



Improvement of heat-induced fibril assembly of soy β -conglycinin (7S Globulins) at pH 2.0 through electrostatic screening

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ABSTRACT

Protein fibril assembly at acidic conditions has recently attracted increasing interests in the fields of biochemical and food science, however, the obtained knowledge about the assembly of the proteins with complex structure (e.g. plant 7S/11S globulins) is still limited. This study investigated the influence of NaCl addition (0–300 mM) on the fibril assembly of soy 7S globulins (β -conglycinin), induced by heating at 80 °C and pH 2.0, with the aim of confirming the improvement of their fibril assembly by electrostatic screening. The formed fibril formation was investigated by using Th T fluorescence and atomic force microscopy (AFM) techniques. Heat-induced structure changes were traced by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and circular dichroism spectroscopy (CD). The results indicated that the whole fibril assembly experienced protein dissociation/hydrolysis and subsequent fibrillization process, and increasing NaCl concentration progressively increased the amount of formed fibrils, and even fibril length. AFM analyses confirmed that the increase in NaCl concentration shortened the time at which the short fibrils were visually observed, and the morphology of formed fibrils, including height of fibrils, width at half-height, and coil periodicity, slightly varied with the added NaCl concentration. The CD analyses indicated that increasing NaCl concentration greatly favored formation of β -type secondary structure, as well as extensive disruption of tertiary and/or quaternary conformations. These results confirmed that the electrostatic screening greatly improved the heat-induced fibril assembly process of soy β -conglycinin at pH 2.0. The improvement of fibril formation was largely attributed to increased extent of conformational changes at higher ionic strength. These findings would provide important information about the fibril assembly of plant oligomeric globulins at acidic pH that have important implications for the development of protein protein-based fibrillar gels.

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1. Introduction

There are growing interests in the fibril assemblies from many food globular proteins, such as β -lactoglobulin (β -LG, a major whey protein), bovine serum albumin (BSA) and ovalbumin, due to their potential applications in the food industry (i.e. thickening agents in food matrixes) (van der Linden, 2010; van der Linden & Venema, 2007). The fibrillization of those important proteins can be induced by heat treatment at acidic (i.e. pH 2.0) or basic environment. During heat treatment, the fibril aggregation will follow thermal denaturation. Once the protein concentration exceeds the critical gelling point, the fibril aggregation will result in the formation of fibrillar gels (Gasal, Clark, Pudney, & Ross-Murphy, 2002; van der Linden & Venema, 2007). Those fibrillar gels are usually transparent, isotropic, and exhibiting unique features such as high thermal stability and gel

strength (at a comparable protein concentration; relative to traditional heat-set gels).

To date, there has emerged a rich variety of articles for addressing the assembly kinetics and microstructure of heat-induced food protein fibrils, including whey proteins (i.e. β -LG; Arnaudov, de Vries, Ippel, & van Mierlo, 2003; Gosal et al., 2004; Arnaudov & de Vries, 2006; Bolder, Vasbinder, Sagis, & van der Linden, 2007; Akkermans et al., 2008; Oboroceanu, Wang, Brodkorb, Magner, & Auty, 2010), BSA (Veerman, Sagis, Heck, & van der Linden, 2003; Vetri et al., 2011), lysozyme (Misha et al., 2007), and legume proteins (i.e. 7S or 11S globulins from soy and kidney beans; Akkermans et al., 2007; Tang & Wang, 2010; Tang, Zhang, Wen, & Huang, 2010). Among those fibril-forming proteins, β -LG is one of the most investigated food proteins. The underlying mechanism for the fibril assembly is still not fully understood. Arnaudov et al. (2003) studied the β -LG fibrillization behavior at low pH and ionic strength by in situ light scattering (LS) and proton nuclear magnetic resonance (^1H NMR). Based on their results of LS and ^1H NMR, they proposed a multistep fibrillization model which involved initial linear aggregates formation and then slow consolidation of existing fibrils (Arnaudov et al., 2003).

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The fibril assembly of the proteins involves acidic hydrolysis of the polypeptides and conformational changes. By tracing the cleaved peptide of β -LG using reversed-phase HPLC, and mass spectrometry (MALDI-TOF), Akkermans et al. (2008) found that the peptides, released from cleavage of the peptide bonds before or after aspartic acid residues of β -LG, shared the similarity for facilitating β -type structure formation. The secondary structure changes from β -LG hydrolysis were then characterized by in situ attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) which confirmed the reduced α -helix and increased β -sheet during fibrillization (Oboroceanu et al., 2010). The phenomenon of peptide hydrolysis occurring also in the fibril assembly of lysozyme (Misha et al., 2007) and 7S/11S globulins from kidney and soy beans (Tang & Wang, 2010; Tang et al., 2010) seems to be a general process during protein fibrillization, which was confirmed by Lara, Adamcik, Jordens, and Mezzenga (2011). In our previous works, we additionally pointed out the fibril assembly of 7S/11S globulins was accompanied by considerable changes in tertiary and/or quaternary conformations (Tang & Wang, 2010; Tang et al., 2010).

Generally speaking, protein fibril assembly is affected by many factors including pH, ionic strength, protein concentration and heating conditions. Once pH, protein concentration and heating conditions are kept constant, the kinetics of fibril assembly will be strongly dependent on ionic strength. For instance, Aymard, Nicolai, and Durand (1999) investigated the aggregation kinetics of β -LG at pH 2.0 by light scattering with multiple sources (laser, neutron, and X-ray). Their small-angle scattering results exhibited a strong decrease trend for persistence length of β -LG fibrils with ionic strength increase, which was also observed by Kavanagh, Clark, and Ross-Murphy (2000) by using transmission electron microscopy (TEM). More recently, Arnaudov and de Vries (2006) even found that ionic strength increase up to 100 mM largely shifted the critical fibrillization concentration to low values, which facilitates fibril formation at low protein concentration. Similarly, Veerman, Ruis, Sagis, and van der Linden (2002) observed that increase of ionic strength from 0.01 to 0.09 M lead to the decrease of critical percolation concentration for the fibrillar β -LG gels at pH 2.0. Besides, their work also verified that fibril assembly rate of β -LG was slower at lower ionic strengths, which was initially stated by Aymard et al. (1999). The impact of ionic strength on fibril assembly is different among distinct proteins, such as human serum albumin (Juárez, López, Cambón, Taboada, & Mosquera, 2009) and kidney bean protein isolate (rich in 7S globulins; Zhang et al., 2010).

On the other hand, soy protein-based ingredients have attracted much attention from food industry due to their health benefits and processing availability. Elaboration of fibril assembly from soy protein will magnificently deepen our understanding of the structure of soy protein under different conditions, which facilitate the scientific design of soy protein-based ingredients. Based upon our previous investigation of 7S/11S globulins fibrils (Tang & Wang, 2010; Tang et al., 2010), we further investigate the influence of ionic strength on the fibril assembly of soy β -conglycinin (7S globulins) at low pH in a systematic way, which is scarcely reported yet. The main objective of this investigation is to study the influence of ionic strength (0–300 mM) on the kinetics and morphology of soy β -conglycinin fibril assembly at pH 2.0. Ionic strength impact on protein hydrolysis and conformation changes were simultaneously evaluated. To the best of our knowledge, this is the first report for addressing the improvement of fibril assembly of plant oligomeric globulins at low pH through electrostatic screening.

2. Materials and methods

2.1. Materials

Defatted soy flakes with low protein denaturation were processed by initial flash desolventization and vacuum drying at 60 °C by

Shandong Yuwang Industrial & Commercial Co., Ltd. The flakes were ground in a Straub mill (Model 4E, Straub Co., Philadelphia, PA, USA) to pass through an 80 mesh sieve. The obtained flour was stored in sealed container at 4 °C until used. The protein content of the flour was $55.5 \pm 0.4\%$ (determined by Kjeldahl method with nitrogen conversion factor of 6.25; on dry basis) and nitrogen solubility index 84.0%. Congo Red, Thioflavin T (Th T), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals applied in the work were of analytical grade.

2.2. Preparation of soy β -conglycinin

The soy β -conglycinin samples were prepared according to the method described in our previous work (Tang & Wang, 2010), which is an improvement based on the work of Nagano, Hirotsuka, and Mori (1992).

2.3. Thermal aggregation experiments

Soy β -conglycinin solutions (1.0%, w/v) with various ionic strengths were prepared by dissolving the freeze-dried soy β -conglycinin samples into 0.1 M HCl solution with different NaCl concentrations (0–300 mM). The solution pH was accurately adjusted to 2.0 by using 6 M HCl. The protein concentrations in the solutions were determined by Lowry assay (Lowry, Rosebrough, Lewis, & Randall, 1951), with BSA as the standard protein. Aliquots (5 μ L) of the protein solutions were poured into glass tubes with hermetic lids. The glass tubes were heated at 80 °C for specific periods of time in a temperature-controlled bath with temperature deviation of less than 1 °C. After heat treatment, the samples were immediately cooled in an ice bath and directly subject to further experiments.

2.4. Thioflavin T (Th T) fluorescence analysis

Th T stock solution was prepared by dispersing 8 mg Th T into 10 mL of phosphate buffer (pH 7.0) containing 150 mM NaCl (LeVine, 1995). The dispersion was filtered with 0.2 μ m syringe filter to remove undissolved Th T. This stock solution should be stored in dark at 4 °C and covered with aluminum foil. The stock solution was diluted 50 times by the same buffer on the day of analysis to generate the working solution (Koscielska-Kasprzak & Otlewski, 2003; Nilsson, 2004). Aliquots (50 μ L) of the tested samples were mixed with 5 mL 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-7000, 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 (Mishra Misha et al., 2007). The fluorescence spectrum of the Th T working solution was subtracted from the fluorescence spectra of the samples to correct the background signal.

2.5. Zeta potential (ζ) measurement

The ζ values of the proteins in the dispersions at varying NaCl concentrations (0–300 mM), as a function of pH were measured by a laser doppler velocimetry and phase analysis light scattering (M3-PALS) technique using a Malvern Zetasizer Nano ZS (ZEN 3600) instrument (Malvern Instruments Ltd., Malvern, Worcestershire, UK), in connection with a multipurpose autotitrator (model MPT-2, Malvern Instruments, Worcestershire, UK). The protein samples were prepared in deionized water containing various concentrations of NaCl (0–300 mM), and diluted to about 0.5% (w/v) with the corresponding same NaCl solutions. The diluted protein dispersions were

filtered with a 0.45 μm Millipore membrane, prior to the analysis. Titration was performed from pH 7.0 to pH 2.0 with 0.1 M and 1 M HCl under constant magnetic stirring. One milliliter of each diluted sample was put in an electrophoresis cell (Model DTS 1060C, Malvern Instruments Ltd., Malvern, Worces-ter-shire, UK). The temperature of the cell was maintained at ambient temperature. The data were reported as the average values and standard deviations of three measurements performed with three individually prepared protein dispersions.

2.6. Atomic force microscopy (AFM) analysis

AFM images were acquired in tapping mode using a Dimension 3000 microscope (Digital Instruments-Veeco, Santa Barbara, CA, USA), equipped with a 'G' scanning head (maximum scan size 10 μm) and driven by a Nanoscope IIIa controller. A 2 μL droplet of unheated and heated soy β -conglycinin solutions (diluted to 25 $\mu\text{g/mL}$) was spread on a freshly cleaved mica disc and dried in air for 10 min at ambient temperature (Ikeda & Morris, 2002). For imaging under ambient condition, single-beam uncoated silicon cantilevers (type OMCL-AC, Olympus, and RTE SP, Veeco) were used. The drive frequency was set at 300 kHz and the scan rate was between 0.3 and 0.8 Hz. Images were processed by the Digital Nanoscope Software (Version 5.30r3, Digital Instruments). For each preparation condition, at least two samples were used for duplication. Approximately 10 images were obtained for each preparation.

2.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on a discontinuous buffered system according to Laemmli's method (Laemmli, 1970) which utilized 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 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) β -mercaptoethanol, in a ratio of 1:2 (v/v).

2.8. Circular dichroism (CD) spectroscopy

Far-UV and near-UV CD spectra were obtained using a MOS-450 spectropolarimeter (BioLogic Science Instrument, France). All unheated and heated β -conglycinin solutions from the aggregation experiments were diluted by the same buffer (pH 2.0) with the corresponding ionic strengths to protein concentrations of 0.1 and 1.0 mg/mL which were suitable for far-UV and near-UV CD measurements, respectively. All the protein samples were centrifuged at 18,000 g for 20 min, prior to the analysis. The far-UV CD spectroscopic measurements were performed in a quartz cuvette of 2 mm and the samples were scanned from 190 to 250 nm with a scan rate of 50 nm per minute. The near-UV CD spectroscopy measurements were performed in a 1 cm quartz cuvette. The sample was scanned over a wavelength range from 250 to 320 nm. For both measurements, the final spectra were an average of 25 scans. The following parameters were used during the CD measurement: 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 $^{\circ}\text{C}$, unless specified otherwise. The protein concentrations were determined by the Lowry assay using BSA as the standard (Lowry et al., 1951). The collected spectra were corrected by subtracting the spectrum of a protein-free buffer. A mean value of 112 for the amino acid residue was assumed in all calculations, and CD measurements expressed as mean residue ellipticity ($[\theta]$) in $\text{deg cm}^{-2}\text{dmol}^{-1}$.

2.9. Statistics

An analysis of variance (ANOVA) of the data was performed, and a least significant difference (LSD) test with a confidence interval of 95% was applied, if needed.

3. Results and discussion

3.1. Zeta potential (ζ) analysis

Since the isoelectric point (pI) of native soy β -conglycinin is about pH 4.5, it will carry positive charges on the protein surface at pH 2.0. In the absence of NaCl, the ζ of native β -conglycinin at pH 2.0 was about 21.5 mV (Fig. 1) which exhibited strong positive charges on the protein surface. When NaCl concentration increased from 0 to 300 mM, the ζ dramatically decreased to 7.7 mV, suggesting the increased electrostatic screening by salt. The electrostatic screening can affect the profile of protein–protein interactions involving electrostatic repulsion and thus the fibril assembly. Positive influences of increasing NaCl concentration or ionic strength on fibrillization have been observed for β -lactoglobulin (Arnaudov & de Vries, 2006; Veerman et al., 2002) and human serum albumin (Juárez et al., 2009) heated at low pH values.

3.2. Fibril formation kinetics

The formation of highly-ordered amyloid-like fibrils can be generally monitored by using Th T fluorescence spectroscopy, since Th T has a high selectivity for amyloid fibrils (Foderà et al., 2008; Nilsson, 2004; Žerovnik et al., 2007). More importantly, Th T does not affect or only slightly affects the early formation of fibrils, making this technique suitable for in situ fibril detection (Foderà et al., 2008). Fig. 2 displays the NaCl concentration impact upon the relative maximum fluorescence intensity profiles of β -conglycinin. The fluorescence intensity of Th T for unheated samples at pH 2.0 was relatively low. However, heating for 0.5 h or above resulted in considerable increase in fluorescence intensity. At any NaCl concentration (0–300 mM), the maximal fluorescence intensity remarkably increased by 6–7 times after a short period of heating (0.5 h). After 3-hour heating, the fluctuation of maximal fluorescence intensity was small compared with the intensity increase in the first 0.5 h period. These results are in consistence with our previous observations (Tang & Wang, 2010) about the fibril assembly of soy β -conglycinin, glycinin, and their mixtures. These data also gave support to the multi-step fibril model put forward by Arnaudov et al. (2003). In their model, fibril formation involves initial formation of linear aggregates and slow consolidation process. For soy β -conglycinin fibrils, most profibrils

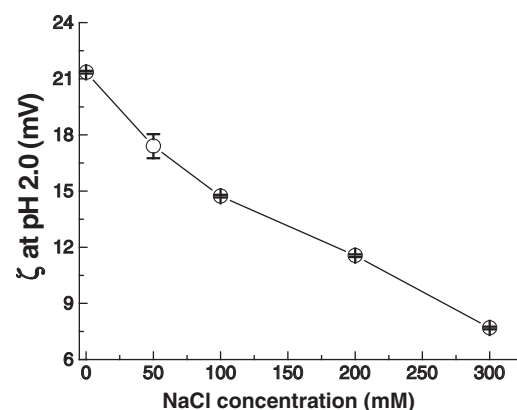


Fig. 1. Zeta potential (ζ) profiles of soy β -conglycinin at pH 2.0 as a function of NaCl concentration. Each data is a mean value with standard deviation of triplicates.

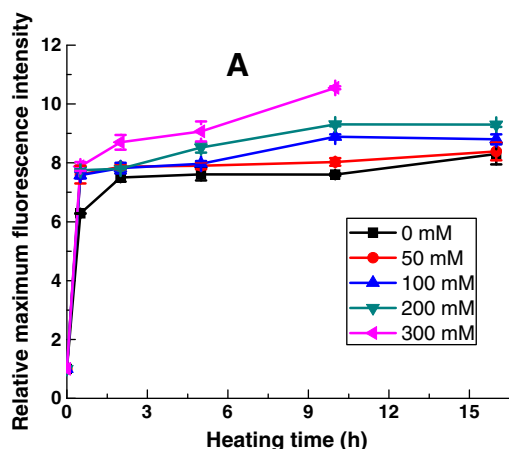


Fig. 2. Heating period evolution (0–16 h) of relative maximal fluorescence intensity of Th T in the presence of soy β -conglycinin (1.0%, w/v) at various NaCl concentrations (0–300 mM NaCl). Each data is a mean value with standard deviation of duplicates which is normalized based on the Th T fluorescence of unheated sample. Each data is a mean value with standard deviation of duplicates.

(building blocks) needed for fibril assembly are generated within a short period (~ 0.5 h) of heating at pH 2.0. Longer period of heating (up to 16 h) facilitates those initially-formed profibrils to further assembly into highly-ordered fibrils with longer length.

On the other hand, it is generally observed that increasing NaCl concentration resulted in an increase in magnitude of maximal fluorescence (Fig. 2). This observation suggests that the decrease of electrostatic repulsion caused by increasing ionic strength was favorable for fibril assembly of β -conglycinin. For example, at 300 mM NaCl concentration, a heating with an incubation period above 10 h almost resulted in fibrillar gel formation of β -conglycinin (1%), which made the fluorescence determination impossible.

3.3. Morphological analysis

Fibril morphologies of soy β -conglycinin at various NaCl concentrations (0–300 mM) upon heating were characterized by tapping mode AFM, as shown in Fig. 3. We can see that the heating resulted in the fibril formation of β -conglycinin at all-tested NaCl concentrations (0–300 mM), if the heating period was enough. However, the minimal time for fibril formation, fibril flexibility, and fibril length varied with different NaCl concentrations (Fig. 3). For example, at 0 mM NaCl concentration, short fibrils were visually observed only after heating up to 10 h, while it just took 5 h at 50–100 mM and 2 h at 200–300 mM, respectively. Thus, less time is needed for initiation of the fibril assembly at higher ionic strength.

Interestingly, the fibrils of soy β -conglycinin that formed at 0 mM NaCl concentration after 16 h heating were in an isolated form with a 'ball of strings' shape. At high NaCl concentrations (50–300 mM), all the fibrils exhibited wormlike shape after a period of heating (i.e. 16 h). The mean fibril length increased with increasing NaCl concentration (in the range of 50–300 mM), e.g. up to 100–200 nm with long heating periods at 300 mM NaCl concentration (Fig. 3). The morphological difference can be attributed to the differences in intermolecular interaction pattern for the formed fibrils. At 0 mM NaCl concentration, intermolecular electrostatic repulsion caused by fibril charges plays a dominant role which might greatly decrease the flexibility of the formed fibrils. As a result, the fibrils themselves coiled to minimize fibril–fibril repulsion. Charge screening by presence of 50–300 mM NaCl resulted in reduction of intermolecular electrostatic repulsion between fibrils and thus increase of

fibril flexibility. The increased fibril flexibility can be verified by long and entangled fibrils observed at 200 or 300 mM NaCl after 16 h of heating (Fig. 3).

In general, the maximal persistence length of the fibrils formed at any prolonged specific period gradually increased with increasing NaCl concentration (Fig. 3). For example, the maximal persistence length of the fibrils formed after 16 h of heating increased from around 400–600 nm to about 2000 nm, as the NaCl concentration increased from 0 to 300 mM. This phenomenon also indicates that increasing ionic strength (or electrostatic screening) improves the fibril assembly of β -conglycinin at pH 2.0 by the heating. Besides, the height (or thickness) of the fibrils varied with NaCl concentration (Fig. 4). The mean height of the formed fibrils at 0 mM NaCl was approximately 2.7–2.8 nm, which were similar to that (about 2.7 nm) at 300 mM NaCl but significantly ($p < 0.05$) higher than that (about 1.7 nm) at 100 mM (data not shown). The height of the fibrils formed at 100 mM NaCl is similar to that (1.4–1.8 nm) previously reported for β -conglycinin fibril at a comparable ionic strength (Tang & Wang, 2010). However, the width at half-height of the fibrils (approximately in the range 23–25 nm) was similar at various NaCl concentrations (data not shown).

On the other hand, all the fibrils formed at various NaCl concentrations exhibited a periodic or twisted coil structure, as evidenced by periodic alteration of height along with the axis of the fibrils, which might account for the diversity of the measured height results, even at a same NaCl concentration (Fig. 4). One typical example of this periodic fibril structure can be observed in Fig. 4 C (b), where the coil periodicity (or the interval of two adjacent peak heights) was approximately estimated to be around 26 nm. A similar periodic fibril structure has been observed using AFM in β -LG at pH 2.0 after 200 min of heating (Oboroceanu et al., 2010). The β -LG fibrils had a periodic structure with a periodicity of 30–40 nm and heights of 2–3 nm. Thus, it can be suggested that the fibril assembly is not directly related to the initial structural state (e.g. tertiary and/or quaternary conformations) of the proteins, but the state of the last products, e.g. released peptides by acid hydrolysis.

3.4. Composition analysis of hydrolyzed peptide

Polypeptide hydrolysis induced by heating at pH 2.0 has been confirmed to play an important role in the fibril assembly of soy 7S/11S globulins (Tang & Wang, 2010). Currently, the hydrolysis of peptide fragments of β -conglycinin during heating at various NaCl concentrations (0–300 mM) was also analyzed by reducing SDS-PAGE. At 0 mM NaCl, the polypeptide components (α , α' and β) of β -conglycinin, with molecular weights of about 80, 75 and 50 kDa, respectively, were progressively hydrolyzed into peptide fragments with molecular weight of 24–26 kDa and even less than 10 kDa as the heating proceeded (Fig. 5 A). It can be observed that among all three polypeptides (α , α' and β), α and α' polypeptides were more easily hydrolyzed by heating at pH 2.0 than the β polypeptide. For instance, the former polypeptides were almost completely disrupted after 5 h heating, while the latter completely disappeared after more than 10 h heating (Fig. 5 A). Similar results have been observed in our previous work (Tang & Wang, 2010). The polypeptide hydrolysis patterns of β -conglycinin by heating were similar at various NaCl concentrations (0–300 mM). Fig. 5 B displays the SDS-PAGE profiles of β -conglycinin formed at various NaCl concentrations after 5 h of heating. The SDS-PAGE patterns of the protein samples at different NaCl concentrations were almost identical. This phenomenon suggested that the polypeptide hydrolysis of β -conglycinin at pH 2.0 was independent of ionic strength. Thus, fibril assembly might be mainly relevant to the hydrophilicity and hydrophobicity balance of the peptides, released during the hydrolysis, which is affected by ionic strength.

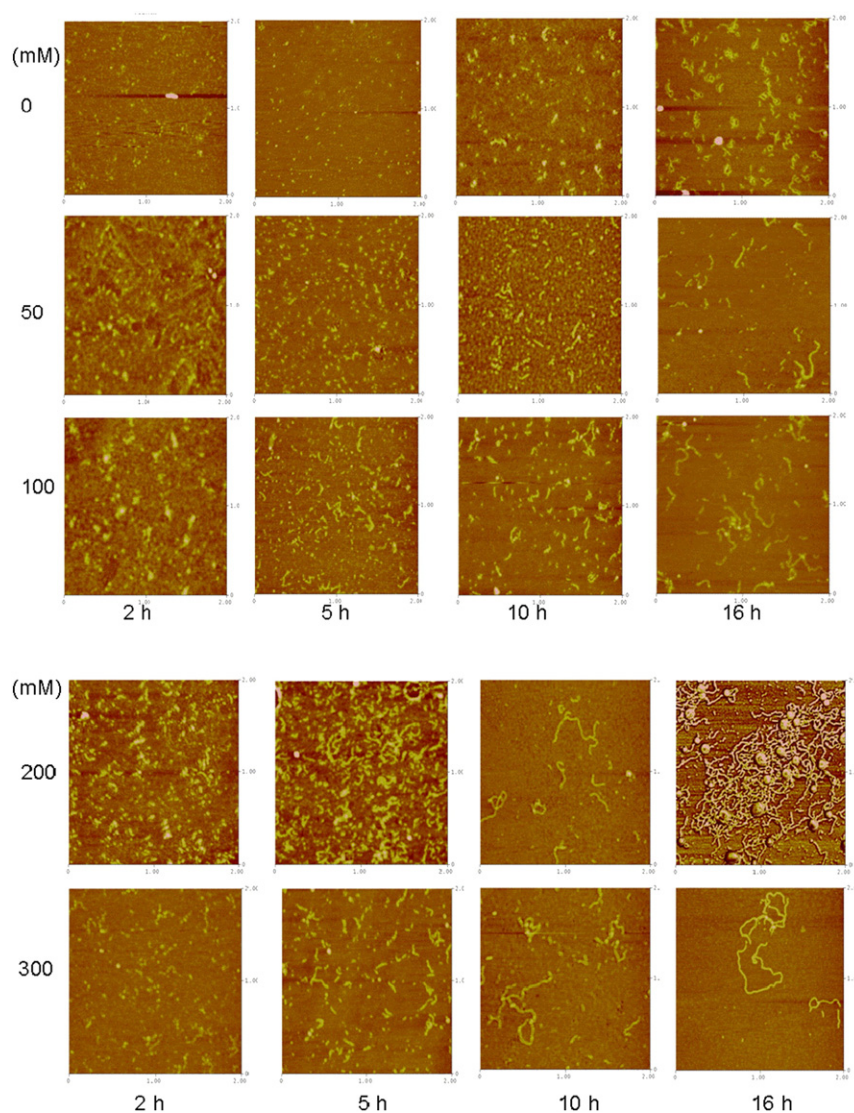


Fig. 3. AFM height images of soy β -conglycinin fibrils formed at pH 2.0 under different conditions combining various NaCl concentrations (0–300 mM) and heating periods (2, 5, 10, and 16 h). The heating temperature was set at 80 °C.

3.5. Conformational analyses

3.5.1. Secondary conformation

The formation of highly-ordered β -type secondary structure (especially β -sheet) is one of the basic features for amyloid fibrils (Nilsson, 2004). The secondary structures of soy β -conglycinin by heating at various NaCl concentrations (0–300 mM) were characterized by using far-UV CD spectroscopy. All the unheated soy β -conglycinin at various NaCl concentrations exhibited similar far-UV spectra with prominent negative bands at 205, 210 and 215 nm, as well as a positive band at 200 nm (data not shown), suggesting the existence of β -type highly-ordered structure (Kelly & Price, 1997; Yang, Wu, & Martinez, 1986). From Fig. 6 A, we can see that heating resulted in remarkable decrease of ellipticity magnitude ($[\theta]$) in the negative bands, indicating the considerable increase in β -type secondary structure of β -conglycinin upon heating. This phenomenon provides indirect evidence to the formation of amyloid-like fibrils during heating at pH 2.0. The changes in $[\theta]$ of the negative band at 215 nm ($[\theta]_{\lambda=215\text{ nm}}$) upon heating at various NaCl concentrations (0–300 mM) are presented in Fig. 6 B. In all cases, the changes during initial heating period (i.e. <2 h) were much more remarkable than further heating (up to

16 h), as shown in Fig. 6 B. The CD results are in consistency with the Th T fluorescence results (Fig. 2), further indicating that most of building peptides with high β -type secondary structure, needed for the fibril assembly, were released during initial heating (e.g. less than 2 h).

With the increase of NaCl concentration till 100 mM, the extent of decrease in $[\theta]_{\lambda=215\text{ nm}}$ within initial 3 h progressively increased. However, the extent of decrease in $[\theta]_{\lambda=215\text{ nm}}$ within initial 3 h dropped when NaCl concentration increased from 100 to 300 mM. The changes of secondary structures at 0–100 mM are consistent with the Th T fluorescence results shown in Fig. 2, suggesting increased formation of β -type rich fibrils at higher ionic strength. However, the changes of $[\theta]_{\lambda=215\text{ nm}}$ at 200–300 mM seem to be inconsistent with the Th T fluorescence results (Fig. 2), and also be contrasting from the viewpoint that the fibril assembly of soy globulins is closely related to the formation of its β -type secondary conformation (Tang & Wang, 2010). Factually, similar observations have been observed human serum albumin (HSA) fibrils which were formed in acidic environment (Juárez et al., 2009), where the $[\theta]_{\lambda=215-220\text{ nm}}$ value of HSA fibril was reduced at high salt concentration (>50 mM). Although electrostatic screening enhanced the fibrillization at 0–100 mM, excessive amount of salt (e.g. 200–300 mM)

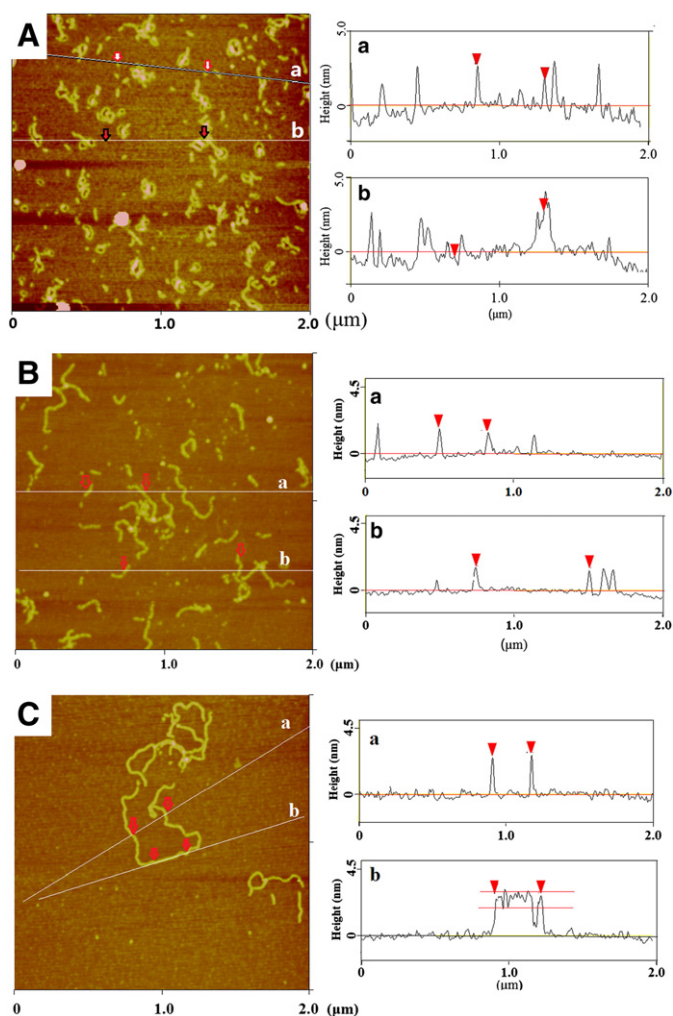


Fig. 4. Typical AFM height profiles of soy β -conglycinin fibrils formed at pH 2.0 and NaCl concentrations of 0 (A), 100 (B) and 300 (C) mM, after 16 h of heating.

greatly favored the fibril-fibril attractive interaction, especially the hydrophobic interactions, thus leading to reduced $[\theta]_{\lambda=215\text{ nm}}$. Thus, the far-UV CD data are still well in accordance with the AFM observations (Fig. 3), reflecting that increase of ionic strength was favorable for the fibril assembly of β -conglycinin.

3.5.2. Tertiary and/or quaternary conformation

Near-UV CD spectroscopy was utilized for probing the tertiary and/or quaternary conformational changes of β -conglycinin by heating at various NaCl concentrations (0–300 mM). The actual shape and magnitude of the near-UV CD spectrum of a protein depend on the amount of aromatic amino acids, their mobility, and their environmental nature (hydrogen bonding, polarity and polarizability) (Yang et al., 1986). The near-UV CD spectrum of unheated β -conglycinin (control) exhibited a prominent positive dichroic band at 280 nm under all NaCl concentration conditions (0–300 mM) (data not shown). This prominent band is a typical near-UV CD feature for Tyr residue (Yang et al., 1986). The magnitude of Tyr residue ellipticity decreased dramatically after a short period of heating (Fig. 7 A). The decreasing rate of Tyr residue ellipticity slowed down upon further heating up to 16 h. The phenomenon indicated progressive exposure of Tyr residue hydrophobic clusters to polar microenvironment, clearly as a result of heat-induced protein structural unfolding, as well as the polypeptide hydrolysis. The tertiary and quaternary

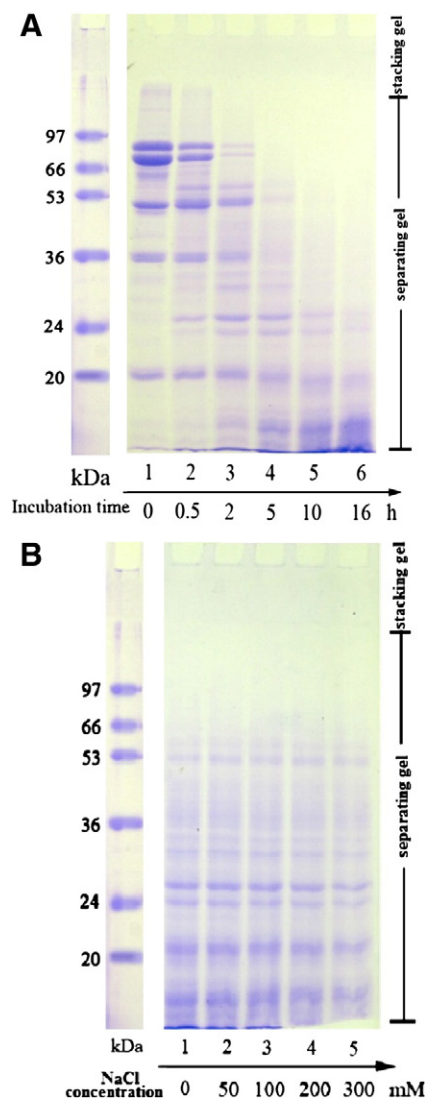


Fig. 5. (A) Reducing SDS-PAGE profiles of soy β -conglycinin at 0 mM NaCl with different heating periods (0–16 h); (B) Reducing SDS-PAGE profiles of soy β -conglycinin heated for 5 h at various NaCl concentrations (0–300 mM). First lane (A or B) displays standard protein markers; Lanes 1–6 (panel A) represent samples heated for 0, 0.5, 2, 5, 10 and 16 h, respectively. Lanes 1–5 (panel B) represent samples treated at NaCl concentrations of 0, 50, 100, 200 and 300 mM, respectively.

conformation changes were similar to the secondary conformation alteration.

With the NaCl concentration increase, the extent of decrease in the ellipticity at 280 nm upon heating progressively increased (Fig. 7 B). Especially at the NaCl concentration of 300 mM, the ellipticity at 280 nm decreased from 20 to $-40\text{ deg cm}^2\text{dmol}^{-1}$ upon 12-hour heating. This observation suggested that the changes in tertiary and quaternary conformations more favorably occurred at higher ionic strength. Together with the results of Th T fluorescence and AFM, it can be reasonably put forward that the formation of highly ordered fibrils of β -conglycinin at pH 2.0 is closely relevant to the extent of changes in its tertiary and quaternary conformations.

4. Conclusions

The fibril assembly of β -conglycinin at pH 2.0 upon heating was greatly improved by electrostatic screening. The increase in

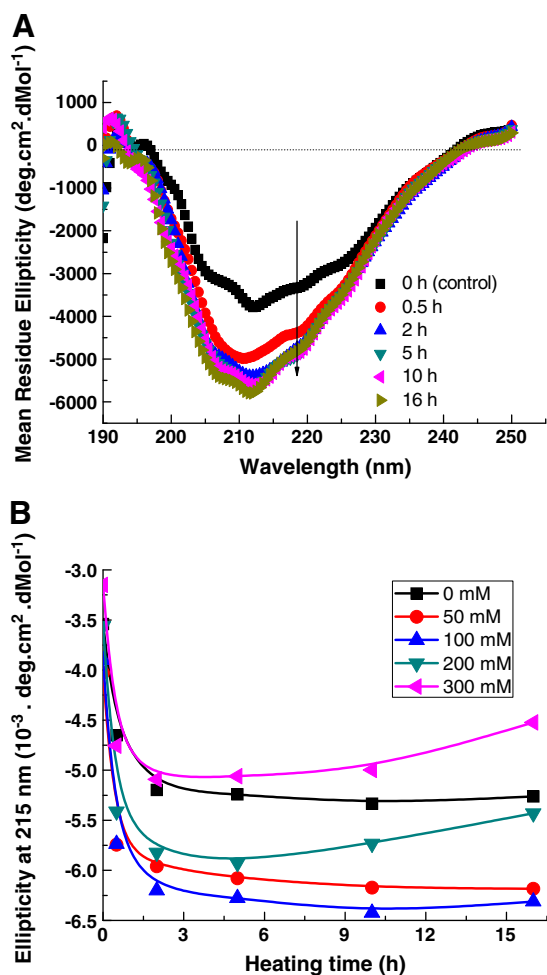


Fig. 6. (A) Typical stacked Far-UV CD spectra of unheated (control) and heated soy β -conglycinin at 0 mM NaCl; (B) Changes in magnitude of the negative ellipticity at 215 nm of soy β -conglycinin at various NaCl concentrations (0–300 mM) upon heating. Heating was carried out at 80 °C and at pH 2.0.

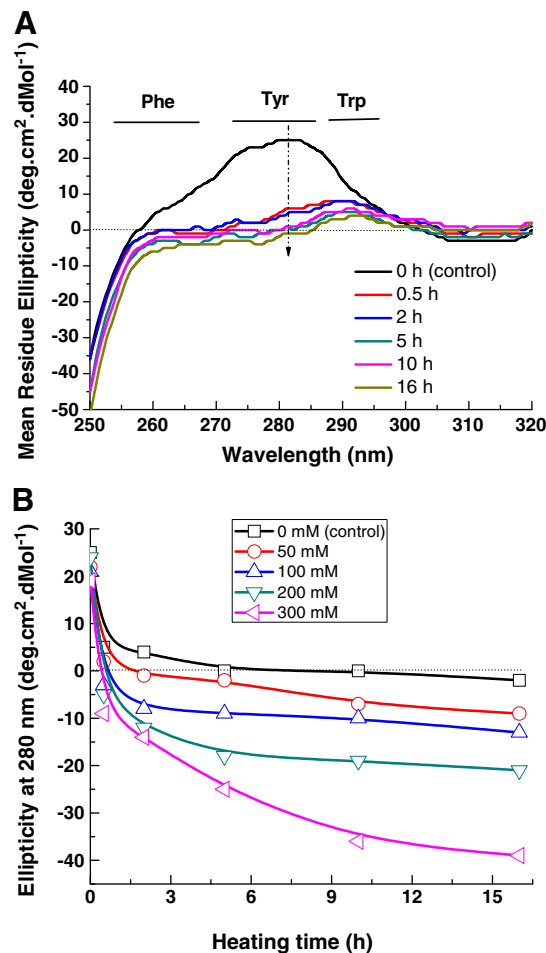


Fig. 7. (A) Typical stacked near-UV CD spectra of unheated (control) and heated soy β -conglycinin at 0 mM NaCl upon different heating period (0–16 h); (B) Heating period evolution of Tyr residue ellipticity at 280 nm upon heating at various NaCl concentrations (0–300 mM).

NaCl concentration within the range of 0–300 mM shortened the time needed to initiate fibril assembly and resulted in the formation of fibrils with larger dimensions (persistence length). The conformational changes during the fibril assembly, especially at tertiary and/or quaternary levels, were more favorable at higher NaCl concentrations. The improvement of the fibril assembly by electrostatic screening could be attributed to decreased intermolecular electrostatic repulsion interactions, as well as increased molecular flexibility (as a result of disruption of tertiary and/or quaternary conformations). These findings provide us with further understanding the fibril assembly of plant oligomeric globulins at acidic conditions that may have great implications for the development of plant protein-based fibril gels.

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