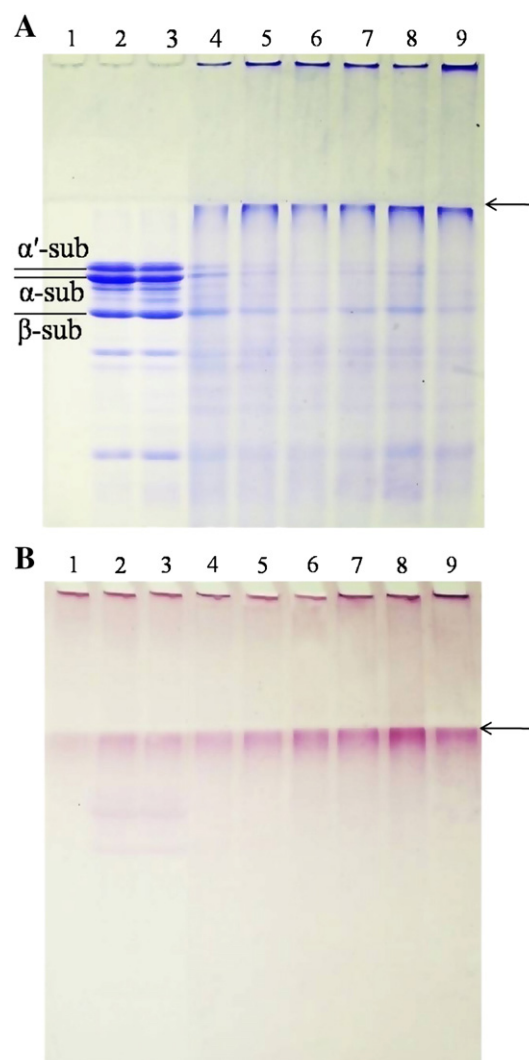


Fig. 3. SDS-PAGE patterns for β -conglycinin/dextran samples: (A) protein stain; (B) carbohydrate stain. Lanes: 1, native dextran; 2, native β -conglycinin; 3, β -conglycinin–dextran mixture (1:3); 4, heated β -conglycinin (95 °C for 6 h); 5–9, β -conglycinin (5%):dextran = 2:1, 1:1, 1:2, 1:3, 1:4, heated at 95 °C for 6 h. Arrows indicate the boundary between stacking and separating gels.

observed for the native β -conglycinin (lane 2) and the mixture at 0 h (lane 3) under protein staining (A). The intensity of characteristic protein bands was almost the same as the native β -conglycinin (lane 2) and mixture at 0 h (lane 3) while faint diffuse band was observed at the top of separating gel for the heated β -conglycinin (lane 4). The reasons may be cross linked protein molecules due to isopeptide or disulfide bond formation during heating (Diftis & Kiosseoglou, 2003). The intensity of the characteristic protein bands of heated β -conglycinin (lane 4) was considerably reduced and β -conglycinin–dextran mixtures heated at 95 °C for 6 h (lanes 5–9) were further reduced in protein stain (A), indicating that most proteins have reacted with the polysaccharide under the employed heating conditions. The native β -conglycinin (lane 2), the mixture at 0 h (lane 3) and the heated β -conglycinin (lane 4) had faint diffuse bands at the top of separating gel under carbohydrate staining (B) because β -conglycinin is a glycoprotein. Compared with the native β -conglycinin (lane 2) and the mixture at 0 h (lane 3), the characteristic glycoprotein bands of heated β -conglycinin (lane 4) and heated mixtures (lane 5–9) disappeared under carbohydrate staining (B). Similar SDS-PAGE was found by Xu, Yang, et al. (2010), Xu, Yu, et al. (2010), and Zhang, Wu, Yang, He, and Wang (2012), who reported that covalent conjugating compounds were produced in β -conglycinin–dextran model system treated by heating.

3.2. Turbidity and particle size of β -conglycinin–dextran conjugate

3.2.1. Turbidity

The solubility was estimated by measuring the turbidity of sample solutions at 540 nm. High turbidity represents low solubility of protein. Table 1 demonstrated the turbidity of various samples at different pH. β -Conglycinin and mixture had high optical density in the pH range from 4.0 to 6.0 and the maximum optical density peak occurred at pH ~4.8, indicating a significant decrease of solubility in this pH region, which was due to the isoelectric point (pI) of β -conglycinin being 4.8. β -Conglycinin–dextran conjugate had very low optical density, indicating that glycation of β -conglycinin increases protein solubility near protein isoelectric point. The result is in accordance with Zhu et al. (2010), who also found that turbidity of conjugate was significantly reduced compared with native protein.

3.2.2. HPSEC

SEC is generally used to characterize the molecular weight of food materials such as proteins, polysaccharides, or their aggregates. The HPSEC elution profiles were displayed in Fig. 4. In the SEC profiles of raw β -conglycinin, there were two major elution peaks (peak I and peak II) eluting at 14.7 and 19.2 min, clearly attributed to the β -conglycinin components. As expected, peak I of heated β -conglycinin disappeared and concomitant appeared a new peak eluting at 10.6 min. The new peak was clearly attributed to the heating induced formation of soluble protein aggregates. For the mixture at 0 h, peak I decreased remarkably, which may be caused by dextran disturbance. In the SEC profiles of conjugate, there were two major elution peaks eluting at 9.2 and 18.7 min respectively. The first peak further moved ahead, compared with heated β -conglycinin, and the peak height obviously

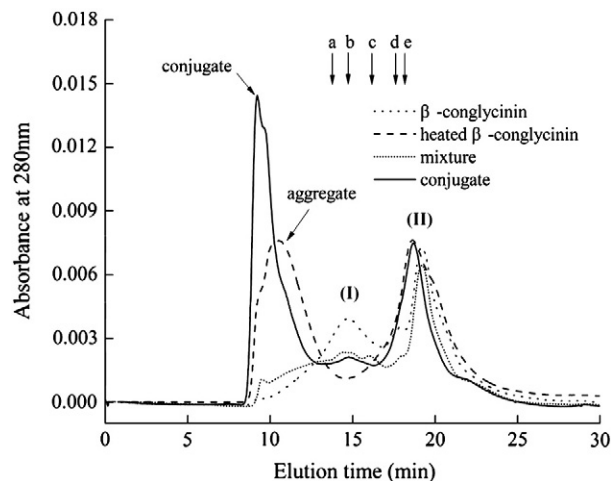


Fig. 4. HPSEC elution profiles of β -conglycinin, heated β -conglycinin, β -conglycinin–dextran mixture and conjugate. The molecular weight markers are as follows: a, thyroglobulin from bovine thyroid (669 kDa); b, ferritin from horse spleen (440 kDa); c, aldolase from rabbit muscle (158 kDa); d, conalbumin from chicken egg white (75 kDa); and e, ovalbumin from hen egg (43 kDa). Arrows identified in the figure indicate the peak time of protein markers.

increased. The phenomena suggest the formation of covalent conjugate. Although heated β -conglycinin formed macromolecule because of self-aggregate, the conjugate formed larger molecular weight macromolecule. This was consistent with the gel electrophoresis (Fig. 3). And similar result was found by Zhang et al. (2012), who reported that a new peak of conjugate appeared at shorter elution time compared with β -conglycinin.

3.2.3. Aggregate size and morphology analysis

DLS as a quantitative technique has been proved to be a sensitive and powerful method in monitoring the formation of aggregates (Lomakin, Benedek, & Teplow, 1999). Due to high magnification with high resolution and low effect on native status of the samples, the AFM has been confirmed to be a powerful tool to investigate the fine structure of many food macromolecules (Ikeda & Morris, 2002; Yang et al., 2007).

Atomic force microscopy and DLS were used to study the effect of glycation in macromolecule crowding environment on the aggregation of β -conglycinin (Fig. 5). β -Conglycinin (A), a globular protein, is a small globule of non-uniform size, and the larger globules might be a result from self-aggregation. For heated β -conglycinin (B), large numbers of self-aggregates of many β -conglycinin molecules were formed obviously. From HPSEC in Fig. 3, it also indicated that heating induced the formation of soluble protein aggregates. This was consistent with DLS. After heating, the mean size of β -conglycinin increased from 14.72 nm to 60.49 nm. The result is in accordance with Xu, Yang, et al. (2010) and Xu, Yu, et al. (2010) for DLS analysis of β -conglycinin size. For mixture (C), big objects and small globules were observed, and the mean size is 22.28 nm. From the AFM images of conjugate (D), there were far less aggregates compared with heated β -conglycinin (B), which maybe attributes to macromolecule crowding environment. Because in the β -conglycinin–dextran system, crowding promotes glycation reaction, the propensity of protein self-aggregation during heating decreases. DLS of conjugate (D) demonstrated that the mean size was 109.4 nm. Thus, in macromolecule crowding environment, glycation reduced the thermal aggregation of β -conglycinin.

3.3. Structural properties of β -conglycinin–dextran conjugate

3.3.1. Surface hydrophobicity (H_0)

Protein surface hydrophobicity was an index of the number of hydrophobic groups on the surface of protein. H_0 was previously used to

Table 1

Turbidity of native β -conglycinin, β -conglycinin–dextran (1:3) mixture and β -conglycinin–dextran (1:3) conjugate heated at 95 °C for 6 h.

Sample	pH						
	3	4	5	6	7	8	9
β -conglycinin	0.154 ^a	0.866 ^b	2.205 ^b	0.174 ^b	0.056 ^a	0.042 ^a	0.064 ^a
Mixture	0.080 ^b	1.591 ^a	2.393 ^a	0.208 ^a	0.056 ^a	0.042 ^a	0.066 ^a
Conjugate	0.082 ^b	0.460 ^c	0.678 ^c	0.064 ^c	0.034 ^b	0.041 ^a	0.032 ^b

Each data point is the mean of at least three determinations. Different letters in the same column indicate significant ($p < 0.05$) differences among samples.

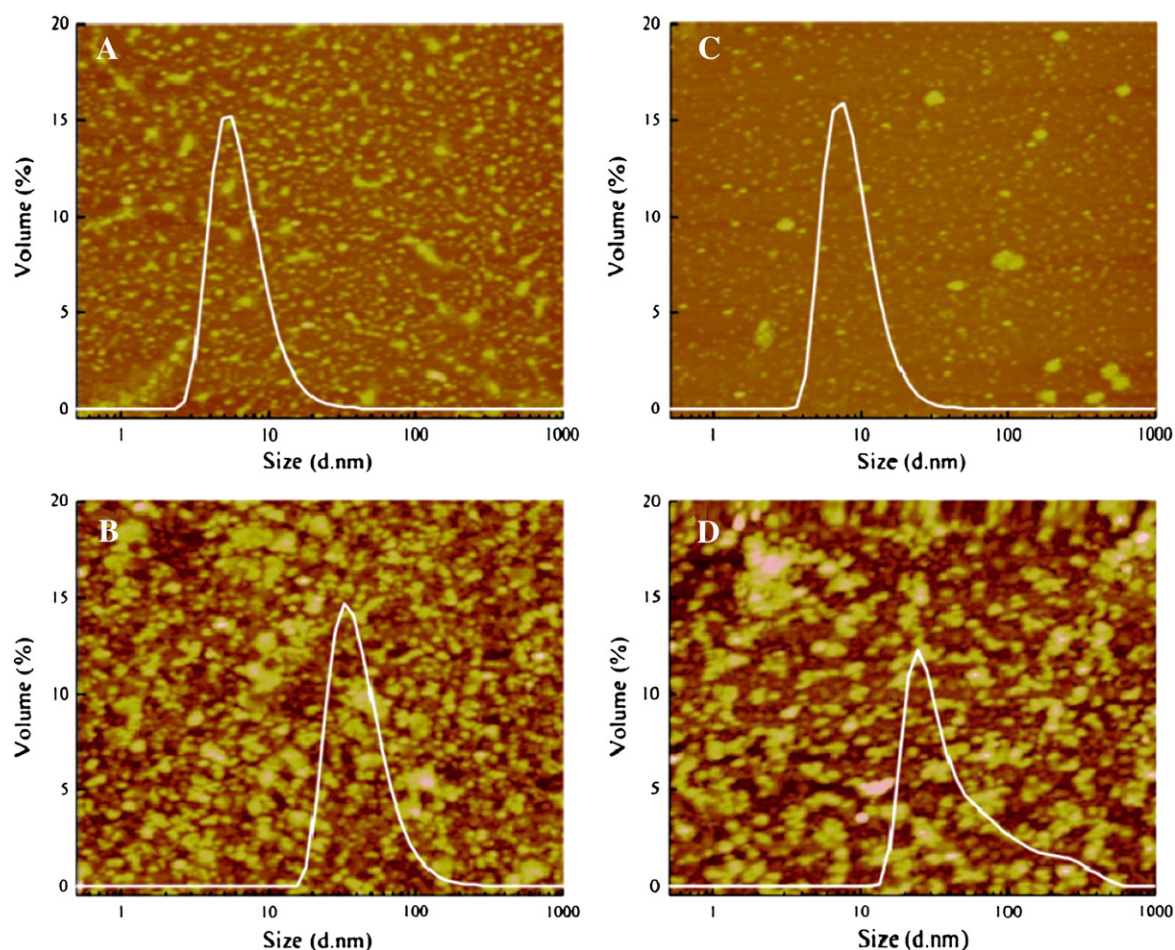


Fig. 5. AFM images and particle size distributions of β -conglycinin (A), heated β -conglycinin (B), β -conglycinin–dextran mixture (C) and conjugate (D). Scan size, $5.00 \mu\text{m} \times 5.00 \mu\text{m}$; data scale height, 20 nm.

identify the structural changes of protein (Puppo et al., 2004). Fig. 6 showed the surface hydrophobicity of various samples. The H_0 of the mixture had no significant difference from that of the native β -conglycinin, indicating that adding of dextran alone will not affect the surface hydrophobicity of protein while the H_0 of heated β -conglycinin was significantly higher than native β -conglycinin, suggesting that more hydrophobic clusters expose on the molecular surface of protein after heating treatment. The H_0 of conjugate was

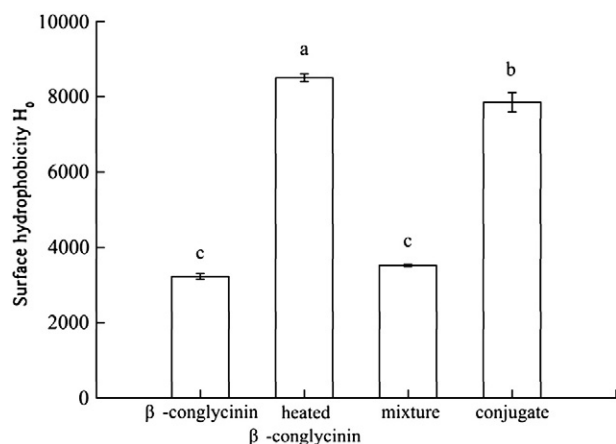


Fig. 6. The surface hydrophobicity (H_0) of β -conglycinin, heated β -conglycinin, β -conglycinin–dextran mixture and conjugate at pH 7.0. Different letters (a–c) on the top of a column indicate significant differences ($p < 0.05$).

lower than heated β -conglycinin, indicating that dextran was covalently bonded with β -conglycinin. On one hand, glycation may reduce exposure of hydrophobic groups buried in the intramolecular; on the other hand, the bonding of hydrophilic straight-chain dextran increases hydrophilicity on the molecule surface. Thus, the H_0 of conjugate reduced in macromolecular crowding environment.

3.3.2. Triple structure of β -conglycinin–dextran conjugate

The tertiary conformations of conjugate were analyzed by near-UV CD spectroscopic technique. The CD spectra in the region 250–320 nm arise from the aromatic amino acid (Kelly, Jess, & Price, 2005). The near-UV CD curves were shown in Fig. 7. The native β -conglycinin had a spectrum with a positive dichroic band at around 272 nm. The CD curve of the mixture had no significant difference from that of the native β -conglycinin. However, the intensity of the bands of heated β -conglycinin decreased. It is likely that the aromatic residues in β -conglycinin are found primarily in the 'α-helical hooks' that hold the subunits together in the trimer, as in phaseolin, the 7S trimer from bean. Thus the loss of the near-UV CD spectra following heating indicates that the 'hooks' probably become denatured. This result was in accordance with Tang, Wang, and Huang (2012), who also reported the loss of the near-UV CD spectra of β -conglycinin after heating. The intensity of the bands of conjugate further decreased, indicating that an increasing number of aromatic side-chains are exposed in heating environment, and these are related to native-like structure changes. This phenomenon also suggested that glycation further changed protein tertiary structure in macromolecular crowding environment.

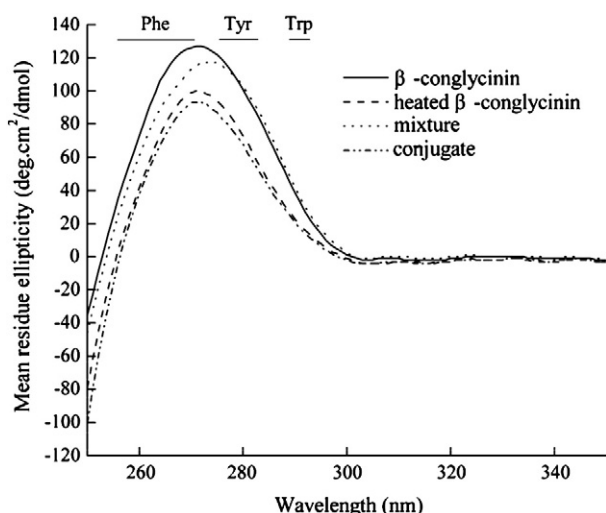


Fig. 7. The near UV CD spectra of β -conglycinin, heated β -conglycinin, β -conglycinin-dextran mixture and conjugate.

4. Conclusion

β -Conglycinin-dextran conjugate was formed by incubation at pH 7.0 and 95 °C for 6 h. Macromolecular crowding environment prompted glycation. Glycation of β -conglycinin increased protein solubility near protein isoelectric point. Glycation changed protein tertiary structure in macromolecular crowding environment. Dextran acted not only as a reactant taking part in the conjugation but also as a crowding reagent in promoting the formation of glycation products and a protective reagent (via macromolecular crowding) in preventing excessive β -conglycinin aggregation. It was an effective and promising method for attaching polysaccharides to proteins in macromolecular crowding environment. The introduction of macromolecular crowding in polysaccharide-protein conjugate in aqueous solutions is a breakthrough from the classical protein glycation means (via dry-heating).

Acknowledgments

All authors appreciate the Chinese National Natural Science Fund (serial number: 31000758 and 31101215), the Fundamental Research Funds for the Central Universities, SCUT of China (fund number: 2012ZZ0086), and Fujian Provincial Major Regional Projects (fund number: 2010N3008) for the financial support.

References

Akhtar, M., & Dickinson, E. (2007). Whey protein-maltodextrin conjugates as emulsifying agents: An alternative to gum arabic. *Food Hydrocolloids*, 21, 607–616.

Damodaran, S., & Kinsella, J. E. (1982). Effect of conglycinin on the thermal aggregation of glycinin. *Journal of Agricultural and Food Chemistry*, 30, 812–817.

Diftis, N., Biliaderis, C., & Kiosseoglou, V. (2005). Rheological properties and stability of model salad dressing emulsions prepared with a dry-heated soybean protein isolate-dextran mixture. *Food Hydrocolloids*, 19, 1025–1031.

Diftis, N., & Kiosseoglou, V. (2003). Improvement of emulsifying properties of soybean protein isolate by conjugation with carboxymethyl cellulose. *Food Chemistry*, 81, 1–6.

Diftis, N., & Kiosseoglou, V. (2004). Competitive adsorption between a dry-heated soy protein-dextran mixture and surface-active materials in oil-in-water emulsions. *Food Hydrocolloids*, 18, 639–646.

Diftis, N., & Kiosseoglou, V. (2006). Physicochemical properties of dry-heated soy protein isolate-dextran mixtures. *Food Chemistry*, 96, 228–233.

Ellis, R. J. (2001). Macromolecular crowding: Obvious but underappreciated. *Trends in Biochemical Sciences*, 26(10), 597–604.

Fujiwara, K., Oosawa, T., & Saeki, H. (1998). Improved thermal stability and emulsifying properties of carp myofibrillar proteins by conjugation with dextran. *Journal of Agricultural and Food Chemistry*, 46, 1257–1261.

Guan, J. J., Qiu, A. Y., Liu, X. Y., Hua, Y. F., & Ma, Y. H. (2006). Microwave improvement of soy protein isolate-saccharide graft reactions. *Food Chemistry*, 97(4), 577–585.

Hall, D., & Minton, A. P. (2003). Macromolecular crowding: qualitative and semiquantitative successes, quantitative challenges. *Biochimica et Biophysica Acta*, 1649, 127–139.

Haskard, C. A., & Li-Chan, E. C. Y. (1998). Hydrophobicity of bovine serum albumin and ovalbumin determined using uncharged (PRODAN) and anionic (ANS) fluorescent probes. *Journal of Agricultural and Food Chemistry*, 46, 2671–2677.

Ikeda, S., & Morris, V. (2002). Fine-stranded and particulate aggregates of heat-denatured whey proteins visualized by atomic force microscopy. *Biomacromolecules*, 3, 382–389.

Jafar, Al-Hakkak, & Fadia, Al-Hakkak (2010). Functional egg white-pectin conjugates prepared by controlled Maillard reaction. *Journal of Food Engineering*, 100, 152–159.

Jing, H., & Kitts, D. D. (2002). Chemical and biochemical properties of casein-sugar Maillard reaction products. *Food and Chemical Toxicology*, 40(7), 1007–1015.

Kato, A., Minaki, K., & Kobayashi, K. (1993). Improvement of emulsifying properties of egg white proteins by the attachment of polysaccharide through Maillard reaction in a dry state. *Journal of Agricultural and Food Chemistry*, 41, 540–543.

Kelly, S. M., Jess, T. J., & Price, N. C. (2005). How to study proteins by circular dichroism. *Biochimica et Biophysica Acta*, 1751(2), 119–139.

Kinsella, J. E. (1979). Functional properties of soy proteins. *Journal of the American Oil Chemists' Society*, 56, 242–258.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680–685.

Larissa, A. M., Elisa, M. C., Vladimir, N. U., & Anthony, L. F. (2004). The effect of macromolecular crowding on protein aggregation and amyloid fibril formation. *Journal of Molecular Recognition*, 17, 456–464.

Laroque, D., Inisan, C., Berger, C., Voulard, E., Dufosse, L., & Guérard, F. (2008). Kinetic study on the Maillard reaction. Consideration of sugar reactivity. *Food Chemistry*, 111, 1032–1042.

Lertittikul, W., Benjakul, S., & Tanaka, M. (2007). Characteristics and antioxidative activity of Maillard reaction products from a porcine plasma protein-glucose model system as influenced by pH. *Food Chemistry*, 100(2), 669–677.

Lomakin, A., Benedek, G. B., & Teplow, D. B. (1999). Monitoring protein assembly using quasielastic light scattering spectroscopy. *Methods in Enzymology*, 309, 429–459.

Nagano, T., Hirotsuka, M., Mori, H., Kohyama, K., & Nishinari, K. (1992). Dynamic viscoelastic study on the gelation of 7S globulin from soybeans. *Journal of Agricultural and Food Chemistry*, 40, 941–944.

Nakamura, S., Kato, A., & Kobayashi, K. (1992). Enhanced antioxidative effect of ovalbumin due to covalent binding of polysaccharides. *Journal of Agricultural and Food Chemistry*, 40, 2033–2037.

Ohara, S., Matsui, Y., Tamesada, M., Saitou, M., Komatsu, R., Yoshino, T., et al. (2007). Serum triacylglycerol-lowering effect of soybean β -conglycinin in mildly hypertriglyceridemic individuals. *e-SPEN, the European e-Journal of Clinical Nutrition and Metabolism*, 2, 12–16.

Puppo, C., Chapleau, N., Speroni, F., Lamballerie, M., Michel, F., Cristina, A., et al. (2004). Physicochemical modifications of high pressure treated soybean protein isolates. *Journal of Agricultural and Food Chemistry*, 52, 1564–1571.

Qi, J. R., Liao, J. S., Yin, S. W., Zhu, J. H., & Yang, X. Q. (2010). Formation of acid-precipitated soy protein-dextran conjugates by Maillard reaction in liquid systems. *International Journal of Food Science and Technology*, 45, 2573–2580.

Shepherd, R., Robertson, A., & Ofman, D. (2000). Dairy glycoconjugate emulsifiers: Casein-maltodextrins. *Food Hydrocolloids*, 14, 281–286.

Singh, R., Barden, A., Mori, T., & Beilin, L. (2001). Advanced glycation end-products: A review. *Diabetologia*, 44(2), 129–146.

Tang, C. H., Wang, S. S., & Huang, Q. H. (2012). Improvement of heat-induced fibril assembly of soy β -conglycinin (7S globulins) at pH 2.0 through electrostatic screening. *Food Research International*, 46, 229–236.

Xu, C. H., Yang, X. Q., Yu, S. J., Qi, J. R., Guo, R., Sun, W. W., et al. (2010). The effect of glycosylation with dextran chains of differing lengths on the thermal aggregation of β -conglycinin and glycinin. *Food Research International*, 43, 2270–2276.

Xu, K. K., & Yao, P. (2009). Stable oil-in-water emulsions prepared from soy protein-dextran conjugates. *Langmuir*, 25(17), 9714–9720.

Xu, C. H., Yu, S. J., Yang, X. Q., Qi, J. R., Lin, H., & Zhao, Z. G. (2010). Emulsifying properties and structural characteristics of β -conglycinin and dextran conjugates synthesised in a pressurised liquid system. *International Journal of Food Science and Technology*, 45, 995–1001.

Yang, H., Wang, Y., Lai, S., An, H., Li, Y., & Chen, F. (2007). Application of atomic force microscopy as a nanotechnology tool in food science. *Journal of Food Science*, 72, 65–75.

Zacharius, R. M., Zell, T. E., Morrison, J. H., & Woodlock, J. J. (1969). Glycoprotein staining following electrophoresis on acrylamide gels. *Analytical Biochemistry*, 30, 148–152.

Zhang, J. B., Wu, N. N., Yang, X. Q., He, X. T., & Wang, L. J. (2012). Improvement of emulsifying properties of Maillard reaction products from β -conglycinin and dextran using controlled enzymatic hydrolysis. *Food Hydrocolloids*, 28, 301–312.

Zhu, D., Damodaran, S., & Lucey, J. A. (2008). Formation of whey protein isolate (WPI)-dextran conjugates in aqueous solutions. *Journal of Agricultural and Food Chemistry*, 56, 7113–7118.

Zhu, D., Damodaran, S., & Lucey, J. A. (2010). Physicochemical and emulsifying properties of whey protein isolate (WPI)-dextran conjugates produced in aqueous solution. *Journal of Agricultural and Food Chemistry*, 58, 2988–2994.

Zimmerman, S. B., & Minton, A. P. (1993). Macromolecular crowding: biochemical, biophysical, and physiological consequences. *Annual Review of Biophysics and Biomolecular Structure*, 22, 27–65.