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Formation of Amyloid Fibrils from Kidney Bean 7S Globulin (Phaseolin) at pH 2.0

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The amyloid fibrils formed by heating 1.0% (w/v) kidney bean phaseolin (7S globulin) solution at pH 2.0 with an ionic strength of 20 mM at 85 °C were characterized using transmission electron microscopy (TEM), atomic force microscopy (AFM), binding of thioflavin T (Th T) and Congo Red dyes, and circular dichroism spectroscopy. The morphology of the formed fibrils was closely dependent upon heating time from 15 to 720 min. The diameters of the fibrils formed at various times were similar, but the mean contour length progressively increased with heating time. The Th T maximum fluorescence also progressively increased with heating time. The heating process caused remarkable changes in secondary, tertiary, and quaternary conformations of the phaseolin, but the extents of the changes were closely related to the heating time. With a short heating time (e.g., 15 min), the β-strand content decreased from 38.7 to 22.9%, but further heating resulted in recovery of β-strand structure. The tertiary and quaternary conformations gradually became flexible and unfolded upon heating. Gel electrophoresis analysis indicated that heating disrupted the polypeptides of phaseolin, leading to the formation of fragments with lower molecular mass (e.g., <10 kDa after 360 min). The results suggest that the amyloid fibril formation of phaseolin (7S globulin) involved the disruption of its polypeptides, as well as conformational changes at secondary, tertiary, and quaternary structural levels. This appears to be the first direct observation of amyloid fibrils from legume 7S storage globulin.

KEYWORDS: Phaseolin; kidney bean 7S globulin; protein aggregation; amyloid fibril; assembly

INTRODUCTION

During the past few years, the assembly of different globular food proteins into amyloid (or amyloid-like) fibrils, as well as ordered fibril aggregates, has attracted increasing attention because these assemblies exhibit unique characteristics that can impart some excellent properties to the systems containing these assemblies. Amyloid and amyloid-like fibrils are usually a few nanometers thick and share some similar structural properties, such as the presence of β-sheets orthogonal to the fibril axis and binding of the dyes thioflavin T (Th T) and Congo Red (*1*). These linear protein aggregates or fibrils have good potential, for example, to be used as thickening or gelling ingredients in food products.

The formation of amyloid fibril structures has been generally considered to be a generic property for any polypeptide chain (*2*). That is, any protein or polypeptide has the potential to form amyloid fibril structures, if appropriate specific conditions are applied. To date, a number of proteins have been confirmed to form amyloid fibrils. The well-studied food proteins with respect to fibril formation are whey proteins and β-lactoglobulin in particular, which aggregate into long, semiflexible fibrils upon heating above the denaturation temperature, at low pH (e.g., pH 2.0) and low ionic strength (*3*−*9*). The length of the fibrils formed usually ranges from 1 to 10 μm, and the thickness is on the order of a few nanometers (*4*, *10*). In a recent work, soy glycinin (11S globulin) and soy protein isolate were also confirmed to form long, fibrillar semiflexible aggregates, with contour lengths of 2.3 μm and the thickness of a few nanometers (*10*). Most properties of the formed soy glycinin fibrils are comparable to those of β-lactoglobulin fibrils. Because plant storage proteins are more sustainable than animal proteins, the protein fibrils of the former would have more potential to be used as functional ingredients in the food industry than those of the latter, especially whey proteins.

There are basically three routes of amyloid-like fibril formation: (a) starting from the native protein, which first has to (partially) unfold, and subsequently assemble into a fibril; (b) starting from natively disordered proteins (including small peptides); and (c) starting from a protein that only locally exhibits a conformational change, which triggers the fibril assembly (*11*). The underlying mechanisms of the fibril assembly corresponding to these three different routes would be different. The formation of amyloid fibrils from many globular proteins would belong to the first class. In this case, the partial unfolding of the native protein structure is a key step in the formation of protofibrils and fibrils (*1*). To date, several models have been used to explain the mechanisms of the fibril formation, especially for β-lactoglobulin.

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For example, Arnaudov and others (*3*) proposed a model of multiple steps during the β-lactoglobulin fibril formation at low pH and ionic strength, which involved at least two steps: the reversible formation of linear aggregates, followed by a slow process of "consolidation". These models seem not applicable to the understanding of the fibril formation of plant oligomeric proteins, such as 7S (trimeric) and 11S (hexameric) globulins. According to our knowledge, the fibril assembly of these oligomeric proteins may occur only when the compact conformations of these proteins are disrupted.

Phaseolin, the major storage protein from kidney bean (*Phaseolus vulgaris* L.), is a typical legume vicilin (7S globulin). The nutritional property and in vitro protease digestibility in particular of this protein have been well recognized, because kidney bean is the most widely produced and consumed food legume in Africa, India, Latin America, and Mexico. The polypeptide constituents of this vicilin are more homogeneous, and the charged amino acid contents are higher than those from other *Phaseolus* legumes, such as mung and red beans (*12*). In our previous work, we investigated the thermal aggregation and gelation of kidney bean protein isolate at pH 2.0 and various low ionic strengths (0−300 mM) and found that heating the protein solution (with low protein concentrations) at 85 °C led to the formation of worm-like and fine-stranded aggregates and that the extent of aggregation progressively increased with increasing ionic strength. When the protein concentration exceeded a critical concentration (depending on the ionic strength), homogeneous, fine-stranded, and transparent (or semitransparent) gels would be formed (*13*). The elastic modulus G' of these protein gels is dependent upon the initial protein concentration and could be well described using both fractal and percolation models. These observations clearly suggest that amyloid or amyloid-like fibrils could be formed from the phaseolin (7S globulin) by heating at low pH and ionic strength.

The main objective of this work is to confirm the formation of amyloid or amyloid-like fibrils from phaseolin (7S globulin) at pH 2.0 and low ionic strength (i.e., 20 mM), using some state-of-the-art techniques, such as transmission electron microscopy (TEM), atomic force microscopy (AFM), and circular dichroism (CD), to characterize the morphological and conformational changes of amyloid fibrils, as well as Th T and Congo Red dyes to carry out the binding studies. The hydrolysis or disruption of the polypeptides of phaseolin during the heating at pH 2.0 was also characterized using gel electrophoresis. The underlying mechanism of the fibril formation for the 7S globulin was proposed.

MATERIALS AND METHODS

Materials. Red kidney beans (*P. vulgaris* L.), cultivated in Shandong province of China, were purchased from a local supermarket (Guangzhou, China). The seeds were soaked in deionized water for 15 h at 4 °C and dehulled manually. The dehulled seeds were freeze-dried, ground, and defatted by Soxhlet extraction with hexane to produce the defatted flour. 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 Purified Phaseolin (7S Globulin). The purified phaseolin sample was prepared according to the method described by Sun and others (*14*) from the defatted kidney seed flour, with slight modifications. An amount of 100 g of the defatted flour was dispersed and extracted with 0.025 M HCl (pH 3.5) containing 0.5 M NaCl for 2 h, at a solid-to-solvent ratio of 1:10 (w/v) at ambient temperature. The dispersion was centrifuged (10000*g*, 20 min) at 4 °C in a CR22G centrifuge (Hitachi Co., Japan), and the resultant supernatant was diluted 5 times with cold deionized water (0−4 °C). The precipitates were collected by centrifugation at the same condition. The pellet obtained was dissolved in 0.5 M NaCl solution and reprecipitated twice as above. The last pellet obtained was further dissolved in 0.5 M NaCl solution, with the pH adjusted to 7.0 using 1 M NaOH. Subsequently, the protein solution was dialyzed against deionized water at 4 °C for 48 h and lyophilized to produce the purified sample. The protein content was determined according to the micro-Kjeldahl method with a nitrogen conversion factor of 5.8.

Aggregation Experiments. Phaseolin solutions with a protein concentration of 1.0% (w/v) at pH 2.0 were prepared by dissolving the freeze-dried sample into HCl solution 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 278 nm according to Lowry's method (*15*), with BSA as the protein standard. Aliquots (about 5 mL) of the phaseolin solutions were put into tubes with hermetic lids. The tubes were heated at 85 °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.

TEM. The microstructures of the aggregates or fibrils formed were observed using TEM. The samples of the aggregate solutions (from 1% protein solution, w/v), applied for the TEM observation were prepared by negative staining. The stained samples were diluted 100 times, and a droplet of the diluted solutions was put onto a carbon support film on a copper grid. After 30 s, the droplet was removed with a filter paper. Then, a droplet of 2% (w/v) uranyl acetate (Sigma, Steinheim, Germany) was put onto the grid and removed after 30 s. The micrographs were made using a Philips CM 12 electron microscope (Eindhoven, The Netherlands) operating at 80 kV.

AFM. 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 phaseolin dispersion (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 (*16*). 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 the Digital Nanoscope software (version 5.30r3, Digital Instruments). Approximately 5−10 images were taken for each preparation using at least two samples.

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. 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 (*17*, *18*). 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). The excitation wavelength was set at 460 nm (slit width = 10 nm), and the emission spectra were obtained from 450 to 600 nm, with a scanning speed of 900 nm/min. 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 was mixed with 1 mL of the working solution and kept at 25 °C for 15 min. The visible spectra (in the range 400−700 nm) of the mixtures, in 1 cm path length cuvettes, were recorded with a scan rate of 120 nm/min on a UV−visible spectrophotometer (Hitachi U-3010). The differential spectra were obtained by subtracting the blank spectrum from the sample spectra.

CD Spectroscopy. Far-UV and near-UV CD spectra were obtained using a MOS-450 spectropolarimeter (BioLogic Science Instrument, France). The far-UV CD spectroscopic measurements were performed in a quartz cuvette of 2 mm with a protein concentration around 0.1 mg/mL in HCl solution (pH 2.0) containing 20 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/min.

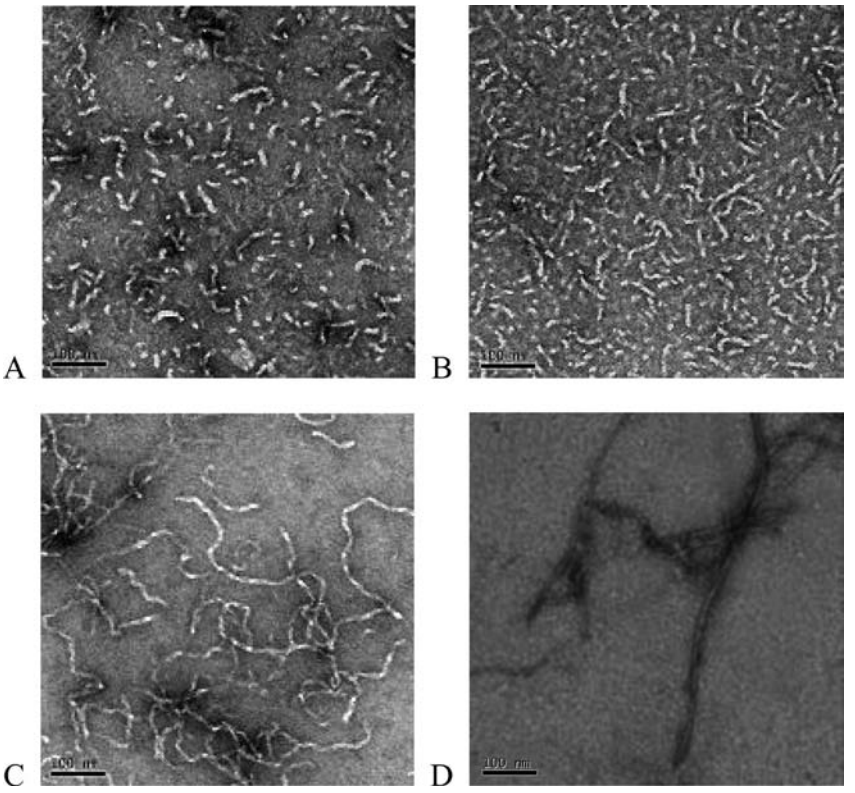

Figure 1. TEM micrographs of amyloid fibrils in phaseolin solutions, heated at 85 °C for 15 (**A**), 60 (**B**), 360 (**C**), and 720 min (**D**), respectively. The bars within the figures indicate the length of 100 nm.

The near-UV CD spectroscopy measurements were performed in a 1 cm quartz cuvette with a protein concentration around 1.0 mg/mL. The sample was scanned over a wavelength range from 250 to 320 nm. For both 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 (*15*) using BSA as the standard. Recorded spectra were corrected by subtraction of 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 were expressed as mean residue ellipticity (θ) in deg cm^{-2} $dmol^{-1}$. The secondary structure compositions of the samples were estimated from the far-UV CD spectra using the CONTIN/LL program in CDPro software, using 43 kinds of soluble proteins as the reference set (*19*). Each datum was the mean of duplicate measurements.

SDS-PAGE Experiments. SDS-PAGE was performed on a discontinuous buffered system according to the method of Laemmli (*20*) using 12% separating gel and 4% stacking gel. The gel was stained with 0.25% Coomassie brilliant blue (R-250) in 50% trichloroacetic acid and destained in a methanol/water solution containing 7% (v/v) acetic acid and 40% (v/v) methanol. The tested samples were prepared by directly mixing the heated phaseolin solutions (1%, w/v) with electrophoretic sample buffer (2×), namely, 0.25 M Tris-HCl buffer (pH 8.0) containing 2.0% (w/v) SDS, 0.1% (w/v) bromophenol blue, 50% (v/v) glycerol, and 10% (v/v) β-mercaptoethanol (2-ME).

RESULTS

TEM and AFM. In our preliminary experiments, we had indicated that at pH 2.0 and low ionic strength, the apparent viscosity of phaseolin solutions (1%) at various specific shear rates in the range of $0.01-100.0\ s^{-1}$ was persistently increased by heating at 85 °C with increasing time of heating, and the intensity of dynamic scattering light signal (and concomitant mean particle size) of the solutions also gradually increased (data not shown). These phenomena suggested the occurrence of heat-induced aggregation of phaseolin. In the present work, the nature of formed phaseolin aggregates, including the morphological and structural characteristics, was characterized.

Table 1. Average Contour Lengths and Secondary Structure Compositions of Protein Fibrils in Phaseolin Solutions (1.0%, w/v) Heated at 85 °C for 0, 15, 60, 360, and 720 min

		secondary structure composition (%)			
heating conditions	contour length[a] (nm)	α-helix[b]	β-strand[c]	turns	random coil
control		5.1	38.7	22.0	34.1
15 min	52 ± 5	8.0	22.9	25.5	43.7
60 min	85 ± 8	8.3	25.6	24.4	41.7
360 min	721 ± 73	8.7	29.5	23.8	38.0
720 min	952 ± 239	8.5	37.5	14.6	38.8

[a] The average contour length and the standard deviation were based on the length of more than 10 single fibrils visible in one TEM micrograph. [b] Combined regular and distorted α-helices. [c] Combined regular and distorted β-strands.

Figure 1 shows the TEM micrographs of the phaseolin aggregates, formed by heating at 15, 60, 360, and 720 min at pH 2.0 and ionic strength 20 mM. Linear ordered aggregates with morphology similar to that of amyloid fibrils were observed. The width (or diameter) of the formed fibrils was nearly unchanged (about 10 nm) throughout the test period, but their average contour length consistently increased from about 52 to 950 nm as the time of the heating increased from 15 to 720 min (**Figure 1** and **Table 1**). It was also observed that the flexibility of the formed fibrils at 720 min seemed to be much less than that at 360 min or less. This might be an indication for the remarkable conformational changes during the formation of the fibrils, when the heating time was increased from 360 to 720 min.

The morphology and microstructural details of protein fibrils were also analyzed by AFM. **Figure 2** shows the tapping mode AFM images of linear aggregates of phaseolin, obtained by heating at the same conditions. In the AFM image of untreated

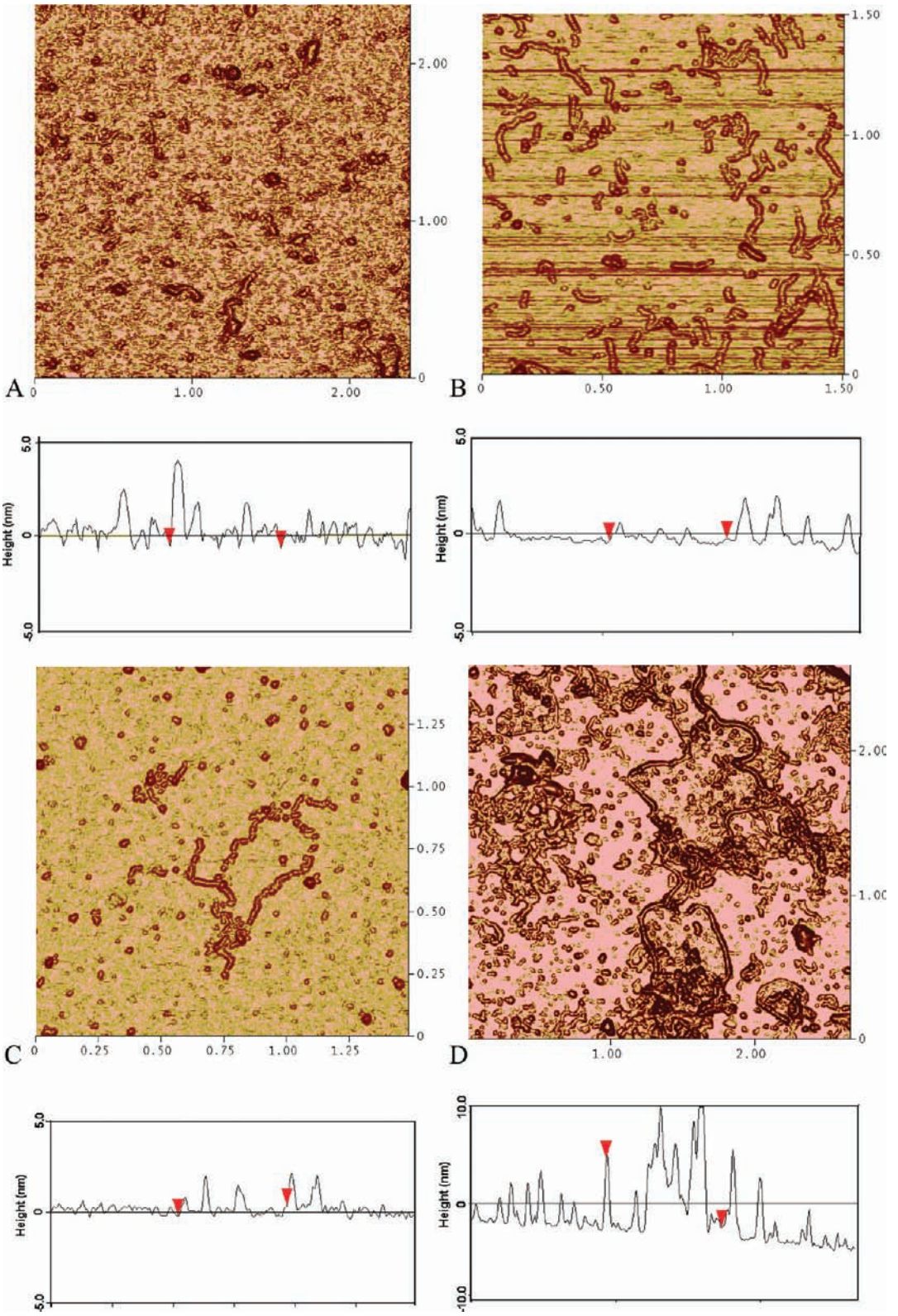

Figure 2. Tapping mode AFM images of phaseolin solutions heated at pH 2 and ionic strength of 20 mM for 15 (**A**), 60 (**B**), 360 (**C**), and 720 (**D**) min, respectively. The curve beneath the images represents the height profile (linear scales) of the cross section highlighted in the image.

phaseolin, relatively evenly distributed particles with approximate mean heights of 1.6 nm were observed (data not shown). The measured heights (< 2.0 nm) were much shorter than those expected from the size of the molecules of trimeric 7S globulins (about 8−12 nm) (*21*). As the sample was dried to facilitate its adhesion to the mica substrate, the measured heights would be much less than those corresponding to fully hydrated conditions (*22*).

The heating resulted in the formation of rod-like aggregates or even flexible fibrils, but the morphology of the formed fibrils was highly dependent upon the extent of heating (**Figure 2**). The dependence of the contour length of the formed fibrils upon heating time is basically consistent with the TEM results. However, the length distribution of the formed fibrils was much more heterogeneous than that observed by TEM (**Figure 1**). The

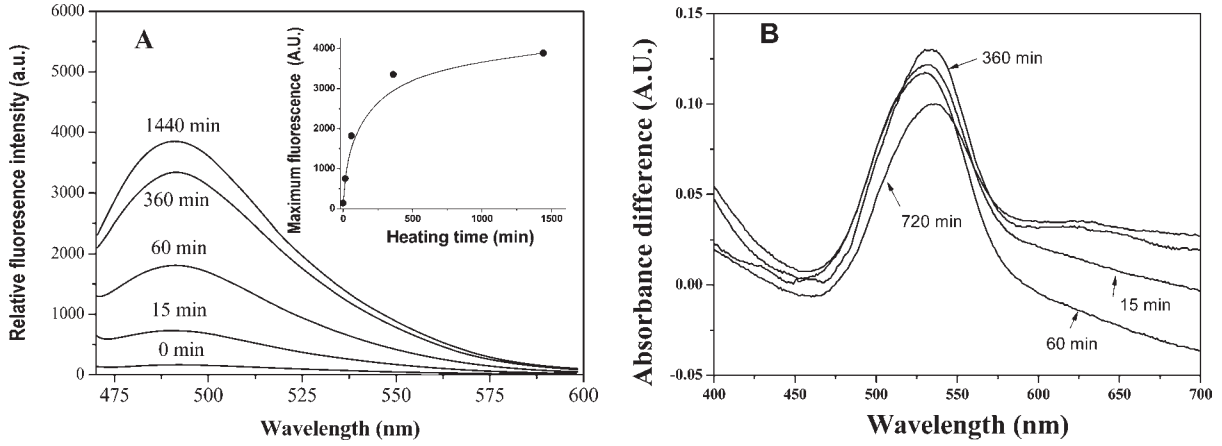

Figure 3. Th T (**A**) and Congo Red (**B**) spectroscopic profiles in the presence of untreated (control) and heated phaseolin solutions. The inset within panel **A** indicates the maximum fluorescence intensity of Th T as a function of heating time. The heated solutions were prepared by heating at 85 °C for 0, 15, 60, 360, 720, and 1440 min, respectively.

difference may be due to the difference in sample preparation. In the AFM experiments, the diluted samples were deposited onto mica by air-drying, and as a result, all of the protein components in the samples would be observed. In the AFM image of the sample heated for 15 min, the formed aggregates with different lengths (~200 nm) and heights (1.0−4.0 nm) were observed (**Figure 2A**). This phenomenon reveals that at pH 2.0, the heating might result in disruption of trimeric phaseolin or its polypeptides into some fragments and, concomitantly, aggregation of trimeric phaseolin or its fragments. The formed linear strands or fibrils appeared to be composed of well-defined "strings of beads", the heights of which are fairly uniform with the size of about 2 nm. Upon further heating for 60 or 360 min, the heights of the formed fibrils were still uniform (at about 2 nm), although their lengths on the whole gradually increased with heating time (**Figure 2B**,**C**). These observations suggest that there might be a distinct transition for phaseolin to form highly ordered fibrils, and this transition involved the disruption of the polypeptides of phaseolin.

In the case of heating for 720 min, the formed long fibrils were further entangled together to form macroaggregates with sizes of several micrometers (**Figure 2D**). In this case, the diameter of each fibril was difficult to measure. The deposition of these fibrils resulted in a multilayer coverage on the mica surface, and our results indicated the existence of large aggregates with heights of 6−10 nm.

Th T and Congo Red Spectroscopic Assays. The nature of the phaseolin aggregates formed at specific time intervals (0−1440 min) was characterized using Th T and Congo Red spectroscopic techniques, as displayed in **Figure 3**. Although Th T fluorescence is not one of the criteria for amyloid fibrils, it is generally accepted as an indicator of the presence of amyloid fibrils (*17*). The fluorescence intensity of untreated sample (control) was very low, but it progressively and considerably increased with heating time increasing from 0 to 1440 min (**Figure 3A**). The increase in maximum fluorescence intensity with heating time was almost exponential (see the Inset, **Figure 3A**). The increasing trend is consistent with that of the mean particle size as estimated by dynamic light scattering technique (data not shown), indicating the formation of amyloid-like phaseolin fibrils at pH 2.0. The kinetics of the fibril formation seemed to be well fitted with a power law equation.

The presence of amyloid-like fibrils can also be indentified using a Congo Red spectroscopic assay. The Congo Red spectrum in the presence of untreated sample (control) did not exhibit any absorbance peak (data not shown), whereaas the spectra in the presence of heated samples exhibited a similar absorbance peak at around 534 nm (**Figure 3B**), which is a typical characteristic of amyloid fibrils (*17*). There were no distinct changes of relative absorbance at 534 nm for heated samples at 15−360 min, possibly reflecting that the basic structural units needed to form amyloid fibrils were similarly released, although the assembled fibrils would be much longer upon prolonged heating. In contrast, the absorbance at 720 min was slightly lower than that at 360 min (**Figure 3B**). The lower absorbance may be due to entanglement of formed fibrils into relatively unordered aggregates, as evidenced by TEM and AFM analyses (**Figures 1** and **2**).

Far-UV and Near-UV Spectra. 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. **Figure 4** shows the far-UV and near-UV CD spectra of phaseolin solutions, heated at 85 °C for 0−720 min. Secondary structure elements such as α-helices and β-sheets have dichroic activity in the far-UV wavelength range from 190 to 260 nm (*23*). The magnitude of far-UV CD ellipticity, as well as the position of the prominent negative band corresponding to the highly ordered β-type structure (*24*, *25*), varied among different samples, indicating the changes in their secondary structure compositions. From the far-UV CD spectra, the secondary structure compositions (including α-helix, β-strand, turns, and random coil) of the phaseolin samples were calculated according to the CONTIN/LL program in CDPro software (*19*), and the results are also displayed in **Table 1**. In unheated sample (control), the secondary structure composition was 5.1% α-helix, 38.7% β-strand, 22.0% turns, and 34.1% random coil. This secondary structure composition is similar to that observed at pH 7.0 (data not shown), indicating that the secondary structure of the phaseolin was highly reserved, nearly independent of pH.

The secondary structure composition of the heated phaseolin samples was highly dependent on heating time (**Table 1**). Heating for 15 min significantly decreased the percentage of β-strand (from 38.7 to 22.9%) and concomitantly increased other secondary structures. However, reversed changes in secondary structure compositions were observed upon further heating up to 720 min. Remarkable changes in the secondary structures occurred when the heating time increased from 360 to 720 min. This phenomenon is consistent with the distinct changes in morphology of the formed aggregates (as heating time increased from 360 to 720 min; **Figures 1** and **2**), suggesting a close relationship between the secondary structure and the morphology of the formed fibrils. Interestingly, the secondary structure composition for the

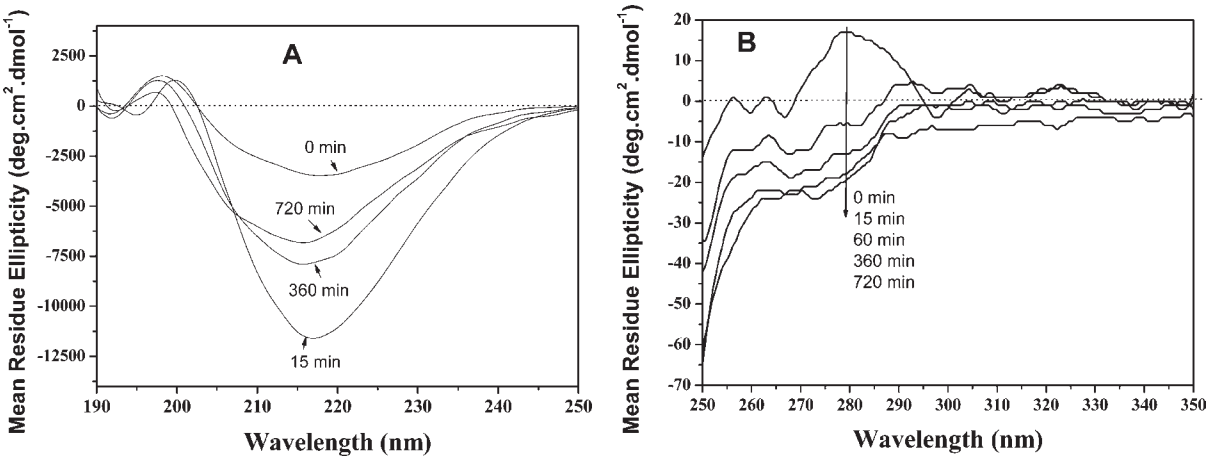

Figure 4. Far-UV (**A**) and near-UV (**B**) CD spectra of untreated and heated phaseolin solutions. Spectra were measured in a 2 mm cuvette (pH 2.0), at a protein concentration of 0.1 (far-UV CD) or 1.0 (near-UV CD) mg/mL. The heated samples were prepared at 85 °C for 0, 15, 360, and 720 min, respectively.

phaseolin sample heated for 720 min was basically consistent with that of untreated sample, except that the former exhibited higher α-helix and lower turn contents. These phenomena clearly suggested that the formation of the linear aggregates or amyloid-like fibrils involved a process of disruption and reorganization of the ordered secondary structures.

The changes in tertiary and quaternary conformations of the phaseolin by heating can be monitored by near-UV CD spectra. The actual shape and magnitude of the near-UV CD spectrum of a protein depend on the number of each type of aromatic amino acid present, their mobility, and the nature of their environment (H-bonding, polar groups, and polarizability), as well as their spatial disposition in the protein (*23*). The near-UV CD spectrum of the untreated sample (control) exhibited a prominent positive dichroic band centered at 278 nm and two minor bands at 260 nm (**Figure 4B**). The prominent band was attributed to the near-UV CD characteristics of Tyr residues, whereas the latter bands were associated with the Phe residues (*23*). Comparison of the near-UV CD spectra indicated that the magnitude of the Tyr and Phe near-UV CD ellipticity (and the Tyr near-UV CD ellipticity in particular) progressively decreased with heating time from 0 to 360 min. The phenomenon indicated that the heating led to gradual unfolding of tertiary and/or quaternary conformations of the proteins. Further heating (720 min) did not significantly affect the near-UV CD spectrum (relative to that at 360 min) (**Figure 4B**).

SDS-PAGE. The hydrolysis of the phaseolin at pH 2.0 was evaluated by SDS-PAGE. **Figure 5** shows the SDS-PAGE image of the phaseolin samples, heated for 0−720 min. In the untreated sample (control), two major bands clearly corresponding to the two major polypeptides of phaseolin (with molecular mass of about 50 kDa) were observed (**Figure 5**, lane 1). Besides the two major bands, a number of bands with lower molecular mass appeared, which might be released fragments of the polypeptides at acidic pH. The relative percentage of the major bands progressively decreased as the heating time increased from 0 to 60 min, and concomitantly, the relative percentage of released polypeptides increased (**Figure 5**, lanes 2−4). As the heating time was increased up to 360 or 720 min, nearly all of the polypeptides (including the major polypeptides and corresponding released products) with molecular masses of > 15 kDa were further disrupted into a kind of polypeptide with molecular masses of < 10 kDa (**Figure 5**, lanes 5 and 6). Similar disruption of the proteins by heating at pH 2.0 has been observed during the preparation of the fibrils from whey protein isolate and its individual pure protein components (*26*).

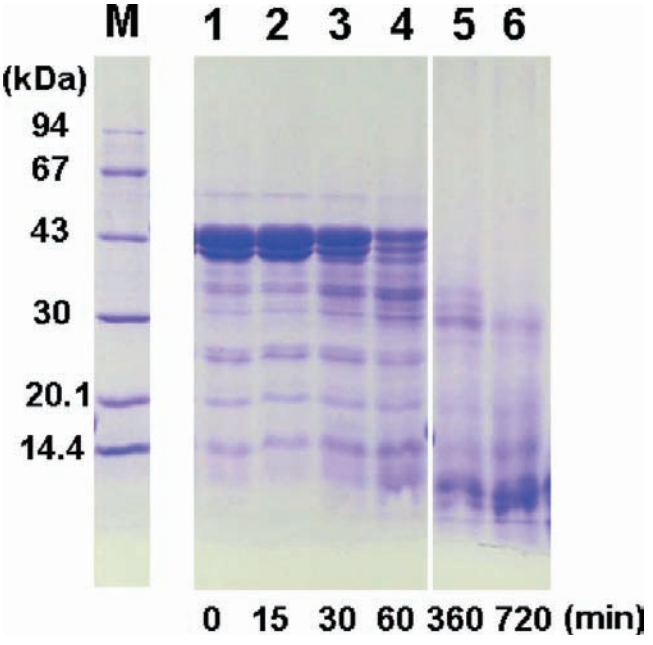

Figure 5. SDS-PAGE profiles of untreated and heated phaseolin samples: lanes 1−6, the phaseolin samples heated at 85 °C for 0, 15, 30, 60, 360, and 720 min, respectively.

DISCUSSION

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 (*17*). 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. In the present work, the phaseolin aggregates, formed by heating (at 85 °C) at pH 2.0, met all of these criteria for amyloid fibrils (although the β-type structure of the phaseolin (7S globulin) aggregates at < 720 min was much less than that of the untreated) and could thus be considered to be a kind of amyloid fibril. The formation of amyloid fibrils from the phaseolin was highly dependent on the period time of heating. Uniform flexible fibrils seemed to be formed only for an appropriate time period, that is, in the range of 60−360 min. This is the first direct observation for the formation of amyloid fibrils from legume 7S globulins (vicilins) at acidic pH.

A large number of recent studies have been aimed at improving our understanding of the mechanisms of fibril assembly or

formation, and it seems that partial unfolding of the native protein structure is a key step in the formation of protofibrils and fibrils (*1*). At pH 2.0 and ionic strength 20 mM, the denaturation temperature of the phaseolin component was about 76 °C (*13*), which is much less than that (85 °C) applied in the present study. Thus, the heating clearly resulted in almost complete denaturation and subsequent aggregation of the denatured proteins. Conformational changes are commonly recognized to play a vital role in the aggregation process, because they lead to the exposure of initially buried hydrophobic clusters, which may promote the formation of initial aggregates or protofibrils (*22*). However, the nature of the aggregation process is affected by the interactions between the proteins or denatured aggregates, especially electrostatic repulsion and hydrophobic interactions. In the present work, considerable changes in secondary, tertiary, and quaternary conformations of the phaseolin were observed, although it was heated only for a short heating time (15 min) (**Figure 4**). Correspondingly, different aggregates with heights ranging from 1.0 to 4.0 nm were formed in this period (**Figure 2A**). The phenomena suggest that the initial aggregates formed at 15 min came from different aggregation pathways, such as amorphous aggregation or amyloid aggregation.

It should be noted that the protein denaturation and/or aggregation process was also accompanied by the hydrolysis of the polypeptides (**Figure 5**). A similar phenomenon during amyloid fibril formation by heating at pH 2.0 and low ionic strength has been observed for whey protein samples (*8*). The acid hydrolysis in the present case seems to be important for the formation of stable and highly ordered amyloid fibrils for the phaseolin. Upon heating up to 60 min, the major polypeptides of the phaseolin were gradually disrupted into polypeptide fragments with lower molecular mass. Concomitantly, the heights of the formed aggregates (e.g., at 60 min) became relatively uniform (around 2.0 nm), and the length of the formed fibrils distinctly increased (**Figure 2B**). The transition of the initially formed amorphous aggregates (with heights of about 4.0 nm) into the fibrils was clearly due to the further acid disruption of the initial aggregates. The initially released fragments were further disrupted into smaller fragments, upon further heating to 360 or 720 min. On the other hand, it was observed that long amyloid fibrils were formed (e.g., at 360 min), and these fibrils were entangled together to form fibril clusters with much greater heights (~10 nm) (**Figure 2C**,**D**).

Interestingly, the β-strand content progressively increased from 22.9 to 37.5% as the heating time increased from 15 to 720 min (**Table 1**), although the hydrolysis of the polypeptides or fragments occurred, and the tertiary and quaternary conformations gradually unfolded, as evidenced by the near-UV CD spectra shown in **Figure 4B**. The increased β-strand content is a direct indication of the formation of amyloid or amyloid-like fibrils, which clearly accounted for the increased Th T fluorescence intensity (**Figure 3A**).

From the above-mentioned statements, it can be proposed that the amyloid fibril formation of phaseolin at pH 2.0 by heating also underwent at least three steps of aggregation process. The first step is the heat-induced denaturation and aggregation (including amorphous and amyloid aggregation) of the phaseolin, and the period of this step is relatively short (about 15 min) because the applied temperature is much higher than the denaturation temperature of the phaseolin at pH 2.0. This denaturation and/or aggregation process is accompanied by significant changes (disruption) in secondary, tertiary, and quaternary conformations. Second, the hydrolysis of the polypeptides or the initially formed aggregates gradually becomes distinct, and as a result, the released polypeptide fragments associate together to form the fibrils, possibly due to balanced hydrophobic and electrostatic repulsion interactions. The gradually increased β-strand content (relative to 15 min) is a reflection of the rebuilding of the ordered secondary structure during the fibril formation. At this step, more released polypeptide fragments are involved in the fibril formation. It seems that the amyloid aggregation predominates the behavior of the whole aggregation at this step. At long heating times (i.e., > 360 min), the formed fibrils entangle together to form fibril clusters with much less flexibility. In all of these steps, a number of polypeptide fragments with heights of less than that (about 1.6 nm) for untreated phaseolin can be observed (**Figure 2**), further suggesting that the hydrolysis of the polypeptides or the release of smaller polypeptide fragments is the determining factor for the amyloid fibril formation from legume 7S globulins at acidic pH. The above-mentioned processes for the amyloid fibril from phaseolin (trimer) at pH 2.0 upon heating are illustrated in **Figure 6**.

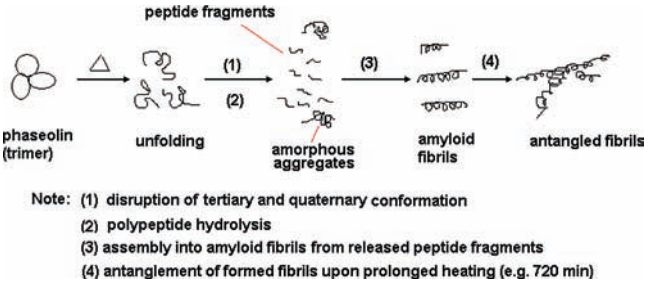

Figure 6. Schematic illustration for the formation of amyloid fibrils from phaseolin (trimer) upon heating at pH 2.0.

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