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Limited Aggregation Behavior of β-Conglycinin and Its Terminating Effect on Glycinin Aggregation during Heating at pH 7.0

Jian Guo, Xiao-Quan Yang,* Xiu-Ting He, Na-Na Wu, Jin-Mei Wang, Wei Gu, and Yuan-Yuan Zhang

Protein Research and Development Center, Department of Food Science and Technology, South China University of Technology, Guangzhou 510640, People's Republic of China

ABSTRACT: In this work, different thermal aggregation behaviors of soy β-conglycinin and glycinin at pH 7.0 were characterized with size exclusion chromatography and low-angle light scattering. Limited aggregation that grew via the consumption of "monomers" was detected in β-conglycinin, forming soluble aggregates. For glycinin, the association between the aggregates that led to the appearance of insoluble materials was observed. Heated with β-conglycinin, the assembly between the glycinin aggregates was terminated and its solubility was recovered. The structure of the soluble and insoluble aggregates was analyzed by small-angle X-ray scattering and dynamic light scattering. Unlike the β-conglycinin soluble aggregates that possessed limited size and less compact conformation, particles with a denser core and a less dense outer shell were found in the glycinin insoluble aggregates. Evidence is presented to reveal the transition between the soluble and insoluble aggregates and the role of β-conglycinin in the solubilization of the soy protein aggregates during heating.

KEYWORDS: *thermal aggregation, soy protein, β-conglycinin, glycinin, soluble aggregates, insoluble aggregates*

■ INTRODUCTION

As a bulk agricultural product, soy protein, which has been shown to possess a variety of health benefits,[1−3] is an important vegetable protein source in human diet nowadays. Thermal treatment is the most common physical agent in soy protein processing. Soybean meals with proper heat treatment can obtain better nutritive value, which had been found in early studies.[4] Heat is also used as an important operation for improving the functional properties of soy protein products, such as abilities for emulsification, foaming, and gel formation.[5−7] The poor solubility and turbid appearance of the products, which were the outcome of the heat processing, are limitations of utilizing soy protein in certain foods.[8] Understanding the kinetics of soy protein aggregation during heating is of importance for maintaining the quality of the products.

β-Conglycinin (7S) and glycinin (11S), which have different structures and properties, are the two major components of soy protein.[9] Both of them are globulins. β-Conglycinin ("vicilin-like" storage protein) is a trimeric glycoprotein consisting of three subunits, α, α′, and β, associated via hydrophobic interactions.[10,11] Glycinin ("legumin-like" storage protein) is a hexamer composed of acidic and basic polypeptides linked by disulfide bonds.[12−14] Different performances were reported when these two globulins were heated individually.[15,16] At neutral pH, heat treatments did not interrupt the solubility of β-conglycinin, but the opposite situation occurred with glycinin. Glycinin was thought to be responsible for the poor thermal stability of soy proteins.[17] However, it was found that the solubility of glycinin was improved when it was heated in the presence of β-conglycinin.[18,19] Existing analysis from different groups reached an agreement: there was a preferential interaction between the β subunit of β-conglycinin and the basic polypeptides of glycinin during heating, which kept glycinin from locating in the precipitates,[20−24] but the driving force for this interaction was still controversial. Electrostatic attraction, formation of disulfide bonds, or hydrophobic interactions were thought to account for the combination in different papers. To elucidate the effect of heat on the soy protein properties, a considerable amount of research has been done on the subunit disassociation/association behavior, but seldom focused on the aggregation kinetics of the denatured soy protein. Information on the absolute molecular mass of β-conglycinin and glycinin during heating that probes into the thermal aggregation behavior is lacking. How the unfolding polypeptide chains of soy protein assemble into the aggregates with different characters is still not clear. The role of β-conglycinin in the thermal aggregation of glycinin should be elucidated. The transition from soluble aggregates to insoluble aggregates has not been captured. The colloidal particle properties of these two aggregates need to be distinguished. In this context, the investigation on the thermal aggregation behavior of β-conglycinin and glycinin at neutral pH is still to be resolved.

Recently, Roberts and co-workers[25−27] proposed the Lumry−Eyring nucleated polymerization (LENP) model that provided a global view for the qualitative and quantitative analysis of protein aggregation mechanisms. This model has been used to infer the aggregation kinetics of α-chymotrypsinogen A,[28−31] bovine granulocyte-colony stimulating factor,[32] IgG1 antibody,[33,34] and anti-CD40 monoclonal antibody.[35] Aggregation is thought to be a convolution of multiple stages in this model. These may include monomer unfolding, prenucleation, irreversible aggregate nucleation, growth via polymerization, and aggregate self-association. The pathway of

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aggregation often involves some or all of the steps and is dependent on the protein and the conditions of interest. According to this model, we speculated that β-conglycinin and glycinin may undergo different aggregation stages during heating, leading to the different appearances after heated. Meanwhile, the existence of β-conglycinin may also change the aggregation kinetics of glycinin, resulting in the increase of solubility. Aggregation includes different stages and may eventually produce aggregates with different properties. However, these inferences still need to be examined through experimental observation.

The present work focuses on the thermal aggregation kinetics of β-conglycinin and glycinin at pH 7.0. Size exclusion chromatography with low-angle light scattering (SEC-LALS) was used to reveal the assembly process of the soy protein unfolding polypeptide chains during heating and expose the role of β-conglycinin in the thermal aggregation of soy protein. The structure of the aggregates is characterized by small-angle X-ray scattering (SAXS) and dynamic light scattering (DLS), so as to tell the difference between soluble and insoluble aggregates from the perspective of conformational information. The interaction between β-conglycinin and glycinin during heating is also discussed. These observations would aid in improving the understanding of the thermal properties of soy proteins and assessing the key step that influences the solubility of soy proteins during processing.

■ MATERIALS AND METHODS

Materials. Defatted soybean flakes were purchased from Shandong Xinjiahua Industrial and Commercial Co. Ltd., China. 1-Anilinonaphthalene-8-sulfonic acid (ANS) reagent was purchased from Sigma (St. Louis, MO, USA). All chemicals used in this work were of analytical or better grade without further purification.

Isolation of Soy Proteins. Soy β-conglycinin and glycinin fractions were isolated from defatted soy flakes as described by Nagano et al.[36] The protein contents of glycinin and β-conglycinin lyophilized powder were 92.20 ± 0.43 and 86.30 ± 1.52% by the Dumas method (N × 5.71).

Preparation of Soy Protein Dispersions and Heating Treatment. The soy protein dispersion was prepared by dispersing 1.0 wt % β-conglycinin or glycinin powder in Millipore water (18.2 MΩ·cm) and stirring at room temperature for 2 h. Buffer condition was 20 mM sodium phosphate at pH 7.0. Afterward, the dispersions were centrifuged at 10000*g* for 20 min to remove insoluble matter. In a typical heat treatment, soy protein dispersions were heated in a water bath at specific temperature for 30 min. After heating, the samples were vibrated vigorously and cooled to room temperature immediately.

Protein contents of various dispersions were estimated according to the method described by Lowry and co-workers,[37] using bovine serum albumin as standard. Each measurement was carried out in triplicate. Protein solubility described in this work referred to the percentage of protein content that remained in the supernatant of the heated samples after centrifugation at 10000*g* for 10 min.

In the speed differential centrifugation experiment, samples were centrifuged at different speeds for 10 min using a himac CS150NX tabletop microcentrifuge with an S140AT rotor (Hitachi Koki Co. Ltd., Tokyo, Japan) before the protein content determinations.

Turbidities of the dispersions were determined at 540 nm using a UV2300 UV−vis spectrophotometer (Techcomp Ltd., Shanghai, China) with 10 mm path length quartz cuvettes. Water was used as the reference. All experiments were replicated at least twice.

Nano Differential Scanning Calorimetry (nDSC). β-Conglycinin or glycinin solutions were diluted to 0.1 wt % with 10 mM sodium phosphate buffer (pH 7.0). Thermodynamic properties of these soy protein samples were determined using a nDSC differential scanning calorimeter (TA Instruments, New Castle, DE, USA) at the scanning rate of 1 °C/min for a temperature range from 25 to 105 °C. The protein dispersion (300 μL) was filled into the sample cell and the reference buffer in the reference cell, and a constant pressure of 2 atm was applied. For the curve analysis, buffer−buffer tracings were recorded under the same conditions and subtracted from the sample endotherms. Subsequently, curve analysis using NanoAnalyze Data Analysis software (TA Instruments) was performed to determine the transition temperature at peak maximum (T_m) as well as the calorimetric enthalpy change (ΔH_m) of the unfolding process normalized for protein content. ΔH_m was obtained by integration of the area of the excess heat capacity endotherm.

Circular Dichroism (CD) Spectroscopy. An MOS-450 spectrometer (BioLogic Science Instruments, Claix, France) equipped with a TCU-250 Peltier temperature controller was used for investigating the thermal unfolding process of β-conglycinin and glycinin. Protein dispersions were diluted to 0.1 wt % with 10 mM sodium phosphate buffer (pH 7.0), which was also used as the reference buffer. A 10 mm path length quartz cuvette was used for detection. To acquire the thermal unfolding curve, the samples were heated from 25 to 85 °C with stirring. The temperature of the samples and the ellipticity at 270 nm (for β-conglycinin) or 283 nm (for glycinin) were recorded.

Size Exclusion Chromatography with Low-Angle Light Scattering. The SEC experiments were conducted using a TSK-Gel G4000SW$_{XL}$ column, 7.8 × 300 mm (Tosoh Corp., Tokyo, Japan), attached to a Waters HPLC system (Waters Corp., Milford, MA, USA; model 1525). A solution containing 50 mM phosphates and 50 mM NaCl at pH 7.0 was used as an eluent buffer. After centrifugation at 10000*g* for 10 min, samples of 20 μL were injected into the column, and the flow rate was 0.5 mL/min. Detection was conducted using an absorbance detector (Waters Corp. model 2487) operating at 280 nm. A Viscotek 270 dual LALS detector (Viscotek Corp., Houston, TX, USA) combined with a differential refractive index (RI) detector (Waters Corp. model 2414) was used to quantify the weight-average molecular mass (M_w) for monomer and soluble aggregates. The RI detector was also used as the concentration detector. The M_w of all samples was calculated using the refractive index increment (dn/dc) of 0.195 mL/g. All data from LALS were processed with OmniSEC software (Malvern Instruments, Worcestershire, U.K.; version 4.7.0).

Dynamic Light Scattering. DLS was adapted to determine hydrodynamic radii for characterizing the growth of the soy protein particles. The measurements were performed using a Nano ZS Zetasizer instrument (Malvern Instruments), which used backscattering detection at an angle of 173°. Samples were diluted to 0.5 wt % with 20 mM sodium phosphate buffer (pH 7.0) before loading to the cuvette (PCS8501). All measurements were carried out at 25 °C in triplicate. A refractive index of 1.450 was used for dispersion (protein) and one of 1.331 for the continuous phase (20 mM sodium phosphate buffer, pH 7.0). The hydrodynamic radius (R_h) was calculated using a cumulant analysis of the autocorrelation function.

Small Angle X-ray Scattering. SAXS experiments were performed using a SAXSess camera (Anton-Paar, Graz, Austria) to collect information about the particle size and shape of the samples. A PW3830 X-ray generator with a long fine-focus sealed glass X-ray tube (PANalytical) was operated at 40 kV and 50 mA. A focusing multilayer optics and a block collimator provide an intense monochromatic primary beam (Cu Kα, $\lambda = 0.1542$ nm). A semitransparent beam stop enables measurement of attenuated primary beam at zero scattering vector. After specific heat treatment, the samples with a concentration of 1.0 wt % were filled into a capillary of 1 mm diameter and 0.01 mm wall thickness. The capillary was placed in a TCS 120 temperature-controlled sample holder unit (Anton Paar) along the line-shaped X-ray beam in the evacuated camera housing. The sample-to-detector distance was 261.2 mm, and the temperature was kept at 26.0 °C. The 2D scattered intensity distribution recorded by an imaging-plate detector was read out by a Cyclone storage phosphor system (Perkin-Elmer, Waltham, MA, USA). The 2D data were integrated into the one-dimensional scattering function $I(q)$ as a function of the magnitude of the scattering vector q ($q = 4\pi \sin \theta/\lambda$, where 2θ is the scattering angle). Each measurement was collected for 30 min. All $I(q)$ data were normalized so as to have the uniform primary intensity

at $q = 0$ for transmission calibration. The background scattering contributions from capillary and solvent were corrected. For compact protein states, the radius of gyration R_g was determined according to the Guinier approximation

$$I(q) = I(0)\exp(-R_g^2 q^2/3) \quad (1)$$

where $I(q)$ is the scattering intensity and $I(0)$ is measured at zero angle. The Guinier region of SAXS patterns is taken in a q range such that $qR_g < 1.3$.

Surface Hydrophobicity. Protein surface hydrophobicity was determined by titration with ANS according to the method of Liu et al.[38] with modifications as described below. Samples (500 μL), which were diluted to 0.02 wt % with 20 mM sodium phosphate buffer (pH 7.0), were loaded in a square quartz cell (path length = 3 mm) and placed in an F7000 fluorescence spectrophotometer (Hitachi Co., Tokyo, Japan). Aliquots (10 μL) of ANS (5.0 mM in 20 mM sodium phosphate buffer, pH 7.0) were titrated to reach a final concentration at 0.1 mM for ANS. The molar coefficient (5000 M^{-1} cm^{-1} at 350 nm) was used to calculate ANS concentration.[38] The fluorescence intensity was measured by excitation at 390 nm and emission at 470 nm. Excitation and emission slits were both 5 nm. All determinations were conducted in triplicate. Data were elaborated using the Lineweaver−Burk equation

$$1/F = 1/F_{max} + (K_d/F_{max})(1/L_0) \quad (2)$$

where L_0 is the concentration of ANS (μM), F_{max} is the maximum fluorescence intensity (at saturating probe concentration), and K_d is the apparent dissociation constant of a supposedly monomolecular protein/ANS complex. F_{max} and K_d can be obtained by standard linear regression fitting procedures. The ratio F_{max}/K_d, corrected for the protein content, represents the protein surface hydrophobicity index (PSH), taking into account both the number and affinity of hydrophobic sites.

Statistics. Analysis of variance (ANOVA) of the data was performed, and a least significant difference (LSD) test with a confidence interval of 95% was used to compare the means.

RESULTS AND DISCUSSION

Thermal Unfolding of β-Conglycinin and Glycinin. In general, thermal aggregation is thought to be the process that the denatured proteins self-assemble into oligomers or polymers in the presence of heat. According to the LENP model,[25−27] the first stage of this procedure is the conformational change of the protein molecules. nDSC was used to monitored the thermal unfolding of β-conglycinin and glycinin. Figure 1 shows the nDSC thermograms of β-conglycinin and glycinin at pH 7.0. The T_m, which was thought to be the denature temperature of protein, and ΔH_m are also summarized in Figure 1. The two globulins both exhibited one main heat adsorption peak, which had a T_m value at about 60.51 °C for β-conglycinin and 75.74 °C for glycinin. As shown in previous papers,[39−43] T_m values of β-conglycinin at neutral pH ranged from 68.0 to 79.4 °C and from 88.0 to 96.4 °C for glycinin. The results we obtained from nDSC were noticeably lower than these published values. Most of these results were obtained via standard DSC equipment, which required the samples to have a relatively high protein concentration to reach the detection limit. Ten weight percent soy protein dispersion, or even 20 wt %, was often employed in these reported experiments. Interactions between the protein molecules played an important role in such a high concentration. Thus, these results might only reflect the thermodynamic profiles of the soy proteins with high concentration. In addition to having a better sensitivity, nDSC with a continuous capillary sample cell is designed to minimize the contribution of aggregation. This suggested that the results obtained by nDSC might be convincing for the cases of low protein concentration.

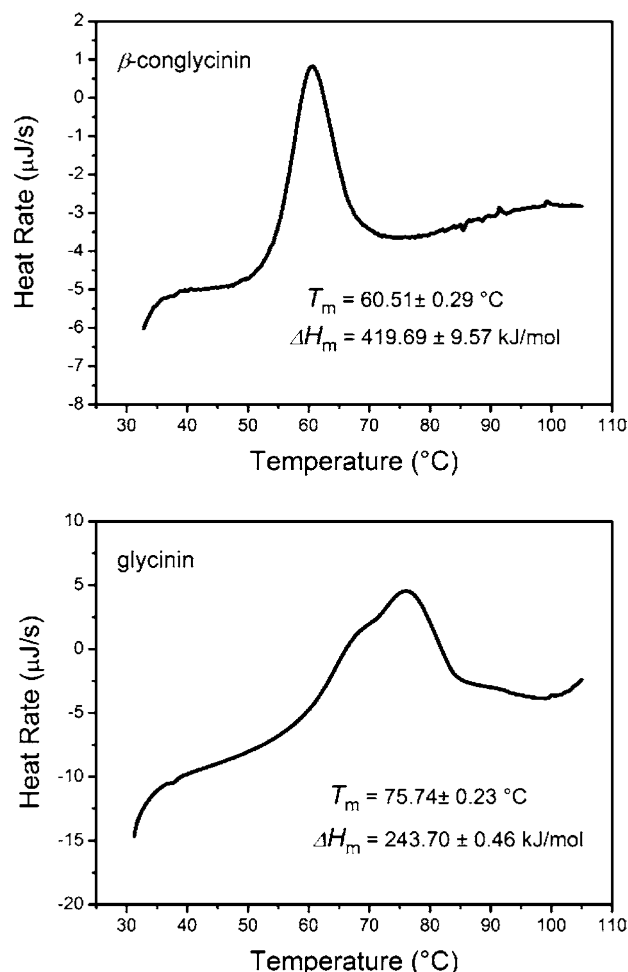

Figure 1. nDSC thermograms of β-conglycinin and glycinin (0.1 wt %) at pH 7.0.

Whereas results from nDSC indicated the thermal transition of the protein quaternary structure, the CD spectra collected in the near-UV region offer information that is related to the protein tertiary structure. To provide further supporting data for revealing the thermal unfolding kinetics of soy proteins, the online observation of the ellipticity at a specific wavelength during the heating process was performed (Figure 2). The maximum ellipticity peaks of β-conglycinin and glycinin CD spectra were 270 and 283 nm in the near-UV region, respectively. Although no exact denature temperatures were received from this thermal unfolding curve, a sudden change of the structure was recorded from about 44 to 67 °C, as shown in Figure 2 for β-conglycinin. This region is similar to the endothermic unfolding region detected by nDSC (about 50−70 °C). For glycinin, the region in which a significant change of tertiary structure was observed ranged from about 65 to 80 °C. This is also similar to the region measured by nDSC (about 60−85 °C). The thermal transition regions gained from the two different techniques were in agreement that β-conglycinin and glycinin might unfold at lower temperatures than those reported before.

Figure 2 also reflects the conformational difference between β-conglycinin and glycinin during heating. When heated over 80 °C, the ellipticity of glycinin was hard to detect. This

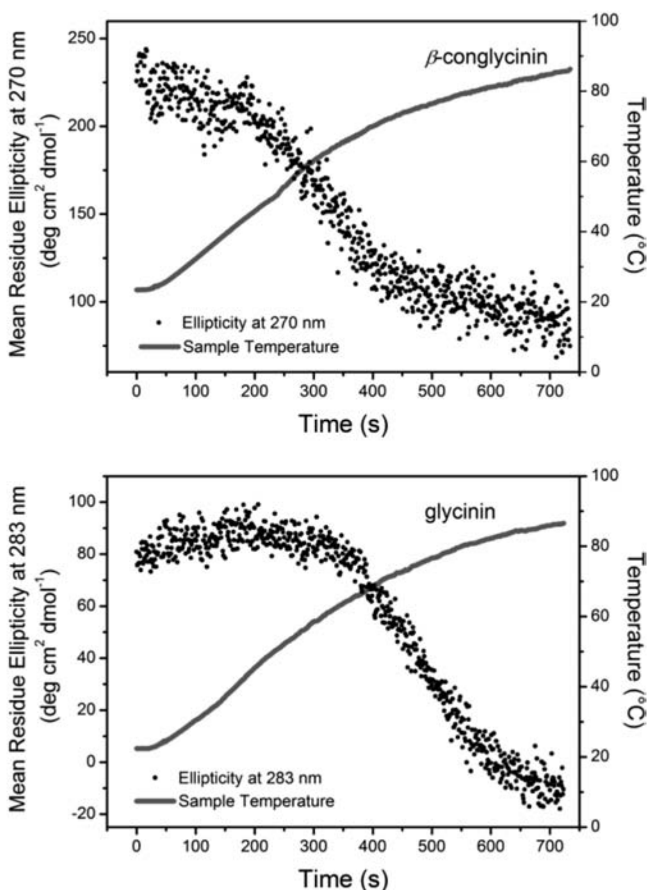

Figure 2. Thermal unfolding kinetics of β-conglycinin and glycinin monitored by CD ellipticity at 270 and 283 nm.

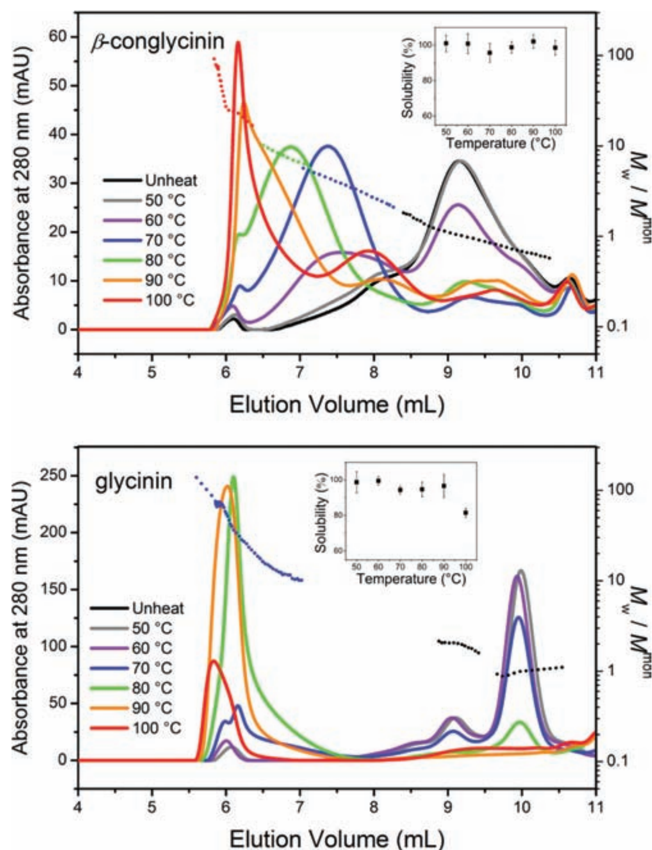

Figure 3. Chromatograms (solid lines) and corresponding M_w/M_{mon} profiles (dotted lines) for 1.0 wt % β-conglycinin and glycinin dispersions that were incubated at different temperatures for 30 min. (Inset) Protein solubility of β-conglycinin and glycinin dispersions after heating at different temperatures.

indicated that flexible or disordered conformations were commonly found in glycinin when heated. Unlike glycinin, β-conglycinin behaved in another way. The ellipticity still maintained a considerable value after a significant decline, indicating that some ordered conformation was preserved during heating. This result was in consistent with the variation in ΔH_m shown in Figure 1. β-Conglycinin had a higher ΔH_m than glycinin. These results demonstrated that compared to β-conglycinin, the conformation of glycinin could be disturbed more easily.

Thermal Aggregation Kinetics. The chromatograms of β-conglycinin and glycinin from SEC are shown in Figure 3. A dominant peak at the elution volume of 9.2 mL was observed in the unheated sample of β-conglycinin. The M_w of this peak determined with LALS was 340 kDa (M^{mon} for β-conglycinin; M^{mon}, the M_w of the monomer). This was consistent with published reports[10,11] that β-conglycinin existed as a 9S form with an estimated molecular mass of 370 kDa at neutral pH in the low ionic solutions. Thus, this fraction was regarded as the monomer form in this study. According to the nDSC and online CD observation (Figures 1 and 2), the transition of the β-conglycinin conformation occurred at about 60 °C. The sample heated below this temperature (50 °C) had a chromatogram similar to the unheated one. Changes of the chromatogram were found in the sample heated at 60 °C. The monomer began to reduce, and the dimers and trimers were detected. Further reducing of the monomer and the increase of the oligomers were displayed in the sample heated at 70 °C. With the increase of the heating temperature, the major peak representing the aggregates moved forward, suggesting that the molecular mass of the aggregates continued to increase. When heated at 100 °C, aggregates with molecular mass over 10 times larger than the monomer were acquired.

For glycinin, a dominant peak that was eluted at approximately 10.0 mL was observed in the unheated sample (Figure 3). This fraction had an M_w of 349 kDa (M^{mon} for glycinin), which was in agreement with the report that glycinin existed as a hexamer with molecular mass of about 360 kDa at neutral pH.[12] Therefore, this fraction was thought to be the monomer form of glycinin. A small amount of dimer was also observed in the unheated sample. In consonance with β-conglycinin, samples heated below the denaturation temperature reported from nDSC and online CD observation (Figures 1 and 2) had chromatograms similar to the unheated one. With heating at 70 °C, the glycinin molecules began to unfold. Monomer of glycinin reduced, and a few aggregates were observed in this sample. A large quantity of aggregates with high molecular mass and a small amount of monomer were detected when samples were heated at 80 °C. The amounts and molecular mass of the aggregates continued to increase in the sample heated at 90 °C. Few monomers could be detected in this sample. Interestingly, the peak that corresponded to the aggregates fell rather grew when samples were heated at 100 °C.

In the case of β-conglycinin, the aggregates kept growing and the protein solubility of all heated samples was almost unchanged during heating (Figure 3). Aggregates with high

Table 1. Structural Parameters of Soy Protein Heated at Different Temperatures Derived from SAXS and DLS Data

soy protein	R_g (SAXS) (nm)	R_h (DLS) (nm)	R_g/R_h	$I(0)$	fractal dimension
7S					
unheated	4.97 ± 0.14	13.55 ± 0.06	0.37	1.37 ± 0.01	1.24
60 °C	6.11 ± 0.19	14.76 ± 0.24	0.41	1.88 ± 0.02	1.52
80 °C	8.06 ± 0.35	18.39 ± 0.13	0.44	3.38 ± 0.04	2.06
100 °C	8.74 ± 0.37	21.50 ± 0.02	0.41	3.68 ± 0.03	2.16
11S					
nnheated	5.83 ± 0.03	26.02 ± 0.01	0.22	2.60 ± 0.02	1.32
60 °C	6.36 ± 0.36	36.16 ± 0.70	0.18	2.89 ± 0.02	1.42
80 °C	9.78 ± 0.04	46.43 ± 0.66	0.21	7.30 ± 0.09	2.69
100 °C	10.97 ± 0.05	119.45 ± 0.14	0.09	8.64 ± 0.11	3.24

molecular mass remained in the supernatant after heating. These aggregates were regarded as soluble aggregates. For glycinin, a decline of the protein solubility was recorded in the sample heated at 100 °C (Figure 3). This explained why the total peak area of this sample was much less than the others in the chromatogram. Part of the aggregates with huge molecular mass in this sample was not able to suspend in the supernatant. These aggregates were thought to be insoluble aggregates. Overgrowth might be the reason for the formation of these insoluble materials.

As the LENP model described,[25−27] protein aggregation included five stages: 1, conformational change; 2, prenucleation; 3, nucleation; 4, polymerization; and 5, condensation. At stages 2 and 3, oligomers might form. As shown in Figure 3, β-conglycinin oligomers were detected in the samples that were heated at 60, 70, and 80 °C. This indicated that the β-conglycinin molecules were in the stage of nucleation when heated below 80 °C. At stage 4 mentioned in LENP model,[25−27] aggregates grew via monomer addition. After the free monomers were run out, aggregation came to stage 5. Interactions between the aggregates with high molecular mass replaced interaction between the aggregates and the monomers. Although aggregates with great molecular mass were found in the sample heated at 100 °C, β-conglycinin monomers were still detected. These monomers might be able to take part in the further polymerization, if necessary. The interaction between the aggregates might not play a dominant role, suggesting that stage 5 did not happen to β-conglycinin. Because the aggregation of β-conglycinin stopped at stage 4, the growth of its aggregates was limited. This made β-conglycinin maintain good solubility during the heating process. In contrast, monomers of glycinin dramatically diminished when heated above the denature temperature (about 75 °C). They were used to produce aggregates and run out when heated at 90 °C. This implied that the aggregation of glycinin came to stage 5 after heating at 90 °C. When heated at 100 °C, aggregates instead of monomers took part in the assembly of the bigger aggregates. The unlimited aggregation and constant growth of the aggregates made them too big to stay in the supernatant after heating. Therefore, insoluble aggregates were present in the glycinin sample that was heated at 100 °C.

Structure Analysis of the Soy Protein Aggregates. As mentioned above, β-conglycinin and glycinin aggregated via different pathways during heating. With different aggregation behaviors, aggregates possessing different solubilities were observed. SAXS and DLS were used to characterize the particle size and shape of the soluble and insoluble aggregates. A summary including the radius of gyration R_g, scattering intensity at zero angle $I(0)$, and fractal dimension of the aggregates determined by SAXS as well as the hydrodynamic radius R_h determined from DLS is given in Table 1. The values of $I(0)$ given in this paper have been corrected by protein concentration, and they are proportional to the molecular mass of the scattering particles. The particle size of the aggregates could be learned from the values of R_g and R_h. The data listed in Table 1 reveal the extent of both β-conglycinin and glycinin aggregation increases as the temperature is elevated. This can be seen in the increases of $I(0)$, R_g, and R_h, which are sensitive to larger aggregates when heated. It is obvious that glycinin had a greater degree of aggregation than β-conglycinin when heated over the denaturing temperature. This once again confirmed that no significant growth of the β-conglycinin and glycinin particles occurred until they were heated beyond their conformational transition temperature.

The ratio of R_g to R_h is frequently used to characterize polymeric architectures. A qualitative, approximate interpretation for the shapes of the aggregates can be provided by this ratio. The value of R_g/R_h is expected to be 0.775 for hard-type spheres.[44] Polymers with unfolded structure could possess higher values. Here the R_g/R_h values of all samples we measured were all well below this value. Although this might also suggest the presence of compact objects with possible structures, the estimation of R_g/R_h from the combination of SAXS and DLS data might not predict the exact shapes of the aggregates. However, it was still possible to predict the conversion trend of the particle shape from the value of R_g/R_h obtained in this study. For β-conglycinin, R_g/R_h was essentially unchanged. The value of R_g/R_h was almost unchanged when glycinin was heated below 80 °C, but a significant decrease was observed in the sample heated at 100 °C when the insoluble materials appeared. The value of R_g/R_h shared the same trend with the protein solubility of β-conglycinin and glycinin after heating. As aggregation stayed at stage 3 or 4, monomer addition made the aggregates grow. For both β-conglycinin and glycinin, aggregates with good solubility formed via polymerization had shapes similar to those of the unheated molecules. Changes of both solubility and structure were recorded when glycinin entered stage 5 during heating at 100 °C. The transition of aggregation stage and particle shape affected the protein solubility of this sample. The condensation of the glycinin aggregates occurred. The significant decrease of R_g/R_h also implied that the density of these particles became more inhomogeneous. With the assembly among the aggregates, the polypeptide chains in the center of the particle aggregated more tightly than the chains on the surface, leading to the particles with a denser core and a less dense outer shell. This implied that aggregates with different solubilities might have different densities.

Fractal dimension determined from the slope of the SAXS scattering curve at high angles is directly related to the compactness of the particles. Densely packed structures give rise to a scattering intensity, which decays more steeply than more open structures with a smaller slope.[45] Particles with higher aggregation degree got denser structure. In contrast, β-conglycinin soluble aggregates were assembled in a less compact way, but a much denser structure was found in the insoluble aggregates that originated from glycinin. As a result of the condensation, which was not experienced for the soluble aggregates, particles grew in size and density. Thus, these particles no longer remained in the supernatant, turning into insoluble materials. This was in accordance with the R_g/R_h analysis. The conformational properties of the polypeptide chain can be assessed with SAXS by representing the scattering pattern in a Kratky plot, that is, $q^2 \times I(q)$ versus q.[46] The Kratky plot of a globular protein displays a typical bell shape, a curve with no clear maximum, and a plateau at large angles indicates the lack of tightly packed core and typical random coil structure. Ordered or molten globule-like conformation could be distinguished from the Kratky plots.[46] As shown in Figure 4,

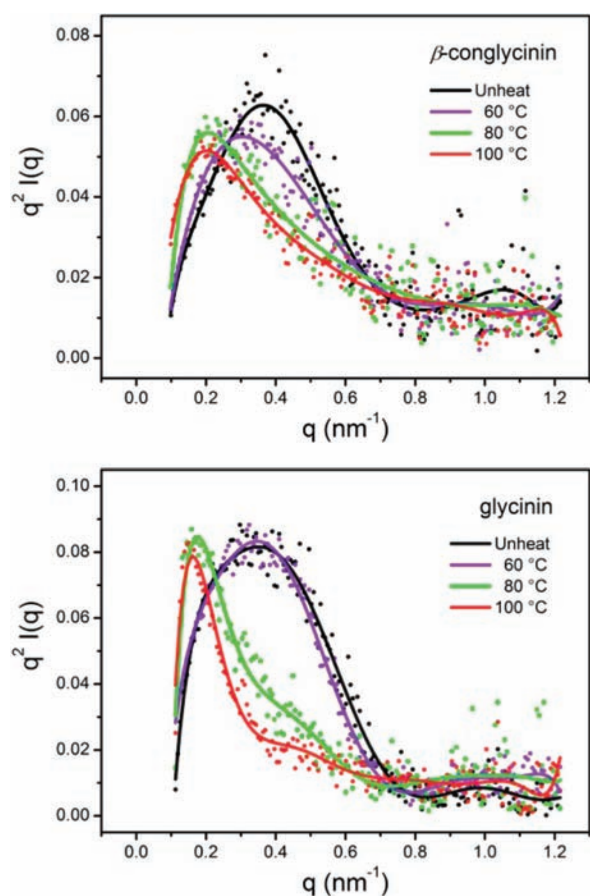

Figure 4. Kratky plots of β-conglycinin and glycinin scattering spectra obtained from SAXS studies.

the curves of all samples displayed a typical bell shape, indicating that both β-conglycinin and glycinin maintain a globular shape after heating. Even though glycinin lost tertiary structure during heating (Figure 2), the core of its aggregates was still tightly packed.

In a summary, there are differences not only in size and molecular mass between soluble and insoluble aggregates but also in the conformation and density. With the increase of radius and density in the core, these aggregates were no longer able to suspend in the supernatant, becoming insoluble matters.

β-Conglycinin and glycinin, the two dominant storage proteins in soybean, show different thermal aggregation behaviors. This may be due to the primary structure of these two types of protein. β-Conglycinin is a trimeric glycoprotein consisting of three subunits, α, α', and β, which are N-glycosylated. The α and α' subunits contain extension regions in addition to core regions common to all subunits.[47] Maruyama and co-workers[47,48] reported that the N-linked glycans and extension regions were not essential for the folding and the assembly into trimmers, but played an important role in preventing protein−protein associations induced by heating. Despite the existence of these hydrophilic groups, thermal aggregation of β-conglycinin was observed in this work. After the unfolding of the polypeptide chains, subunits disassociated, and they reassociated with the hydrophobic interaction that came from the exposed residues. These hydrophobic residues could be considered as active sites for aggregation. If these sites were covered by the sites from another molecule, aggregates were formed. The distance between the protein particles was determined by the struggle between the repulsion contributed by hydrophilic residues and the attraction provided by hydrophobic residues. In the case of β-conglycinin, once the hydrophobic residues were covered and buried in the core of the aggregates, the carbohydrate moieties and hydrophilic groups that were placed on the surface provided enough repulsive force for inhibiting the approach of other monomers and the aggregation was stopped. In other words, the thermal aggregation of β-conglycinin is limited. However, glycinin showed another case. Few glycans that linked to the polypeptide chain were found in glycinin. The repulsive barrier supplied by the acid polypeptides seemed to be not strong enough to prevent the approach of other protein particles. Furthermore, more hydrophobic amino acids than any other subunits of soy proteins were found in the primary structure of basic polypeptides.[49] Once the unfolding occurred, more sites available for aggregation were exposed. Although part of the active sites was covered in the process of aggregation, hydrophobic residues rather than hydrophilic glycan were still located on the surface of the aggregates. The number of active sites did not reduce during heating, leading to the continual aggregation. Therefore, rapid growth and condensation of the aggregates were observed in the thermal aggregation of glycinin.

Effect of β-Conglycinin on the Thermal Aggregation of Glycinin. Formed in different pathways, β-conglycinin and glycinin aggregates had different sizes and structures. As the aggregation behaviors of β-conglycinin and glycinin were characterized, 1.0 wt % glycinin dispersions in the presence of 0.25, 0.50, or 1.0 wt % β-conglycinin were used to investigate the effect of β-conglycinin on the thermal aggregation of glycinin in the following section. Fractions of the samples heated at 100 °C for 30 min were separated by speed differential centrifugation. The protein content of each fraction is shown in Figure 5. Most of the β-conglycinin soluble aggregates with high molecular mass remained in the supernatant even when centrifuged at 20000*g*. These aggregates gradually departed from the supernatant as the centrifuge speed was further elevated. Most aggregates were collected after centrifugation at 800000*g*. The insoluble materials found in the glycinin dispersion (heated at 100 °C) could be isolated easily

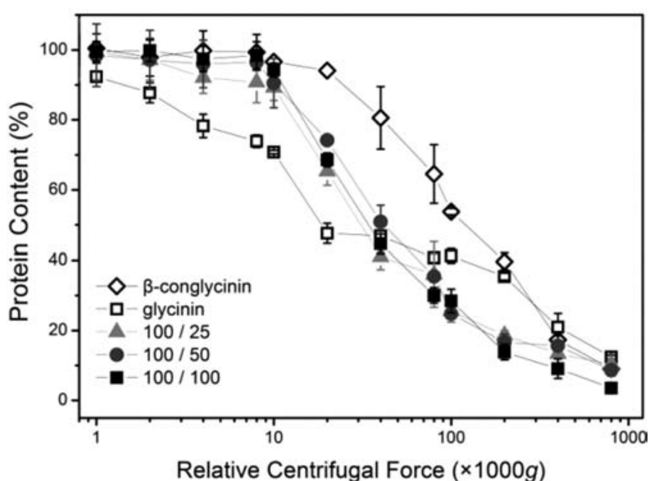

Figure 5. Protein content in supernatant after speed differential centrifugation. β-Conglycinin, glycinin, and their mixtures were incubated at 100 °C for 30 min. The open symbols correspond to the samples with only β-conglycinin or glycinin. The solid symbols correspond to mixtures of glycinin and β-conglycinin of different ratios. Lines are guides for the eye.

by centrifuging at 10000*g*. The remaining aggregates might possess a much smaller particle size distribution, leading to a slight decrease of the protein content in the range from 20000 to 100000*g*. This part of the protein could be obtained when centrifuged at a speed of >100000*g*. However, the ultracentrifuge pattern of heated glycinin was changed with the addition of β-conglycinin. The patterns for the mixtures seemed to be closer to β-conglycinin. A slight decline of protein content instead of a steep decay was seen when centrifuged at a speed of <10000*g*. Compared to β-conglycinin, the mixtures possessed significantly lower protein content after centrifugation at 20000*g*. This might be due to the formation of β-conglycinin−glycinin complexes as confirmed by Figure 6. With greater particle size, the complexes might have a greater centrifugal sedimentation velocity than the β-conglycinin aggregates. These results were in accordance with previous papers.[18,19] With the help of β-conglycinin, the solubility of glycinin was improved. Dissociation of the subunits of β-conglycinin happened with the unfolding of the polypeptides.[50] The preferential interaction between the β subunit and the basic polypeptides, which occurred in the reassociation of the subunits, generated the β-conglycinin−glycinin aggregates with high molecular mass.[20−24]

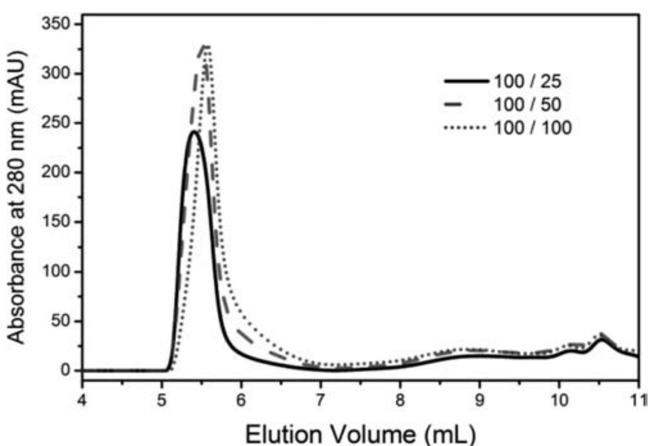

Figure 6. SEC chromatograms of glycinin dispersions with different additions of β-conglycinin after heating at 100 °C for 30 min.

SAXS and DLS were used again to investigate the structure of the β-conglycinin−glycinin aggregates. The conformational information is summarized in Table 2 and Figure 7. The aggregates still show a globular shape when glycinin is heated with β-conglycinin (Figure 7). After heating at 100 °C, densely packed aggregate particles with an R_h of nearly 120 nm were formed in the glycinin dispersion. With the increase of β-conglycinin, a decrease of the particle size was noted. Meanwhile, the aggregation extent and compactness of the particles also decreased. This implied that the complex aggregates were smaller and possessed more flexible structures with the addition of β-conglycinin. In other words, the more β-conglycinin, the more the complex particles behaved like β-conglycinin aggregates. As mentioned previously, active sites for aggregation were exposed with the unfolding of the polypeptide chains during heating. When heated alone, interactions of these sites occurred between the β-conglycinin chains or the glycinin chains. When heated together, interactions between the β-conglycinin and the glycinin chains might happen. In the presence of β-conglycinin, what covered the surface of the glycinin aggregates was no longer a hydrophobic residue after another. The hydrophilic groups brought by the β-conglycinin might occupy the surface of the complex aggregates, leading to the termination of the condensation among the aggregates that was supposed to happen between the aggregates. Because the aggregation was restricted by β-conglycinin, the growth of particles was also limited. In such circumstances, soluble aggregates rather than insoluble aggregates were formed in the dispersion. This is schematically illustrated in Figure 8.

Interaction between the subunits that led to the formation of the β-conglycinin−glycinin complexes was still in doubt. Electrostatic attraction, linking via disulfide bonds, or hydrophobic interaction was thought to account for the combination in different studies.[18−20,22,23] The understanding of this interaction could help to enhance the ability of β-conglycinin that improved the solubility of the heterogeneous proteins in thermal processing. To evaluate this ability, basic polypeptides of glycinin with the worst solubility in soy proteins were selected as the object for improvement. Ten millilmolar dithiotreitol (DTT) was added to the glycinin dispersion, cleaving the disulfide bonds that linked acid and basic polypeptides during heating. The dissociation of basic polypeptides made the dispersion become milky. As described above, β-conglycinin had different molecular mass distribution, particle size, and conformation after heating at different temperatures (Figure 3 and Table 1). The decline of the turbidity to various degrees was recorded when different preheated β-conglycinins were added to the basic polypeptide dissociation dispersions (Figure 9). This suggested that β-conglycinin with different properties had different effects on improving the solubility of basic polypeptides. PSH of β-conglycinin heated at different temperatures is also shown in Figure 9. With heating at 50 °C, the unfolding of β-conglycinin did not happen and the PSH was similar to that of the unheated sample. A substantial increase of PSH appeared when β-conglycinin was heated beyond the denaturing temperature. As aggregation happened, some hydrophobic residues were covered and buried, resulting in the decrease of F_{max} and PSH when heated over 70 °C. The change in turbidity of the samples shared the same trend with F_{max} and PSH of β-

Table 2. Structural Parameters of Glycinin and β-Conglycinin Mixtures Heated at 100 °C for 30 min Derived from SAXS and DLS Data

11S/7S	R_g (SAXS) (nm)	R_h (DLS) (nm)	R_g/R_h	$I(0)$	fractal dimension
100/25	9.93 ± 0.05	56.05 ± 0.35	0.18	6.53 ± 0.09	2.76
100/50	9.70 ± 0.05	47.25 ± 0.12	0.21	6.05 ± 0.08	2.49
100/100	9.02 ± 0.05	42.10 ± 0.10	0.21	4.78 ± 0.07	2.27

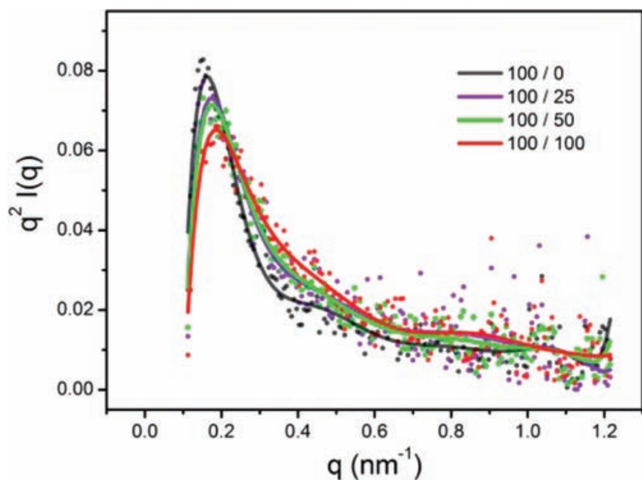

Figure 7. Kratky plots of glycinin and β-conglycinin mixture (heated at 100 °C for 30 min) scattering spectra obtained from SAXS studies.

conglycinin. The ability that improved the solubility of heterologous protein might be related to the structure of β-conglycinin. More flexible structure and more exposed hydrophobic groups allowed β-conglycinin to attach to the target protein more easily. Surrounded by more hydrophilic groups, the aggregation of the target protein was restricted in a more effective way. This implied that hydrophobic interaction might be of great importance in the interaction between β-conglycinin and glycinin during heating.

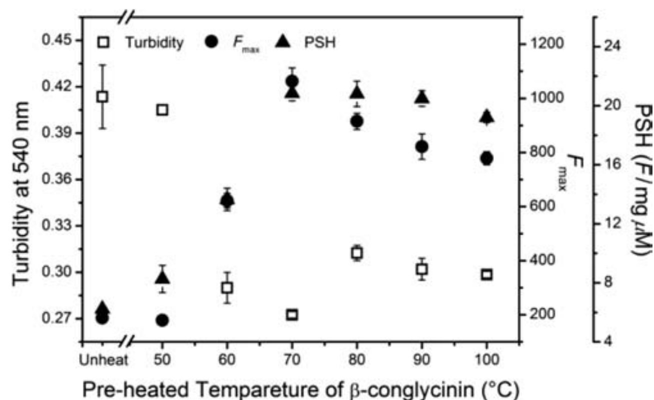

Figure 9. Turbidity of the glycinin dispersions in the presence of the preheated β-conglycinin and PSH of β-conglycinin heated at different temperatures. 2.0 wt % β-conglycinin was preheated at different temperatures for 30 min. 1.0 wt % glycinin dispersions with 1.0 wt % preheated β-conglycinin and 10 mM DTT were heated at 100 °C for 30 min.

AUTHOR INFORMATION

Corresponding Author

*Phone: +86 20 87114262. Fax: +86 20 87114263. E-mail: fexqyang@163.com or fexqyang@scut.edu.cn.

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Notes

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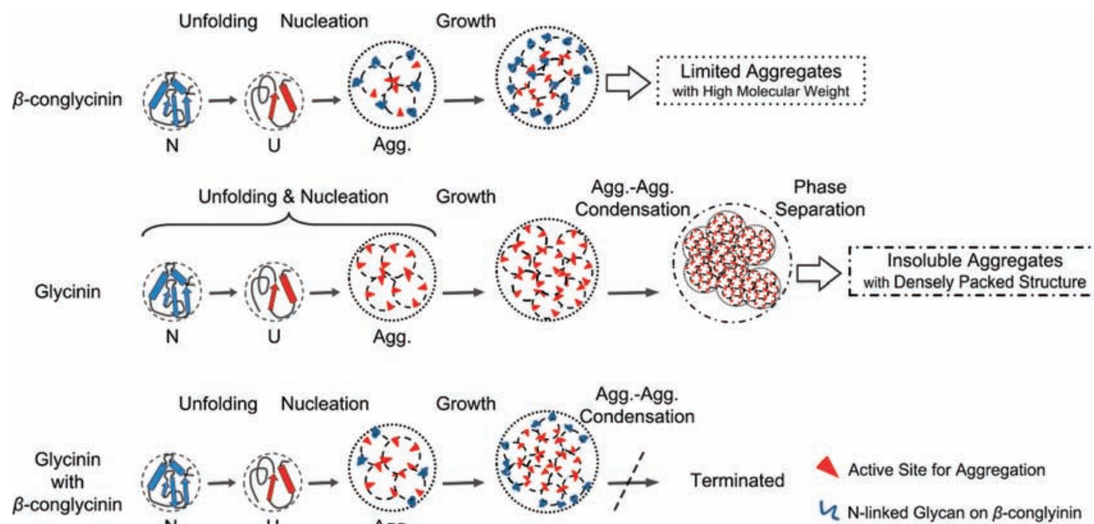

Figure 8. Diagrammatic depiction of β-conglycinin and glycinin thermal aggregation behavior at pH 7.0. N, native state; U, unfolded state; Agg., aggregates.

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