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ARTICLE

Formation and Characterization of Amyloid-like Fibrils from Soy β-Conglycinin and Glycinin

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The fibrillar aggregation at pH 2.0 of soy β-conglycinin, glycinin, and the 1:1 mixture thereof, induced by heating at 80 °C for various periods of time, was investigated using Th T and Congo Red spectroscopic techniques. The morphology of the formed fibrillar aggregates was characterized using atomic force microscopy (AFM), whereas the conformational changes and the polypeptide hydrolysis of the proteins upon heating were also evaluated. Th T fluorescence analysis indicated that β-conglycinin had a much higher potential to form heat-induced amyloid-like aggregates than glycinin. AFM analyses showed that all of the soy globulins could form twisted screw-structure fibrils with heights of 1.4−2.2 nm, but the morphology of the amyloid-like fibrils considerably varied among various soy proteins. Significantly lower width at half-heights and higher coil periodicity values were observed for the β-conglycinin fibrils than the glycinin counterpart. Far-UV CD spectral analysis indicated that upon heating, the secondary conformations of the proteins changed considerably, especially during initial heating (e.g., <4 h), and the changes were much more distinct in the β-conglycinin case than in the glycinin case. Furthermore, reducing electrophoresis analyses indicated that progressive polypeptide hydrolysis occurred upon heating, but the polypeptide hydrolysis for the β-conglycinin was much more severe than that of glycinin. The data suggest that soy β-conglycinin exhibited a much higher potential to form thermally fibrillar aggregates than glycinin, and the differences seem to be mainly associated with the differences in their conformational changes and extent of polypeptide hydrolysis by the heating. The results would be of vital importance for the utilization of soy proteins to produce thermally induced fibrillar gels with excellent properties.

KEYWORDS: Soy protein; β-conglycinin; glycinin; fibrillar aggregate; amyloid fibril; assembly

INTRODUCTION

Recently, the assembled structures of food globular proteins, especially fibrils with a thickness of ~4 nm and a length between 1 and 10 μm, have attracted much attention due to their potential applications in the food industry, for example, as a weight-effective thickeners for food products (*1−3*). These self-assembled fibrils are generally induced by heat treatment at acidic pH (e.g., pH 2.0 in particular) and at low ionic strengths, at which the proteins would be positively charged and linear aggregates favorably occur. The most investigated food proteins to form fibrils are whey proteins and β-lactoglobulin in particular (*3−9*). It is now appreciated that the fibrillar structures are usually constructed in a multistep process from "partially folded still globular entities", which vary with the nature or properties of assembled fine strands or fibrils. Even the fibril formation itself at low pH and low ionic strength also involves a multistep step; for example, Arnaudov and colleagues proposed a model of multi-steps during the β-lactoglobulin fibril formation at low pH and ionic strength, including reversible formation of linear aggregates and a slow process of "consolidation" (*3*).

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Another reason for the interest in protein fibril research, from the point of view of the food industry, is that food protein fibrillar gels based on the fibril assembly usually exhibit some unique textural, structural, and sensory characteristics (*1*, *10*). Under pH and ionic strength similar to those of fibrils, the fibrillar gels for a globular protein can be formed if the protein concentration exceeds a given critical concentration (*11*). To date, the rheological and structural properties of fibrillar gels formed by heating at acidic pH (e.g., pH 2.0) and low ionic strength from many food globular proteins, including whey proteins (and β-lactoglobulin, in particular), bovine serum albumin, ovalbumin, and egg white lysozyme have been well recognized (*3*, *5*, *12−17*). The fibrillar gels are usually transparent and isotropic, and the turbidity increases with increasing ionic strength. The elasticity or elastic modulus (G') of heat-set fibrillar gels for a globular protein can be well described using a percolation model, especially the temperature and protein concentration dependence of the elasticity, far from the isoelectric point and at low ionic strength (*18*).

In contrast, the formation and properties of fibrillar gels of plant oligomeric proteins are to a much lesser extent investigated than those of whey proteins. One major difference between plant oligomeric proteins and whey proteins is their complexity of tertiary and/or quaternary structure, which clearly leads to the

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more difficult understanding of the fibril assembly. Glycinin and β-conglycinin are the major globulins in soy proteins, which usually account for about 70% of total protein. Native glycinin (11S globulin; hexamers) is an oligomeric protein having a molecular weight (MW) of approximately 350 kDa and consisting of six subunits $(AB)_6$, of which one acidic (MW 37−42 kDa) (A) polypeptide and one basic (MW 17−20 kDa) polypeptide are linked by a disulfide bridge. β-Conglycinin (7S globulins) is a kind of trimer composed of three major subunits (α', α, and β) associated in various combinations by noncovalent interactions (*19, 20*). Due to increasing interest in soy protein-containing products, it would be interesting to investigate the formation and properties of fibrillar gels of soy proteins, induced by heat treatment.

In a recent work, Akkermans and colleagues (*9*) reported that when heated at pH 2.0 and at low ionic strength, soy glycinin (11S globulin) and soy protein isolate could be transformed into long, fibrillar semifexible aggregates, with a contour length of 2.3 μm and a thickness of a few nanometers, and most properties (e.g., flow properties) of the formed soy glycinin fibrils were even comparable to those of β-lactoglobulin fibrils. In this work, the underlying mechanism for fibril formation from soy glycinin at pH 2.0 is not addressed, and there is also no information about fibril formation from soy β-conglycinin. In another previous work, it was shown that heating at 100 °C and pH 7.6 also led to the formation of fibrous aggregates from β-conglycinin, with a height (diameter) of 8−11 nm (*21*). The nature of the formed fibrillar aggregates at pH 7.6 is distinctly different from those formed at low pH and low ionic strength (*21*).

More recently, in our laboratory, we investigated the thermal gelation of phaseolin (7S globulin)-rich kidney protein isolate at pH 2.0 and at low ionic strengths (0−300 mM) and found that heating at a temperature above the denaturation temperature of phaseolin resulted in the formation of wormlike fibrillar aggregates and a contour length highly dependent on the applied ionic strength (*22*). In a further work, we characterized the formation of amyloid-like fibrils from purified phaseolin at 85 °C and at pH 2.0, using transmission electron microscopy (TEM), atomic force microscopy (AFM), and spectroscopic techniques, and found that the morphology of the formed fibrils was closely dependent upon the heating time, and the heating resulted in remarkable conformational changes of the protein, as well as polypeptide hydrolysis (*23*).

The main objective of this work was to investigate and compare the formation of heat-induced fibrils from soy glycinin, β-conglycinin, and their mixture (1:1, w/w), at pH 2.0 and low ionic strength, and characterize the properties of these formed fibrils. AFM and circular dichroism (CD) were applied to characterize the morphological and conformational changes of the proteins during the fibril formation, and fluorescence techniques using thioflavin T (Th T) and Congo Red dyes were used to evaluate the formation of highly ordered amyloid-like fibrils. The hydrolysis or disruption of the polypeptides of soy globulins during heating at pH 2.0 was also characterized using gel electrophoresis. The underlying mechanism of the fibril formation for soy globulins was proposed.

MATERIALS AND METHODS

Materials. Defatted soy flakes with low extent of protein denaturation, obtained by flash desolventization and then drying at 60 °C under vacuum, were provided by Shandong Yuwang Industrial & Commercial Co., Ltd. The flakes were ground in a Straub mill (model 4E, Straub Co., Philadelphia, PA) to pass through an 80 mesh sieve. The obtained flour was stored in a sealed container at 4 °C until used. The protein content of the flour was 55.5 ± 0.4% (determined by Kjeldahl method with a nitrogen conversion factor of 6.25; on a dry basis) and the nitrogen solubility index 84.0%. Congo Red and Th T, as well as bovine serum albumin (BSA), were purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals applied in the work were of analytical grade.

Preparation of Soy β-Conglycinin and Glycinin. The soy β-conglycinin and glycinin were prepared according to the method of Nagano and others (*24*), with a few modifications. The defatted soy flour was mixed with a 15-fold volume of distilled water, and then the pH of the dispersion was adjusted to 7.5 using 0.5 M NaOH. The supernatant containing the extracted soy proteins was obtained by centrifugation at 9000*g* for 30 min. Sodium bisulfite was then added to the supernatant and the pH adjusted to 6.4 using 0.5 M HCl; the mixture was kept in an ice bath overnight. The precipitate, mainly composed of the glycinin component, was obtained by centrifugation (6500*g*, 20 min) at 4 °C. The pH of the resultant supernatant was further adjusted to pH 5.0 using 0.5 M HCl and, simultaneously, NaCl was gradually added to reach a concentration of 0.25 M. The insoluble was removed by centrifugation as mentioned-above. The last obtained supernatant was diluted 2-fold with ice-cold water and the pH adjusted to pH 4.8, and the precipitate mainly containing β-conglycinin was obtained by centrifugation (6500*g*, 20 min) at 4 °C. The obtained β-conglycinin and glycinin fractions were washed with cold water and dispersed (with the pH adjusted to 7.5) and then freeze-dried to produce the β-conglycinin and glycinin samples.

Thermal Aggregation Experiments. Various soy globulin solutions with a protein concentration of 1.0% (w/v) at pH 2.0 were prepared by dissolving the freeze-dried samples into HCl solution (about 0.1 M) of pH 2.0 and then accurately adjusting the pH of the solutions to 2.0 with 6 M HCl. The protein concentrations of the solutions were determined at 750 nm according to Lowry's method (*26*), with BSA as the protein standard. Aliquots (about 5 mL) of the protein solutions were put into tubes with hermetic lids. The tubes were heated at 80 °C for specific periods of time, in a temperature-controlled bath with a temperature deviation of <1 °C. After the heat treatment, the samples were immediately cooled in an ice bath and directly subjected to further experiments.

Determination of Apparent Viscosity (η). Flow curves were evaluated on a stress-controlled HAAKE RS600 rheometer (HAAKE Co., Karlsruhe, Germany) with parallel plates (d = 27.83 mm). The protein dispersions were placed between two parallel plates, and the gap between two plates was set to 1.0 mm. Steady shear apparent viscosities were measured for shear rate increasing from 0.01 to 100 s^{-1}. Each shear rate was applied for 30 s up to a shear rate of 1 s^{-1}. Each data point was obtained when a steady state was reached after 30 s.

Th T Fluorescence Spectral Analysis. Th T stock solution was prepared by dispersing 8 mg of Th T into 10 mL of phosphate buffer (pH 7.0) containing 150 mM NaCl (*26*). The dispersion was filtered with a 0.2 μm syringe filter to remove undissolved Th T. This stock solution should be stored in the dark at 4 °C and covered with foil. The stock solution was diluted 50 times in the same buffer on the day of analysis to generate the working solution (*27, 28*). Aliquots (50 μL) of the tested samples were mixed with 5 mL of Th T working solution and allowed to stand for at least 1 min. The fluorescence spectra of the mixtures were measured using a fluorescence spectrophotometer (Hitachi F-4500, Tokyo, Japan). The excitation wavelength was 460 nm (slit width = 10 nm), and the emission wavelength was 490 nm (slit width = 5 nm), with a scanning speed of 240 nm/min. The excitation wavelength at 460 nm (rather than at 440−450 nm as applied in many previous studies) was chosen to minimize light-scattering problems (*29*). The fluorescence spectrum of the Th T working solution was subtracted from the fluorescence spectra of the samples to correct the background signal.

Congo Red Spectral Analysis. Congo Red stock solution (10 mM) was prepared by dissolving the dye in 10 mM potassium phosphate buffer (pH 7.4) containing 150 mM NaCl under continuous stirring. It was then filtered with a 0.2 μm Millipore filter. A fresh working solution was prepared by diluting the stock solution 100 times. Aliquots (100 μL) of the samples were mixed with 1 mL of the working solution and kept at 25 °C for 15 min. The visible spectra (in the range of 400−600 nm) of the mixtures, in 1 cm path length cuvettes, were recorded with a scan rate of 120 nm per minute on a UV−vis spectrophotometer (Shimadzu 1800, Kyoto, Japan). The differential spectra were obtained by subtracting the blank spectrum from the sample spectra.

Atomic Force Microscopy. AFM images were acquired in tapping mode using a Dimension 3000 microscope (Digital Instruments-Veeco, Santa Barbara, CA), equipped with a 'G' scanning head (maximum scan size = 10 μm) and driven by a Nanoscope IIIa controller. A 2 μL droplet of heated soy protein dispersions (diluted to 25 μg/mL) was spread on a freshly cleaved mica disk and allowed to dry in air for 10 min at ambient temperature (*30*). For imaging in air, single-beam uncoated silicon cantilevers (type OMCL-AC, Olympus, and RTESP, Veeco) were used. The drive frequency was around 300 kHz, and the scan rate was between 0.3 and 0.8 Hz. Images were treated with Digital Nanoscope software (version 5.30r3, Digital Instruments). Approximately 5−10 images were taken for each preparation using at least two samples.

Circular Dichroism Spectroscopy. Far-UV CD spectra were obtained using a MOS-450 spectropolarimeter (BioLogic Science Instrument, Grenoble, France). The measurements were performed in a quartz cuvette of 0.2 cm with a protein concentration around 0.1 mg/mL in HCl solution (pH 2.0) containing 100 mM NaCl. All of the protein samples were centrifuged at 18000*g* for 20 min, prior to the analysis. The samples were scanned from 190 to 250 nm, with a scan rate of 50 nm per minute. For the measurements, the spectra were an average of 25 scans. The following parameters were used: step resolution, 1 nm; acquisition duration, 1 s; bandwidth, 5.0 nm; sensitivity, 100 mdeg. The cell was thermostated with a Peltier element at 25 °C, unless specified otherwise. The concentrations of the proteins were determined according to the Lowry method (*25*) using BSA as the standard. Recorded spectra were corrected by subtraction of the spectrum of a protein-free buffer. Each data point was the mean of duplicate measurements.

SDS-PAGE Experiments. SDS-PAGE was performed on a discontinuous buffered system according to the method of Laemmli (*31*) using 12% separating gel (pH 8.8) and 4% stacking gel (pH 6.8). The gel was stained with 0.1% Coomassie brilliant blue (R-250) in 10% acetic acid and 45% methanol and destained in a methanol−water solution containing 10% (v/v) acetic acid (methanol/acetic acid/water = 1:1:8, v/v/v). The tested samples were prepared by directly mixing the protein solutions (1%, w/v) with electrophoretic sample buffer, namely, 0.25 M Tris-HCl buffer (pH 8.0) containing 10.0% (w/v) SDS and 2.5% (v/v) 2-ME, in a ratio of 1:2 (v/v).

Statistical Analysis. An analysis of variance (ANOVA) of the data was performed, and a least significant difference (LSD) test with a confidence interval of 95 or 90% was used to compare the means.

RESULTS AND DISCUSSION

Apparent Viscosity (η). In our preliminary experiments, we investigated the changes in η of protein solutions (pH 2.0; 1%, w/v) of β-conglycinin, glycinin, and their mixture (1:1) during heating at 80 °C for a period of up to 18 h and observed that the η of all tested protein solutions at various specific shear rates in the range 0.01−100.0 s^{-1} was persistently increased upon heating for > 10 h (data not shown). The thickening effect upon heating is clearly attributed to thermally induced structural unfolding of protein molecules and subsequent aggregation between unfolded proteins. Typical η profiles of various protein solutions heated for a specific period time of 15 h as a function of shear rate are presented in **Figure 1**. All of the dispersions exhibited shear-thinning behaviors upon increasing shear rate; however, the change pattern of η upon shear rate varied with the type of tested soy globulins. From **Figure 1**, it can be observed that there seemed to be a shear rate range (around 0.3−0.5 s^{-1}) within which all of the dispersions exhibited similar viscosity (about 0.7 Pa·S). Below this range, the η at specific shear rates increased in the order β-conglycinin < glycinin ≈ the mixture, whereas above the range, the increasing order was reversed. The differences in these flow behaviors between various heated soy globulin solutions may reflect the differences in nature of the formed aggregates, for example, the extent in flexibility of fibrillar aggregates.

A similar phenomenon of the increase in η has been observed for 2% (w/v) glycinin solution at pH 2 upon heating at 85 °C for 20 h (*9*). By comparison, the η at specific shear rates of the heated glycinin solution (2%, w/v) in this previous work was even much lower than that of 1% (w/v) in the present work.

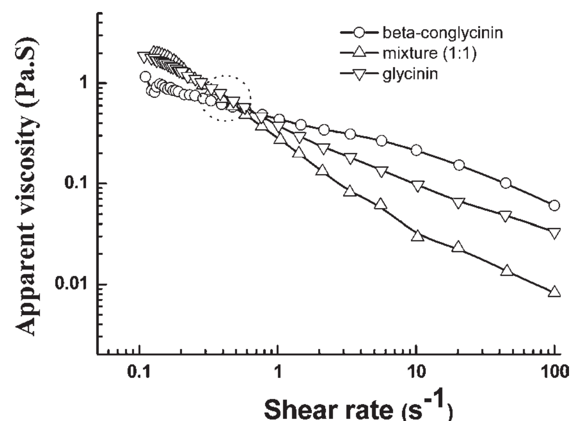

Figure 1. Apparent viscosity profiles of various heated soy globulin dispersions (1%, w/v) at pH 2.0. The heating was carried out at 80 °C for a period of 15 h.

Characterization of Formed Fibrillar Aggregates of Soy Globulins at pH 2.0. It is well recognized that heating at temperatures above the denaturation temperature of a protein, at pH 2.0 and at low ionic strengths, usually results in fibrillar aggregation of the protein and even the formation of amyloid or amyloid-like fibrils (*3*, *5*, *12*−*15*). The formation of amyloid fibrils is generally defined by three criteria: green birefringence upon staining with Congo Red, fibrillar morphology, and β-sheet secondary structure (*27*). Although Th T fluorescence is not one of the criteria for amyloid fibrils, it is relatively accepted as an indicator of the presence of amyloid fibrils (*27*). In the present work, the binding nature with Th T and Congo Red dyes of various soy globulin aggregates formed by heating for various periods of time was characterized, in addition to their morphological and conformational characteristics.

Th T and Congo Red Binding Spectroscopic Assay. **Figure 2** shows the Th T fluorescence spectra in the presence of various soy globulin dispersions (pH 2.0), untreated (control) and heated for various period times in the range of 0−12 h. The fluorescence intensity of untreated samples (controls) was relatively low; however, as expected, it progressively and considerably increased with heating time, increasing from 0 to 12 h. The progressive increase in fluorescence intensity indicated the formation of amyloid-like fibrils, upon heating. The changes in maximum fluorescence intensity of Th T with heating time are displayed in **Figure 3**. It was generally observed that in all of the cases, the maximum fluorescence intensity increased quickly during a very short period of heating (e.g., about 0.5 h), and then the rate of increase gradually decreased, and at last, a plateau was reached. However, the maximum fluorescence intensity at any a period of heating considerably varied among various soy globulins (**Figure 3**). A similar change in fluorescence intensity with heating time has been observed for hen egg white lysozyme at pH 1.6 (65 °C), although in this case, Nile Red dye was applied instead of Th T (*29*).

At any specific heating time, the maximum fluorescence intensity for β-conglycinin was highest, followed by the mixture (1:1) and glycinin (**Figure 3**). The data suggest that β-conglycinin had a much higher potential to form amyloid-like fibrils by heating than glycinin. Interestingly, the maximum fluorescence intensity at any a period time of heating for the 1:1 mixture was approximately intermediate between the β-conglycinin and glycinin, possibly suggesting that the formation of amyloid-like

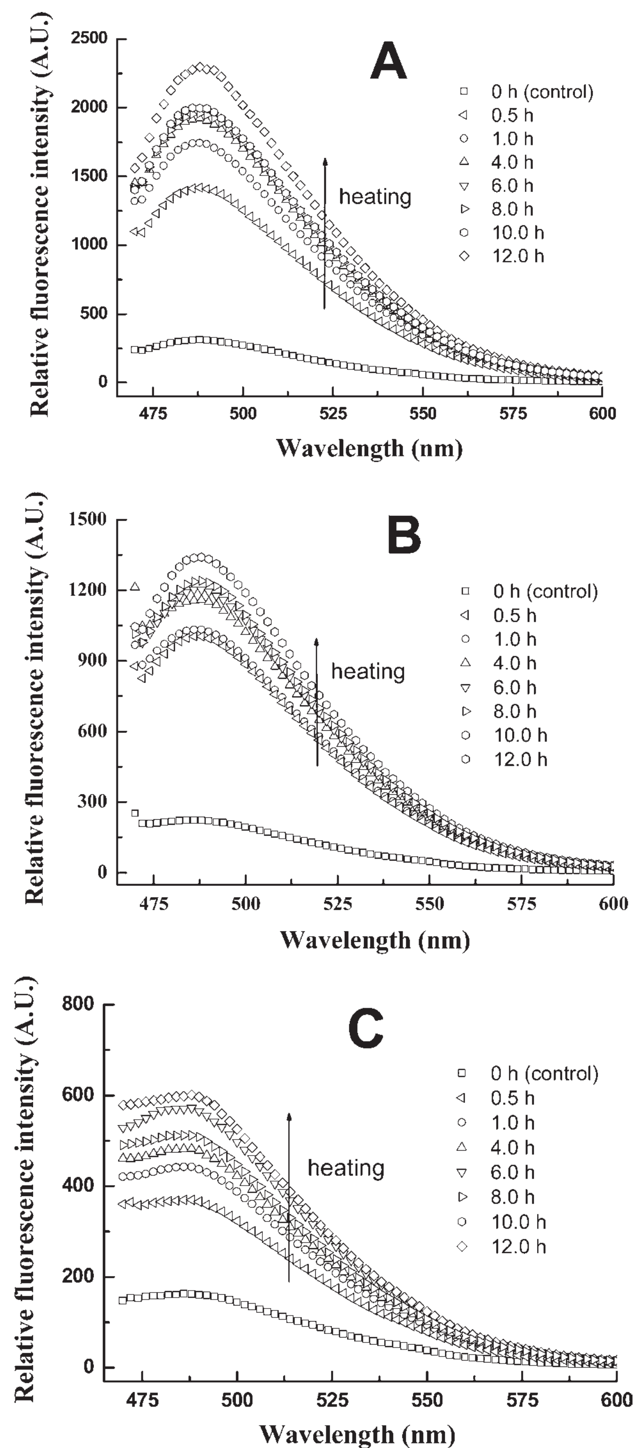

Figure 2. Th T fluorescence emission spectroscopic profiles in the presence of untreated (control) and heated soy globulin dispersion at pH 2.0: (**A**) β-conglycinin; (**B**) 1:1 mixture; (**C**) glycinin. Heating was carried out at 80 °C for 0, 0.5, 1.0, 4.0, 6.0, 8.0, 10, and 12 h, respectively.

fibrils for β-conglycinin and glycinin might evolve in different ways and that fibril formation in the presence of both β-conglycinin and glycinin might be mainly dependent upon the assembly of the β-conglycinin component. In a previous work investigating the fibrillar aggregates from soy glycinin and soy protein isolate (mainly consisting of glycinin and β-conglycinin in a similar ratio), a much higher Th T fluorescence intensity (at 486 nm) was similarly observed for heated soy protein isolate solution than for glycinin solution (at protein concentration 2%, w/v; pH 2.0; heating conditions: 85 °C for 20 h) (*9*).

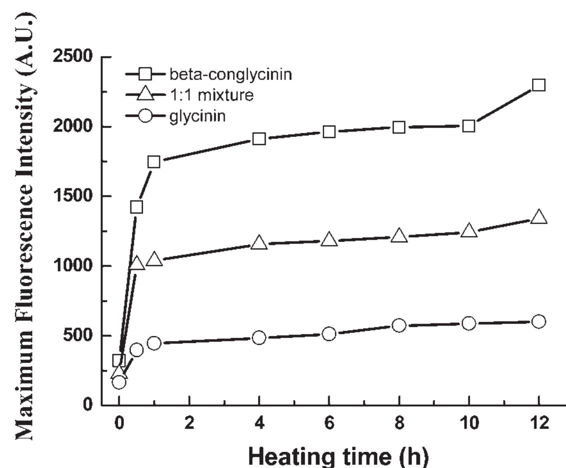

Figure 3. Maximum fluorescence intensity of Th T in the presence of soy globulin dispersions as a function of heating time (0−12 h). Each data point is the mean of duplicate measurements.

The presence of amyloid or amyloid-like fibrils can also be indentified using Congo Red spectroscopic assay. The Congo Red spectra in the presence of untreated samples (control) did not exhibit any absorbance peak, whereas the spectra in the presence of heated samples exhibited a similar absorbance peak at 529−534 nm (**Figure 4**), which is a typical characteristic of amyloid fibrils (*27*, *32*). The magnitude and location of the peak varied with the type of applied soy globulins, as well as the period of heating (**Figure 4**). In the case of β-conglycinin, the magnitude of the peak at 6 h was lowest at three selected periods of heating (1, 6, and 12 h), but the location was similar. In the case of glycinin, the magnitude of the peak at 6 h was considerably higher than that at 1 h, whereas prolonged heating (12 h) slightly decreased the magnitude relative to that at 6 h. In the case of the mixture, the magnitude of the peak at 12 h was even lower than that at 1 h. The differences in changes might suggest differences in structural properties of the formed fibrils among various soy globulins. The diversity in Congo Red spectra may be partially related to low solubility of this dye at acidic pH (*29*).

Morphological Analysis of Formed Fibrils. In the present work, the AFM technique was applied to characterize the structural characteristics of the formed fibrils. **Figure 5** shows typical tapping mode AFM height images of the amyloid-like fibrils of β-conglycinin, glycinin, and the 1:1 mixture thereof, formed by heating at pH 2.0 and 80 °C for 12 h. Herein, one point should be clarified that the meaning of the term "height" widely used in AFM characterization should vary depending on the practical application; for example, in the present work, it is more like the "thickness" of formed aggregates or fibrils. However, the term "height" is still adopted in this work to maintain consistency between AFM analysis and the related statement in the text.

In the AFM images of untreated soy globulins, relatively even distributed particles with mean heights of approximately 1.2−1.8 nm were observed (data not shown). This height value is similar to that (around 2.0 nm) observed for β-conglycinin heated at 60 °C (*28*). The measured heights ($<$2.0 nm) were much shorter than those expected from the size of the β-conglycinin or glycinin molecules, for example, 3.3 nm for trimeric β-conglycinin determined by transmission electron microscopy (TEM) (*33*). The underestimation of the thickness of the proteins has been attributed to vertical compression during scanning (*11*, *21*) and adhesion to the mica substrate (*34*). Measurements in air-drying using AFM might result in a loss in height of ~25% compared to the values obtained for measurements in liquid (*35*).

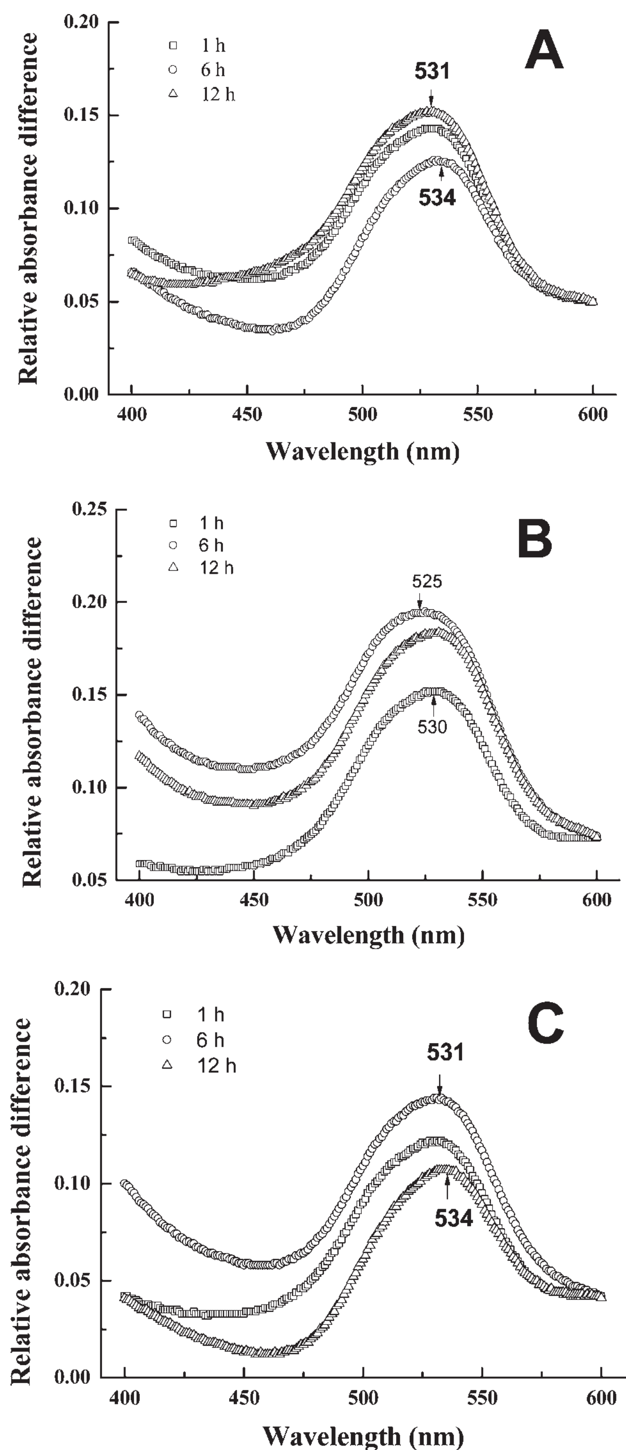

Figure 4. Congo Red spectroscopic profiles in the presence of untreated and heated soy β-conglycinin (**A**), glycinin (**B**), and their 1:1 mixture (**C**) dispersions at pH 2.0. Heating was carried out at 80 °C for 1, 6, and 12 h, respectively.

Heating (80 °C, 12 h) resulted in the formation of amyloid-like fibrils in all cases, but the morphology of the formed amyloid-like fibrils varied distinctly among various soy proteins (**Figure 5**). In general, regular fibrils with uniform width were formed in β-conglycinin and glycinin, and in the mixture case, the formed fibrils were relatively irregular and the widths not uniform (for example, some nodes within the fibrils were distinctly observed). In the latter case, plenty of irregular short fibril debris together with long fibrils was also observed.

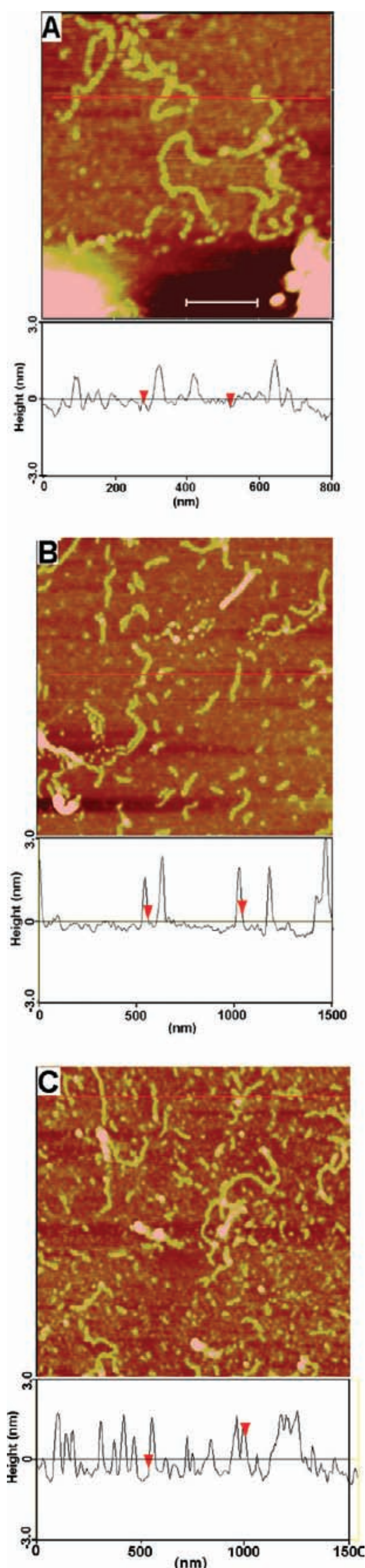

Figure 5. AFM images of the formed amyloid-like fibrils of soy β-conglycinin (**A**), glycinin (**B**), and their 1:1 mixture (**C**). The fibrils were obtained by heating at 80 °C for a period of 12 h. The height profiles of the fibrils located at red lines (within the figures) are presented below the AFM images.

Table 1. Some Characteristics for the Formed Amyloid-like Fibrils of Various Soy Globulins[a]

	characteristics of the formed fibrils	
soy protein	width at half-height (nm)	coil periodicity (nm)
β-conglycinin	20.5 ± 2.8 b	26.3 ± 2.4 ab
glycinin	25.8 ± 1.4 a	22.5 ± 4.6 b
mixture (1:1)	21.1 ± 0.5 b	33.0 ± 6.6 a

[a] The characteristics of the formed fibrils were obtained from the AFM images. Each data point is the mean and standard deviation of at least eight determinations. Different letters (a, b) represent the significant difference at the P < 0.10 level.

All tof he formed fibrils exhibited similar linear morphology and periodic or twisted coil structure, but the related structural characteristics, including the average contour lengths, fibril heights, and coil periodicity, as well as the width at half-heights, varied considerably (**Figure 5**). In the β-conglycinin case, the maximal contour length of the formed fibrils was around 600−800 nm, and many of these wormlike fibrils were present in the twisted form (**Figure 5A**). In this case, the heights of the formed fibrils were in the range of approximately 1.4−1.8 nm. Most of the fibrils from glycinin were in a radial pattern, and some of the fibrils even associated into bundles. The fibril heights for the glycinin were in the range of about 1.6−2.2 nm. In this case, particulate or strand-like aggregates with heights of about 5.0 nm were observed (**Figure 5B**), indicating that heating led to both fibrillar and amorphous aggregation. In contrast, very long fibrils with maximal contour lengths of about 0.8−1.0 μm and heights of 1.6−1.8 nm were observed in the 1:1 mixture case, for which particulate aggregates with heights of about 4.5−6.0 nm were similarly observed (**Figure 5C**). The formed fibrils seemed to be more easily coiled to form a yarn-like structure than those from β-conglycinin. The width at half-height (20.5 nm) of the formed fibrils for β-conglycinin was significantly lower than that (25.8 nm) for glycinin, whereas in the 1:1 mixture case, the half-height widths were similar to that of β-conglycinin (**Table 1**). The coil periodicity (33.0 nm) for the 1:1 mixture was significantly larger than that (22.5 nm) for glycinin, but insignificantly larger than that (26.3 nm) for β-conglycinin (**Table 1**). These phenomena regarding fibril morphology further suggest that various soy globulins might undergo different thermal aggregation mechanisms. The differences in fibril characteristics can largely account for the differences in flow behaviors (**Figure 1**).

A similar periodic fibril structure has been observed using AFM in β-lactoglobulin at pH 2.0 and 80 °C after 200 min of heating (*35*). The β-lactoglobulin fibrils had a periodic structure with a periodicity of ∼30−40 nm and heights of ∼2−3 nm. Thus, the present observation partially supports the viewpoint that some of the properties of soy protein fibrils are comparable to those of whey protein fibrils (*9*). On the other hand, the morphology of amyloid-like fibrils formed at pH 2.0 is distinctly different from that of the β-conglycinin aggregates formed at pH 7.6 and ionic strengths > 0.1 M; for example, the heights (1.4−2.2 nm) of the former are much less than that (8−11 nm) of the latter, although their half-height widths (20−30 nm) are similar (*21*). Furthermore, the half-height widths obtained in the present work are much higher than that (a few nanometers) observed by TEM for soy glycinin or soy protein isolate fibrils (*9*). The difference may be caused by the full hydration of the proteins on the mica.

Secondary Conformational Analysis. The formation of amyloid fibrils for a protein is usually accompanied by changes in their molecular conformations, especially for the formation of highly ordered β-type secondary structure. The secondary conformational changes of various soy globulins (1%, w/v) by heating at 80 °C and pH 2.0 were characterized using a far-UV CD spectroscopic technique. **Figure 6** shows typical far-UV CD spectra of unheated and heated (1−16 h) soy β-conglycinin, glycinin, and the 1:1 mixture thereof, at pH 2.0. Usually, secondary structure elements such as α-helices and β-sheets of the proteins have dichroic activity in the far-UV wavelength range from 190 to 260 nm (*36*). All of the unheated soy proteins exhibited similar far-UV spectra with prominent negative bands centered at around 205, 210, and 215 nm, as well as a positive band at 200 nm (**Figure 6**). These features are sufficient indicators of a highly ordered structure, most probably of the β types (*37*, *38*).

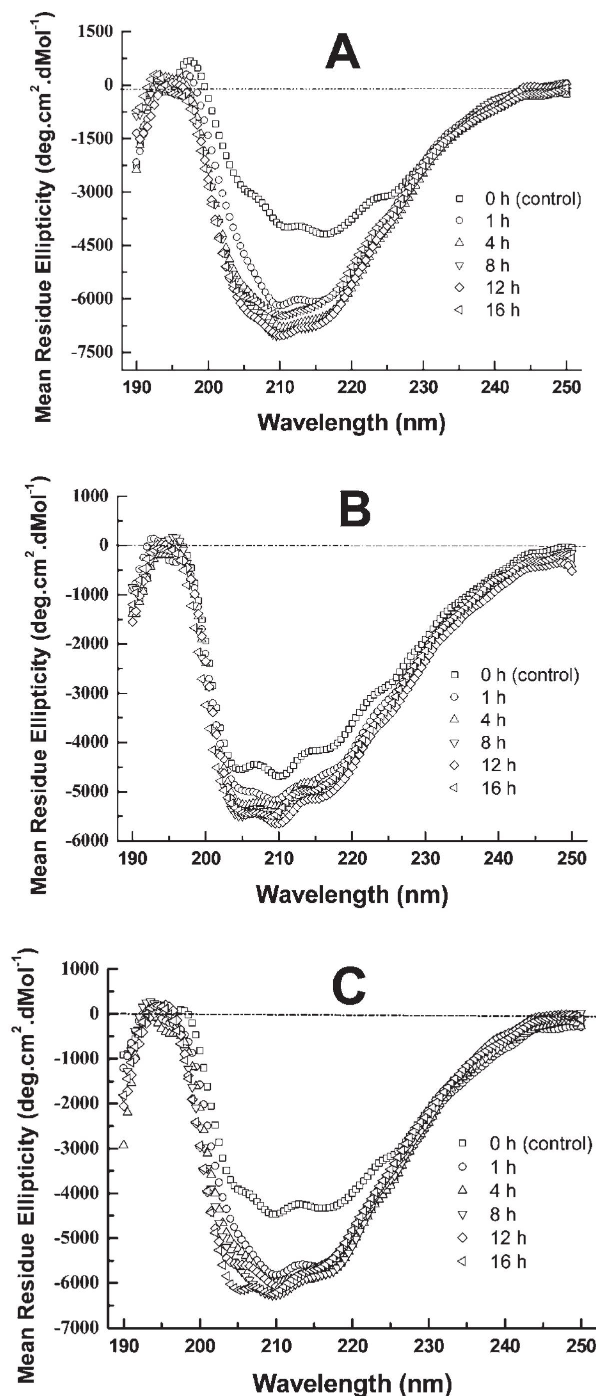

Figure 6. Stacked far-UV CD spectra of unheated and heated soy β-conglycinin (**A**), glycinin (**B**), and the 1:1 mixture thereof (**C**), at pH 2.0 and 100 mM ionic strength. The protein concentration was 1.0% (w/v), and heating was carried out at 80 °C for various periods of time (0−16 h).

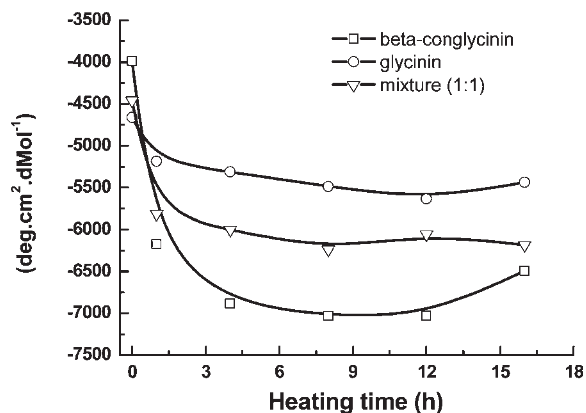

Figure 7. Changes in magnitude of the negative ellipticity band at 210 nm of various soy globulins with heating time. Heating was carried out at 80 °C for 1−16 h.

The heating resulted in remarkable decreases in the magnitude of the negative bands, but the extent of the decrease varied considerably among various soy globulins. The changes in the magnitude of the negative band at 210 nm with heating time, which can reflect the formation of β-type secondary structure, are presented in **Figure 7**. In all cases, the changes during initial heating (e.g., for less than 4 h) were much more prominent and then slowed upon further heating up to 12 h, indicating formation of β-type secondary structure upon heating. In the β-conglycinin case, the extent of the decrease in the magnitude for the negative band at 210 nm was much higher than that for glycinin, whereas the extent of the changes for the 1:1 mixture was intermediate (**Figure 7**). The data suggest that β-conglycinin underwent much easier changes in secondary conformational changes than glycinin, during heating at pH 2.0. The change patterns of secondary conformations for various soy globulins are well consistent with the formation of amyloid or amyloid-like fibrils as evidenced by the Th T fluorescence technique (**Figures 2** and **3**), suggesting a close relationship between the fibril formation of soy globulins and the secondary conformational changes by heating. Upon prolonged heating up to 16 h, the magnitude of the negative bands for β-conglycinin and glycinin, on the contrary, increased (**Figures 6** and **7**). The increases may be attributed to the disruption of highly ordered secondary conformations by the severe heating, possibly as a result of extensive hydrolysis of the polypeptides.

Polypeptide Hydrolysis. The hydrolysis of the polypeptides of various soy globulins by heating at pH 2.0 was monitored using reducing SDS-PGAE, and the results are presented in **Figure 8**. Under reducing conditions, all of the unheated soy globulins exhibited typical polypeptide electrophoresis bands, among which β-conglycinin consisted of α, α′, and β subunits with MW of about 80, 75, and 50 kDa, and the glycinin was composed of acidic and basic subunits with MW of about 36 and 22 kDa, respectively. As expected, distinct hydrolysis occurred in all tested soy globulins, upon heating. However, the extent of polypeptide hydrolysis considerably varied among various soy globulins, and even within the same soy globulin sample, the hydrolysis of various polypeptide constituents varied distinctly (**Figure 8**). By comparison, the polypeptides of β-conglycinin were generally much more easily hydrolyzed by heating at pH 2.0 than those of glycinin. In β-conglycinin, the α and α′ subunits were more easily disrupted than the β subunit, whereas in glycinin, the acidic subunits were much more easily disrupted than the basic subunits. In the case of β-conglycinin, various subunits progressively decreased and, concomitantly, a number of polypeptides

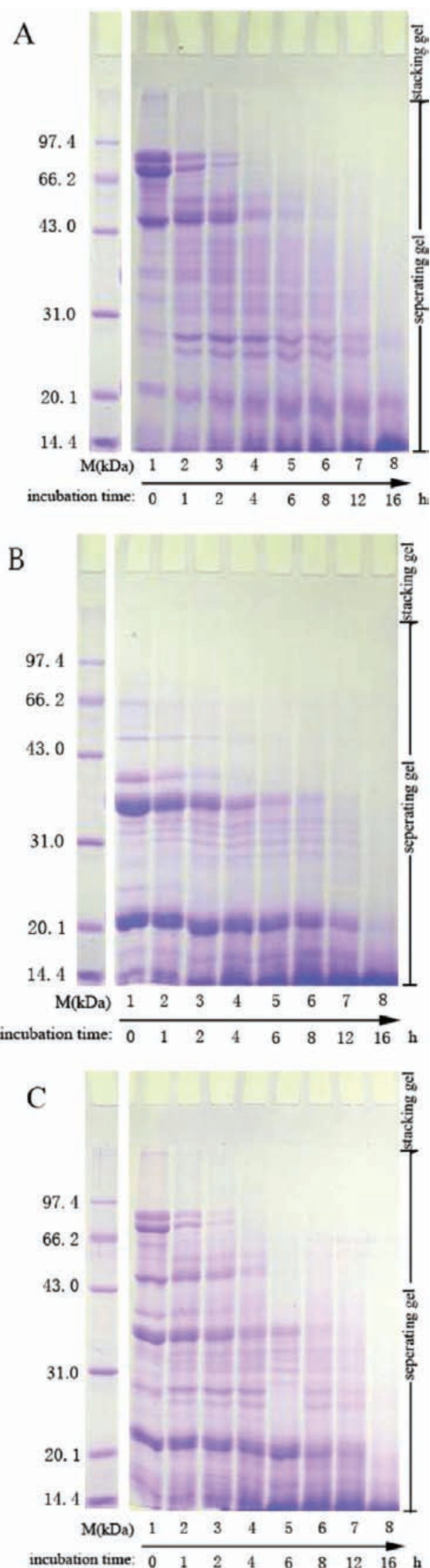

Figure 8. Reducing SDS-PAGE profiles of unheated and heated soy β-conglycinin (**A**), glycinin (**B**), and the 1:1 mixture thereof (**C**). Heating was carried out with a protein concentration of 1% (w/v), at 80 °C for various periods time in the range of 0−16 h.

with MW of <30 kDa increased, upon heating (**Figure 8A**). Similar disruption of the proteins by heating at pH 2.0 or 1.6 has

been observed for whey protein isolate (and its individual pure protein components (*39*, *40*)), lysozyme (*29*), and phaseolin (*23*). For example, β-lactoglobulin was found to be hydrolyzed into peptides with MW between 2000 and 8000 Da after heating (20 h at 85 °C) at pH 2.0 (*40*).

The combined charged amino acid content (acidic + basic amino acids) of β-conglycinin is considerably higher than that of glycinin; among individual subunits of β-conglycinin, the combined charged amino acid content of acidic α' or α subunits is also much higher than that of the β subunit (*41*). Thus, the differences in extent of hydrolysis by heating can be explained by the differences in the contents of charged amino acid compositions, especially the acidic amino acids among these globulins, because the charged amino acids or amides such as Gln and Asn would easily undergo deamidation at pH 2.0 upon the heating. This is further confirmed by the following facts: Asp was reported to be preferably hydrolyzed under acid conditions when the β-carboxyl group is protonated; in the β-lactoglobulin case, the majority of the peptides (both aggregated and nonaggregated) are a result from cleavage of the peptide bonds before or after Asp residues (*40*).

These phenomena of acid hydrolysis seem to be consistent with the conformational changes by heating (**Figures 6** and **7**), indicating that the conformational changes were accompanied by polypeptide hydrolysis. Thus, the results suggest that the formation of amyloid or amyloid-like fibrils for soy globulins by heating at acidic pH involves both conformational changes and disruption of their polypeptides, and the extent of the conformational changes or polypeptide disruption largely decides the potential of these proteins to assemble into highly ordered fibrils. Akkermans et al. had clearly indicated that peptides are building blocks of heat-induced fibrils of β-lactoglobulin formed at pH 2.0, and the fibrils were composed of a part of these peptides and not intact β-lactoglobulin (*40*).

General Discussion of the Formation Mechanism of Amyloid-like Fibrils from Soy Globulins. In the present work, the formed aggregates of various soy globulins meet all three criteria for the amyloid fibril (*27*) and, thus, can be considered to be a kind of amyloid-like fibril. Heat-induced self-assembly into fibrils is usually controlled between electrostatic and hydrophobic interactions (*2*). At pH 2.0 and 100 mM ionic strength, the zeta potential data for unheated soy β-conglycinin, glycinin, and their 1:1 mixture are 14.7, 11.8, and 14.3 mV, respectively (data not shown). Thus, the differences in fibril formation among various soy globulins cannot be ascribed to the difference in their electrostatic repulsion (between unheated proteins). Considering the Th T binding data (**Figures 2** and **3**) and the polypeptide hydrolysis (**Figure 8**), it can be suggested that the fibril formation of soy globulins might be closely related to their amino acid composition. The importance of amino acid composition for the thermal aggregation has also been observed in legume protein isolate, although the aggregation was carried out at pH 7.0 (*42*).

It is generally recognized that partial unfolding of the native protein structure is a key step in the formation of protofibrils and fibrils (*43*). At present, the aggregation experiments at pH 2.0 were carried out at a temperature of 80 °C, which is just above the denaturation temperature of soy glycinin at the same conditions (data not shown). Thus, it can be considered that all of the globulins would be partially and even completely denatured before the initiation of fibril assembly. Besides the denaturation, the fibril assembly process is also accompanied by progressive and remarkable changes in secondary conformation, as well as disruption of polypeptides (**Figures 6** and **8**). Most importantly, it is observed that the extent of conformational changes and the extent of polypeptide disruption are closely related to the β-conglycinin and glycinin ratio. If the data of Th T binding experiments are taken together, these phenomena suggest that the conformational changes and polypeptide disruption are requisites for the amyloid-like fibril assembly for soy globulins, and higher extents of conformational changes or polypeptide hydrolysis are more favorable for fibril formation. This is consistent with the viewpoint of Akkermans et al. (*40*) that released peptides during hydrolysis are building blocks of β-lactoglobulin fibrils formed at pH 2.0.

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