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REGULAR ARTICLE

Antioxidant ferulic acid prevents the aggregation of bovine β-lactoglobulin *in vitro*

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Abstract. Amyloids, a well-ordered β-sheet-enriched structural network, can be broadly defined as insoluble protein aggregates that are linked to a wide variety of diseases including systemic amyloidosis and some neurodegenerative disorders. Ferulic acid (FA), a phenolic acid, abundant in antioxidant and efficient pharmaceutical has beneficial effects against several ailments. Based on this, we have investigated the protective role of FA on amyloid formation of bovine β-lactoglobulin (β-lg), a model globular protein. Using a set of *in vitro* biophysical methods, such as UV-Vis spectroscopy, fluorescence, circular dichroism, transmission electron microscopy, etc., our research group has concluded that FA significantly inhibits the heat-induced amyloid formation of β-lg and this inhibitory effect is dose-dependent. Exposed surface hydrophobicity of β-lg amyloid fibrils decreased significantly in the presence of FA. Docking study revealed that ionic and hydrogen bonding interactions between FA and β-lg prevented protein conformational changes leading to fibrillation. We anticipate that our finding would give an insight into the protein aggregation inhibited by the antioxidant compound, FA and pave the way for finding and developing other new small molecules (protein misfolding inhibitors) that give similar result against amyloid fibril formation and its allied neurodegenerative disorders.

Keywords. Antioxidant; ferulic acid; β-lactoglobulin; aggregation.

1. Introduction

In the present area of research, a very interesting topic is the alteration of native (often soluble) proteins into non-native folded fibrillar structures; these are often not soluble in various solvents as well as in water. These protein fibrils are usually called amyloid fibrils and are the hallmark for numerous ailments, including Alzheimer's, Huntington's, type II diabetes, Parkinson's, prion-associated encephalopathy diseases and others.[1–6] Amyloid fibrils are highly organised polypeptide aggregates and are rich in β-sheet secondary conformation.[7] These fibrils are stable against temperature,[8] hydrolytic pressure,[9] proteolytic enzymes[10] and denaturants.[11] Several proteins and peptides, e.g. amyloid β-peptide (Aβ), β-lactoglobulin (β-lg) islet amyloid polypeptide (IAPP), insulin, α-synuclein, and transthyretin have been identified as amyloidogenics,[12–16] but it has been observed that there is no similarity in primary structure among them.[17] The aggregation pattern of such peptides and proteins differs owing to their differential forms.[18–20]

In recent research work, a number of endeavours have been applied to find or design the compounds which can prevent the formation of these toxic oligomeric species or break up the pre-formed fibrils. Several working parameters, e.g. concentration of protein, pH of experimental solution, ionic strength of the reaction medium, reaction temperature, existence of co-solvents, etc., can be altered to modulate the aggregation process of β-lg into the oligomers or fibrils.[21–23]

Small organic molecules (either from natural origin or synthetically derived) play a significant role in

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amyloid inhibition. Nanoparticles,[24] small molecules having antioxidative potency, e.g. vitamin C,[25] nicotine,[26] kaempferol,[27] and vitamin B12[28] have been found to reduce oxidative strain associated with neurodegenerative disorders. Curcumin, a well-known phenolic molecule, showed anti-amyloid efficacy and has been pondered for the Alzheimer's disease (AD) therapy.[29] Borana *et al*., showed that curcumin prevents lysozyme fibril formation, which is not related to any neurodegenerative disorders.[30] A polyphenolic compound, epigallocatechin gallate has been shown to inhibit the formation of noxious amyloid of Aβ[31] and α-synuclein.[32] A well-established fact is that these phenolic compounds can reduce the risk of developing age-related neurodegenerative disorders, e.g. AD, Parkinson's, and Huntington diseases, and also, they prevent chronic ailments, for instance, cancers and cardiovascular diseases. Literature reports have demonstrated that they bind with proteins[32,33] and form complexes through weak forces, including hydrogen bonding and hydrophobic interactions. Along with the polyphenols, the phenolic acid present in the plant foods and beverages also shows anti-amyloid role against proteins/peptides linked with diseases, e.g. Alzheimer's[34] and diabetes mellitus.[35] Ferulic acid (FA), a ubiquitous phenolic acid compound, present in plants as well as in many foods has a potent antioxidant activity.[36] The structure of FA (4-hydroxy-3-methoxycinnamic acid), shown in Figure 1 is similar to the structure of curcumin, which directly affects the Aβ peptide aggregation[29,37] as well as can dissociate the pre-formed Aβ fibrils.[38] Likewise, FA has been shown to inhibit the Aβ fibril formation[34] and destabilize the pre-formed fibrils.[39] Accumulating evidence showed that FA has potential therapeutic role in cancer and cardiovascular disorders as well as neurodegenerative disorders.[40–43]

Beta-lactoglobulin, (β-lg), the principal protein constituent in the whey of ruminant milk (MW 18.4 kDa), is a compactly folded small globular protein having 162 amino acid residues. In this study, our research group has chosen the bovine β-lg as it is extensively studied owing to its availability and high dietary importance relevant to the food industry. This

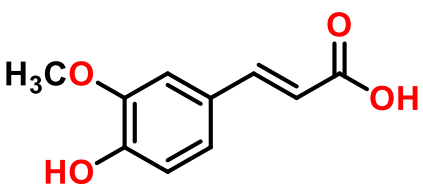

Figure 1. Chemical structure of *trans*-ferulic acid.

macromolecule is water soluble, consisting nine antiparallel β-strands and one α-helix segment, in which the hydrophobic chains are mainly buried.[44,45] Additionally, β-lg has two intra-molecular disulfide linkages (Cys66-Cys160 and Cys106-Cys119) and one free –SH unit (Cys121) which is buried between the major α-helix and β-barrel.[46] β-lg exists as a dimmer at room temperature with pH ranging 5.5–7.5. However, in acidic medium (pH<3.5), it dissociates into monomeric forms due to electrostatic repulsions between the subunits. The heat-induced aggregation of β-lg has been widely documented, but the mechanism is not hitherto fully known.[47–49] Based on the experimental factors, β-lg forms either amorphous aggregates or amyloid fibrils.[49,50] In acidic medium, β-lg molecule can form fibrils above 75°C. Nonetheless, prolonged heating at 75°C instigates the oxidation reaction endorsing the exchange between –SH groups and S–S linkages.[51]

Although FA can inhibit the formation of several amyloid fibrils, the inhibitory action of FA against β-lg has not hitherto been accounted. In this present study, we investigated the inhibitory effects of FA on heat-induced fibril formation of β-lg *in vitro*. We studied the influence of FA on the morphology of β-lg fibril by using transmission electron microscopy (TEM). The inhibition of β-lg amyloid fibril formation has been confirmed by Congo red (CR) and Thioflavin T (ThT) binding studies, utilizing UV-Vis and fluorescence spectroscopy. The secondary conformational changes during fibril formation and its inhibition upon addition of FA have been directly monitored by CD and FTIR spectroscopic measurements. Moreover, the outcomes of molecular docking study showed the considerable mode of interaction and sites of interaction between β-lg and FA.

2. Experimental

2.1 *Reagents and chemicals*

Isolation and purification of bovine β-lg from cow's milk was performed as previously described.[52] Total purified material was lyophilized and kept at 4°C for further use. As the extinction coefficient of β-lg (0.959 mg^{-1} mL^{-1} cm^{-1} at 280 nm) is known, different concentrations of β-lg solutions were made by dissolving lyophilized protein in Milli-Q water and checking the absorbance at 280 nm. Sodium dihydrogen phosphate (NaH_2PO_4), *trans*-ferulic acid (FA, 128708) were obtained from Sigma Aldrich (USA), and different fluorescent probes, namely 8-anilinonaphthalene-1-sulfonic acid ammonium salt (ANS), Congo red (CR) and Thioflavin T (ThT) were purchased

from Sigma Chemical (St. Louis, USA) and utilized as such. All the other reagents utilized in our experiments were of analytical grade or high purity reagent available.

2.2 *Protein sample preparation*

Stock solution of β-lg, used in each experiment was made in NaH_2PO_4 buffer (10 mM) at pH 7.4 to get a final β-lg concentration of 3 mM. Stock solution of FA, because of its poor aqueous solubility was prepared freshly by first dissolving it in ethanol and then diluting with NaH_2PO_4 buffer in order to get the final FA concentration in solution of 3 mM. Appropriate volumes of β-lg, FA stock solutions and 10 mM buffer were mixed to get 1:0, 1:1, 1:3, 1:4, 1:5 and 1:10 molar ratio of β-lg:FA. β-Lg fibril formation was monitored by heating these β-lg-FA mixtures at 75°C over a period of 1 h prior to all experiments. The required β-lg concentrations were attuned for all the experiments.

2.3 *Intrinsic fluorescence study*

Intrinsic fluorescence was measured by using a Shimadzu spectrofluorometer (Shimadzu 5301 PC) at an excitation wavelength of (λ_{ex}) 295 nm. The spectral resolution for both excitation and emission was set at 5 nm. Fluorescence experiments were conducted with all protein solutions as depicted above in 10 mM buffer, pH 7.4 and at 25°C. β-lg (concentration 13.6 μM) intrinsic fluorescence emission were measured by using a quartz cell with a path length of 1 cm in the wavelength ranging from 300 to 400 nm, and all measurements were done in triplicate.

2.4 *1-Anilinonaphthalene-8-sulfonate (ANS) fluorescence study*

Surface hydrophobicity of β-lg fibrils in the absence and presence of FA was determined by 1-anilinonaphthalene-8-sulfonate (ANS), a polarity-sensitive fluorescent probe.[53] For this, a stock solution of ANS was added to each aliquot of β-lg solution to get a final ANS concentration of 30 μM. Typically, ANS concentration was 50 molar excess of protein concentration. The ANS-fluorescence emissions (400–650 nm) were measured using Shimadzu RF-5301 PC with excitation at 380 nm. Slit widths were set at 5 nm for both excitation and emission. Each spectrum was blank corrected. The data reported were means of three replicates.

2.5 *Thioflavin T (ThT) fluorescence assay*

Thioflavin T, a dye bound to amyloid fibrils, shows enhanced fluorescence emission at 480 nm.[54] To explore and compare the fibrils formed by protein (β-lg) in the absence and presence of FA, the following assay was employed using ThT as a probe. Concisely, 250 μL of sample solutions (1 mg mL^{-1}) were added separately to 20 μL ThT solution (stock 3.13 mM ThT in buffer (10 mM), pH 7.4) containing 1.73 mL of buffer (pH 7.4, 10 mM), mixed and incubated for 30 min. The final ThT and protein concentration were 30 μM and 6.8 μM, respectively. After 30 min of incubation with ThT dye, the ThT fluorescence of working solutions were studied by a Shimadzu spectrofluorometer (Shimadzu 5301 PC) with an excitation wavelength of 450 nm[22] and emission recorded from 460 nm to 650 nm. Excitation and emission slit widths were same (5 nm). All solutions were measured at least thrice, and the data taken were averaged. All measurements were done at 25°C.

2.6 *Rayleigh light scattering (RLS) study*

The heat-induced β-lg aggregation in the absence and presence of FA was quantified by RLS experiments. After exciting the working solutions at 350 nm, mentioned in figure caption 3B, the fluorescence intensities were recorded at 350 nm in a Shimadzu spectrofluorometer (Shimadzu 5301 PC). All sample solutions were prepared in NaH_2PO_4 buffer (10 mM) at pH 7.4, and we used a quartz cell and 1 cm path length for measurements. Emission and excitation slits were 5 nm. These experiments were performed in triplicate.

2.7 *Congo red (CR) assay*

To evaluate the influence of FA on formation of heat-induced β-lg aggregates, CR assay was applied. The CR absorption spectra were recorded from 400 to 600 nm. Samples for aggregation experiments were prepared as follows. For this, 60 μL heated working solutions (5 mg mL^{-1}) was mixed with 500 μL of CR solution (100 μM) in buffer (10 mM, pH 7.4). Requisite volume of buffer solution was added to get a total volume of 2 mL for each solution.[55] Concentrations of CR and protein were 25 μM and 8.2 μM, respectively.

2.8 *Circular dichroism (CD) spectroscopy*

Far-UV CD spectra of heat-treated β-lg in the presence and absence of different concentrations of FA in 10-mM phosphate buffer (pH 7.4) were completed using Jasco Spectropolarimeter (J-815) with a quartz cell (path length=1 nm) at 20°C. β-lg concentrations (0.25 mg mL^{-1}) were kept constant for far-UV CD measurements. A far-UV CD spectrum was also recorded for a 0.25 mg mL^{-1} native protein solution in the same phosphate buffer. All data reported here was average of three sequential experiments. Averaged scans of solvent spectrum were subtracted from protein (β-lg) scans to obtain the final spectrum. The far

UV–CD spectra were fixed into a curve-fitting program CDNN 2.1 to ascertain the percent (%) of secondary structures present in β-lg under different experimental conditions.

2.9 *Dynamic light scattering (DLS) measurements*

The diffusion of small particles in solution induces fluctuations in the intensity of the scattered light. DLS detects these fluctuations using an autocorrelator on a microsecond time scale and is used to analyze the distribution of molecules and supramolecular aggregates as it is very sensitive to particle size.[56] DLS measurements were conducted with heated β-lg solutions in the absence and presence of different concentration of FA using Zetasizer Nano (Malvern Instrument, UK). The sample solutions were illuminated by a 633-nm laser. A rectangular 2-mL cuvette was used for holding samples having 10 mm path length. All experiments were performed at 20°C, taking 2 mL solutions (0.25 mL of sample and 1.75 mL buffer). The time-dependent autocorrelation function was obtained with 12 acquisitions for every run. The data reported were means of five experiments.

2.10 *Fourier transform infrared (FTIR) spectroscopy*

FTIR spectra were collected by utilizing a Spectrum 100 FT-IR spectrometer (Perkin-Elmer) at a nominal resolution of 2 cm^{-1} in N_2 atmosphere. For FTIR experiments, 50 μL native and heattreated β-lg solutions (in the absence and presence of the FA) 20 mg mL^{-1} were taken in a Microcon filter device and diluted with D_2O (200 μL). Then, the solution was immediately centrifuged (4000× g, 10 min) until the volume reached up to 50 μL. Another 200 μL of D_2O was added and centrifuged. This D_2O exchange process was repeated thrice. Finally, the D_2O exchanged protein solutions were taken for FTIR scans (1550–1750 cm^{-1}). Spectrum of D_2O background at pD 7.0 was taken and subtracted from the raw sample spectrum. The band positions were then assigned according to the standard protocol.[57]

2.11 *High resolution transmission electron microscopy (HRTEM)*

A HRTEM (JEOL-HRTEM-2011, Tokyo, Japan) with an accelerating voltage of 80–85 kV in different magnifications was applied using 5 μL of sample solutions to observe the images of β-lg aggregates (β-lg concentration 10 μM). Prior to HRTEM assay, all heat-treated sample solutions were sonicated for 1 h and diluted 50–150 times with buffer (pH 7.4). Two drops of each diluted sample were spotted onto a carbon-coated copper grid (300 C mesh, ProSciTech). After 20 s, excess sample was soaked away from the grid using filter paper and subsequently, a drop of 2% uranyl acetate (Sigma, Steinheim, Germany) in H_2O was added for 15 min. Excess reagent was removed, left for air drying and air-dried specimens were utilized for imaging study. All samples were left to equilibrate for 6 h before getting the image.

2.12 *Molecular docking study*

The AutoDock 4.2.0 based docking studies of FA molecule with β-lg (2BSY) were carried out. The structure of FA used in docking after minimized its energy by DFT optimization using Gaussian 09W. Lamarckian genetic algorithm (LGA) was utilized for molecular docking. In this calculation, 126 × 126 × 126 grid box was used.

3. Results and Discussion

3.1 *Microenvironment change of β-lg investigated by intrinsic fluorescence*

To study the effect of FA on the heat-induced conformational alteration of β-lg, intrinsic fluorescence spectral measurements were performed. The inhibitory effect of FA was examined at five different molar ratios of FA, as stated in the caption of Figure 2A. Native β-lg has two tryptophan moieties and the intrinsic fluorescence property of β-lg is almost exclusively due to the tryptophan 19 (Trp 19) residue.[58] It exhibits the characteristic fluorescence emission maximum at 334 nm by fixing the excitation wavelength at 295 nm. As evident from Figure 2A (profile b), in the absence of FA, heated β-lg exhibited a distinct fluorescence spectrum as native β-lg. The red shift of the tryptophan fluorescence spectrum of heat-exposed β-lg reflected significant enhancement of the accessibility of tryptophan moiety (Trp 19) of protein to the solvent. Figure 2A (profiles c–g) showed the fluorescence intensity of β-lg decreased regularly with gradual addition of FA into a fixed concentration of β-lg solutions. This suggested a concentration-dependent change in the intrinsic fluorescence of the heat-exposed β-lg in presence of FA (Figure 2A). Additionally, much lower fluorescence intensities were found in β-lg:FA (1:5 and 1:10) systems (profiles f&g of Figure 2A) than other ratios of β-lg:FA systems. The decrease in fluorescence intensities, in fact, may be due to the change in polarity of microenvironments around the tryptophan moieties in presence of FA. At 1:10 molar ratio of β-lg to FA, the highest inhibitory effect of FA was noticed from the lowest fluorescence intensity of the solution, shown in profile g

(Figure 2A). Thus, FA showed inhibitory effect on temperature-induced amyloid formation of β-lg.

At this point, the protein-structure deformation effect of ethanol, which comes from the ethanolic solution of FA needs to be discussed here. In these experiments, the stock solutions of FA and the protein are 10 mM and 183 μM, respectively. Overall, to prepare 2 mL β-lg (45 μM) and FA solution of 1:10 ratio, the required ethanol is 4.5% ethanolic solution. Previous studies showed that the ethanol has the ability to form aggregates of β-lg under similar heating conditions at 30% and above ethanol concentrations.[59a–c] Thus, the organic solvent ethanol accelerates only the aggregation of β-lg at higher concentrations. In our cases, the concentration of ethanol used was sufficiently low. Hence, it is clear that no β-lg aggregation or disaggregation effect was possible with this low ethanol concentration.

3.2 *ANS-fluorescence study to observe the hydrophobicity change of β-lg*

The change of hydrophobicity of β-lg during thermal aggregation in the absence and presence of FA was monitored by collecting the ANS emission spectra. Evidently, ANS, a fluorescent hydrophobic probe, binds to molten globule state of proteins and shows fluorescence intensity.[60] The interaction of ANS with β-lg may be due to both electrostatic and hydrophobic interactions.[61] Figure 2B shows the ANS fluorescence spectra of heat-treated β-lg in sodium phosphate buffer (pH 7.4). Generally, heat-induced β-lg showed ANS fluorescence signal at around 480 nm at pH 7.4 (profile b, Figure 2B) with a greater ANS intensity than native β-lg (profile a, Figure 2B). This rise of fluorescence intensity may be ascribed to further entrance of ANS to hydrophobic loops present in thermally exposed β-lg compared to native β-lg. Thus, increase in hydrophobic loops enhances the protein–protein interactions leading to more thermal aggregation of β-lg.[62] However, we obtained significant different results when β-lg was incubated (75°C, 1 h) in the presence of different FA concentrations, mentioned in figure caption (profiles c–g, Figure 2B). The β-lg fluorescence intensity was found to be decreased considerably, indicating less disclosure of hydrophobic loops of β-lg, and thus, FA efficiently inhibited the amyloid fibril formation of β-lg. Further, the lowest fluorescence intensity of β-lg-FA solution was observed at much higher concentrations of FA (10-fold molar excess) (profile g, Figure 2B), demonstrating a minimum binding of ANS. This was strongly supported by our ThT data given below.

3.3 *Formation of aggregates monitored by ThT assay*

To determine the inhibitory activity of FA on temperature-induced β-lg amyloid fibrillation, we used Thioflavin T (ThT) dye which is a non-covalent reporter of amyloid aggregates and often utilized to report the aggregation behavior of proteins. Usually, ThT binds to amyloid aggregates and as a consequence, the dye shows enhanced fluorescence emission significantly.[63] Formation of a hydrogen bond between ThT and amyloid fibrils is reported to play a

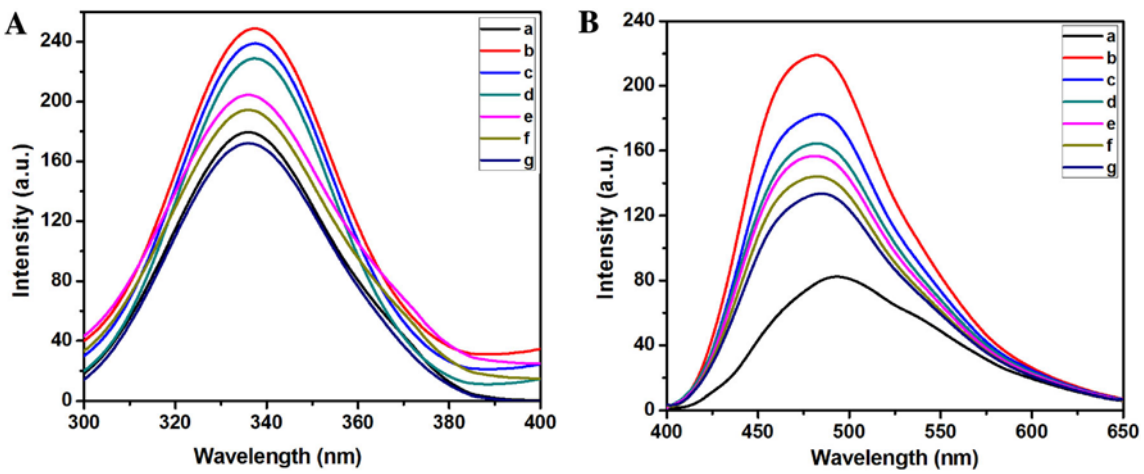

Figure 2. (A) Intrinsic tryptophan fluorescence and (B) ANS fluorescence emission spectra of (a) native β-lg, heat exposed (75°C), (b) β-lg, (c) β-lg:FA (1:1), (d) β-lg:FA (1:3), (e) β-lg:FA (1:4), (f) β-lg:FA (1:5) and (g) β-lg:FA (1:10). β-lg concentrations throughout all emission experiments were kept at 0.25 mg mL^{-1}. Results were the mean of three different experiments.

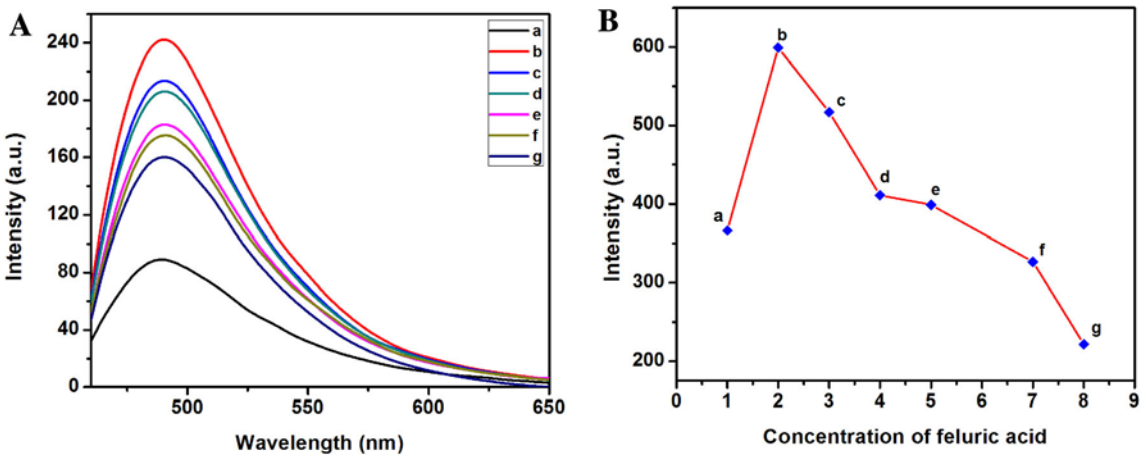

Figure 3. (A) ThT fluorescence spectral changes of heated (75°C for 1 h) β-lg in the absence and presence of FA. Line (a) native β-lg, line (b) heat treated β-lg, lines (c–g) correspond to heated β-lg in presence of FA: (c) β-lg:FA (1:1), (d) β-lg:FA (1:3), (e) β-lg:FA (1:4), (f) β-lg:FA (1:5), and (g) β-lg:FA (1:10). β-lg concentrations were 0.25 mg mL^{-1}. (B) Effect of increasing concentration of FA on the fibrillation of β-lg heated at 75°C in NaH_2PO_4 buffer (pH 7.4, 10 mM) as monitored by RLS measurement. β-lg concentration was 0.25 mg mL^{-1}. Points a–g: (a) native β-lg, heat treated: (b) β-lg:FA (1:0), (c) β-lg:FA (1:1) (d) β-lg:FA (1:3), (e) β-lg:FA (1:4), (f) β-lg:FA (1:5), and (g) β-lg:FA (1:10). Results were the mean of three different experiments.

role in the enhancement of ThT fluorescence intensity at maximum wavelength (490 nm).[64] Figure 3A displays the ThT fluorescence intensities of different β-lg samples. We have observed a strong increase in ThT emission intensity of heated β-lg at 490 nm (Figure 3A, curve b), typical alter for amyloid fibrils. Even though the interaction between ThT and amyloid fibrils is not yet known clearly, the literature reports[65,66] suggest that this interaction depends on the amyloidogenic protein along with binding pattern.[67] Nonetheless, enhanced fluorescence intensity at 490 nm confirms the formation of β-lg amyloid fibrils. ThT intensity of β-lg decreased regularly with the variation of FA concentration (Figure 3A, curves c–g), suggesting the efficient concentration-dependent inhibitory effect of FA on the fibril formation of β-lg. In β-lg–FA mixture with 1:10 molar ratio, the ThT showed lowest fluorescence emission intensity and this indicated that the maximum inhibitory effect of FA on β-lg fibrillation appeared at 10 times excess concentrations. Thus, FA played a significant role in the protective effect of β-lg fibrillation. This result also corroborated with TEM imaging of different β-lg fibrils.

3.4 *Rayleigh light scattering (RLS) detected β-lg aggregates*

RLS is commonly utilized to detect aggregation of proteins.[68] The result in Figure 3B showed the RLS data of heat-induced β-lg aggregates in the absence and presence of FA. Usually on thermal denaturation, the structure of a protein is lost and it forms aggregates. An increase in scattering intensity was observed in thermally exposed β-lg (point b) than native β-lg (point a), signifying the formation of β-lg aggregates. Additionally, points c–g showed the RLS intensities of β-lg in the presence of different molar ratios of FA. In these points, the fluorescence intensity decreased gradually with increasing FA concentration. Lowest scattering intensity of the solution (one-third) was observed when β-lg to FA molar ratio reached 1:10, indicating the maximum inhibition of aggregation as evidenced by ThT analysis (point g, Figure 3B). Hence lowering RLS intensities of the incubated β-lg-FA samples corroborated the thermal disaggregation of β-lg by FA.

3.5 *CR assay*

The amyloid fibril aggregates present in solutions can also be detected by CR binding analysis. CR, a secondary diazo dye interacts with β-sheet structures[21] and exhibits a bathochromic shift in the absorbance maximum (480–490 nm). The heated β-lg without FA at pH 7.4 showed absorbance maximum at 490 nm (Figure 4A, profile b). This shift in the absorbance maximum of heated β-lg without FA might have occurred due to the formation of amyloid-like aggregates. The addition of FA to β-lg significantly affected

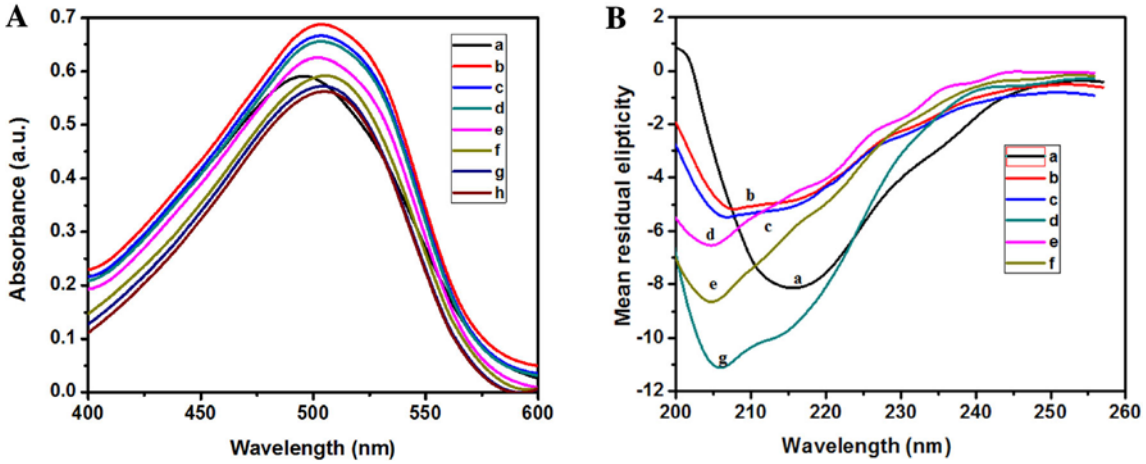

Figure 4. (A) CR absorption spectral alteration demonstrating the inhibition of thermal (75°C, 1 h) aggregation of β-lg by FA. Line (a) native β-lg, lines (b–g) correspond to heated β-lg in the presence of different concentration of FA, (b) β-lg:FA (1:0), (c) β-lg:FA (1:1), (d) β-lg:FA (1:2), (e) β-lg:FA (1:3), (f) β-lg:FA (1:4) (g) β-lg:FA (1:5) and (h) β-lg:FA (1:10). Final concentrations of β-lg in solutions were 0.5 mg mL^{-1}. (B) Far-UV CD spectra (200–260 nm) of (a) native β-lg, heated (75°C, 1 h), (b) β-lg along, (c) β-lg:FA (1:1), (d) β-lg:FA (1:3), (e) β-lg:FA (1:5) and (f) β-lg:FA (1:10) showing secondary structural changes in thermal fibrillation of protein.

the thermal aggregation profile of the protein in a dose-dependent way, and a decrease in absorption intensity was noticed (Figure 4A, profiles c–h). The decrease in absorption intensity of incubated β-lg samples measured in the presence of FA indicated that FA inhibited the formation of β-sheet structures. Adding a high molar excess of FA (1:10 molar ratio of β-lg to FA) resulted in the lowest absorbance for β-lg-FA mixture (profile g, Figure 4A), indicating the maximum inhibitory effect of FA at this particular molar ratio. Thus, at 1:10 molar ratio of β-lg to FA, FA stabilized more for the monomeric and dimeric structures of β-lg, and thus, can significantly suppress the formation of fibrillar aggregates. This result supported the ThT fluorescence measurements.

3.6 *Changes in secondary structure of β-lg in the presence of FA*

Far-UV CD technique was employed to investigate the potential effect of FA on the secondary structural transformation of β-lg. β-lg was incubated with or without FA for 1 h (75°C) and the presence of β-sheet was determined by CD spectroscopy. Generally, CD spectra provided the typical information about β-sheet structure linked with amyloid fibril. CD spectra of incubated solutions were carried out by scanning the spectra in the spectral region of 200–260 nm at different concentrations of FA. CD spectra of β-lg in the presence and absence of FA is represented in Figure 4B. CD spectra of native β-lg (profile a, Figure 4B) showed two negative bands at 207 and 216 nm. These two bands of β-lg represented the existence of ordered secondary structural content that contained α-helix and β-sheet.[69] After heating at 75°C, the CD spectrum of β-lg showed a significant shift of the band position (profile b, Figure 4B). The heated β-lg showed a decrease in α-helix content and an increase in the β-sheet (Table 1). We have also analyzed the change of secondary structure of heated β-lg with FA at different molar concentrations (profiles c–g, Figure 4B). It has been shown that at 1:1 molar ratio of β-lg:FA, a very small change of peak position with negative ellipticity value differing from heated β-lg was observed. CD spectra of β-lg:FA (1:3) showed lower MRE values at 215 and 207 nm (profile d, Figure 4B). It is of importance that the shape of the CD spectra for β-lg at higher ratios of FA (1:5 and 1:10, molar ratio) showed more negative MRE values with the bands between 206 and 207 nm, respectively and the disappearance of band near 215 nm, indicating the structural transitions leading to the disaggregation of fibrillar structure. The difference in MRE values could be related to the different concentrations of FA, an inhibitor of protein aggregation. One interesting observation was the maximum inhibitory effect of FA at 1:10 molar ratio on the thermal fibrillation of β-lg. The calculated results obtained from CD (Table 1) also revealed that the secondary structure of β-lg was strongly affected by the addition of FA. Our result thus support that ferulic acid (FA), a natural oxidant, can inhibit the fibrillation of β-lg in a concentration-dependent way.

Table 1. Percentage of α-helix, β-sheet, β-turn and random coil of native β-lg, heated β-lg with or without FA as determined by CD calculations.

Sample	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random coil (%)
Native β-lg	11.6	38.43	12.46	37.51
Heated β-lg	10.67	41.33	12.24	35.76
Heated β-lg:FA (1:1)	10.77	40.22	11.59	37.42
Heated β-lg:FA (1:3)	11.98	39.67	12.80	35.55
Heated β-lg:FA (1:5)	11.69	39.14	12.52	36.65
Heated β-lg:FA (1:10)	11.56	38.16	12.84	37.34

3.7 *Dynamic light scattering (DLS) revealed the size of β-lg aggregates*

To corroborate our above-mentioned result, we employed DLS experiments. The β-lg aggregates were analyzed through DLS experiments to analyze the effects of various FA ratios on the average hydrodynamic radii of β-lg aggregates. Scattered light intensity versus size in radius of different protein solutions was displayed in Figure 5A. Curve 'a' in Figure 5A showed the size distribution of native β-lg. The size distribution curves (Figure 5A, b–f) of heated (75°C) β-lg with and without FA (1–10 molar ratios) showed the formation of different size aggregates of protein. DLS result showed a greater light scattering intensity of heated β-lg (Figure 5A, curve b) as a result of greater particle size.[70] With the addition of FA, the scattering intensity of β-lg solution decreased. At β-lg:FA = 1:1 molar ratio, the size of the aggregates decreased minimally in the range of 30–350 nm and small-size diameter became prominent (Figure 5A, curve c). Further increase in the molar concentration of FA showed the size of the aggregates decreased gradually (Figure 5A, curves d–f). From this analysis, we again showed that at 1:5 and 1:10 molar concentration ratio of β-lg:FA, FA strongly suppressed the fibrillation of β-lg and the size of aggregates decreased largely in the Figure 5A, curve e and f respectively. Thus, on heating β-lg with FA at 75°C, large size β-lg particles (aggregates) decreased and the light scattering was controlled by FA with smaller diameter. Therefore, according to the results, it can be concluded that FA inhibited the formation of fibrillar aggregates of β-lg.

3.8 *Secondary structural composition revealed by ATR-FTIR analysis*

IR technique is commonly employed to understand the secondary structural composition of protein. Thus, this technique was employed to confirm the β-sheet

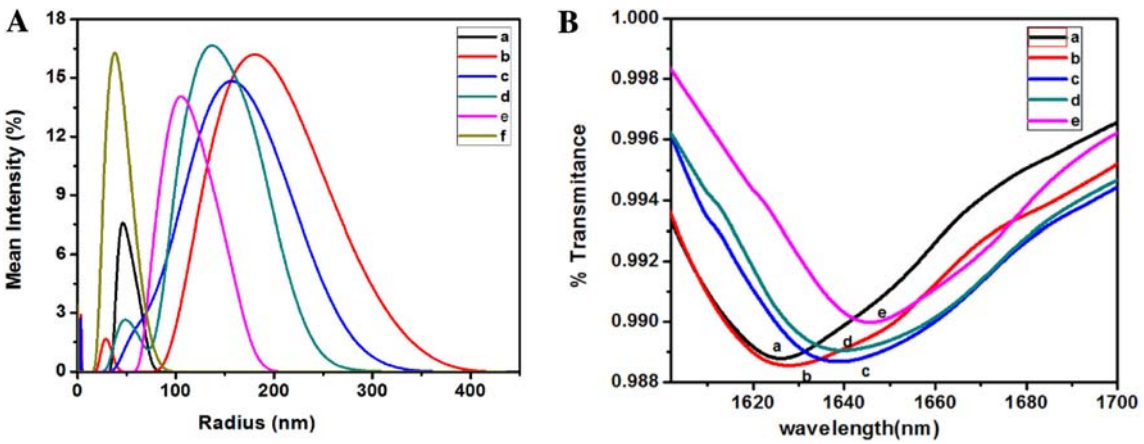

Figure 5. (A) A plot of mean intensity versus radius of aggregates of native β-lg (curve a), β-lg:FA (1:0) (curve b), β-lg:FA (1:1) (curve c), β-lg:FA (1:3) (curved d), β-lg:FA (1:5) (curve e) and β-lg:FA (1:10) (curve f). Samples (curve b–f) were treated at 75°C in 10 mM sodium phosphate buffer (pH 7.4, 1 h) and concentration of β-lg was kept at 54.35 mM. Every spectrum is mean of 48 scans. (B) FT-IR spectra of amide-I region of β-lg:FA mixture at different molar ratios [native (a), heated: (b) 1:0, (c) 1:1 (d) 1:5 and (e) 1:10]. Sample solutions were incubated in buffer at 75°C. Concentrations of β-lg were 20 mg mL^{-1}. Reported every spectrum was a mean of 32 scans in D_2O measured at 25°C.

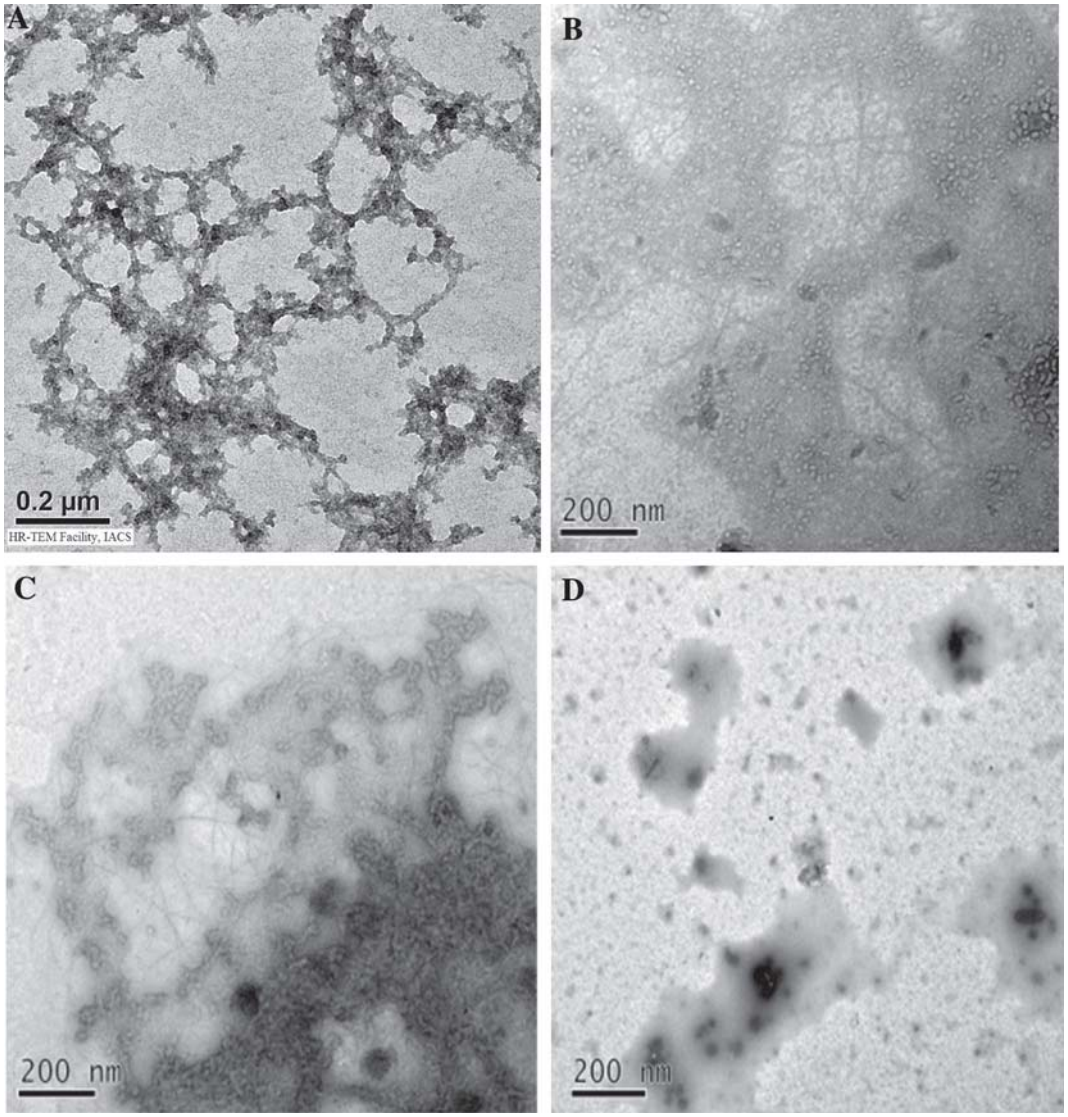

Figure 6. Selected TEM images of β-lg fibrils. (A) Heat-treated β-lg, (B) heat-treated β-lg:FA (1:1), (C) heat-treated β-lg:FA (1:5), and (D) heat-treated β-lg:FA (1:10). All samples were heated at 75°C for 1 h.

structure formation during fibrillation of β-lg (75°C) in the presence of FA. The secondary structure of β-lg can be acquired on the basis of infrared assignment of the amide-I band ranging from 1600 to 1700 cm^{-1} of ATR-FTIR spectra. The amide-I band of native β-lg molecule appears at around 1629 cm^{-1} (Figure 5B, profile a) which is the typical feature for the protein like β-lg having predominant β-sheet structure.[62] In the absence of FA, the IR spectrum of heated β-lg exhibited a band roughly at 1633 cm^{-1} (profile b) that indicted an increase in β-sheet structure in comparison to native β-lg owing to self-assembly of the protein. In presence of FA at 1:1 molar ratio with β-lg, FTIR showed a peak centerd at 1642 cm^{-1} (profile c) which indicated deformation or structural transition of β-sheet structure. At β-lg to FA molar ratio of 1:5 and 1:10, the peak positions were shifted between 1640 cm^{-1} and 1646 cm^{-1}, respectively (profiles d and e). Thus, the increased concentration of FA shifted the peak positions due to the structural transitions towards random coil structure. Therefore, it may be concluded that FA at higher concentration strongly inhibited the heat-induced fibrillation of β-lg. This IR results were in harmony with the conclusion obtained from our CD experiment.

3.9 *TEM results distinguished the morphology of β-lg aggregates with FA*

The HR-TEM study was also performed to ascertain whether FA affected the heat-induced aggregation and fibril formation of β-lg (Figure 6). The heat-exposed β-lg without FA formed the self-assembly (Figure 6A)

Table 2. Comparison of the classes of compounds showing protein aggregation inhibitiory activity.

Entry	Compounds (C)	Protein (P)	Mole ratio (P:C)	References
1	Curcumin	β-lg	1:1	[71]
2	Curcumin	Aβ	1:8	[72]
3	FA	Aβ	1:4	[73]
4	FA	β-lg	1:10	Present study
5	Phenol red	Aβ	1:9.6	[72]
6	Indomethacin	Aβ	1:21	[72]
7	Quinacrine mustard dihydrochloride	Aβ	1:46	[72]
8	Pherphenazine	Aβ	1:56	[72]
9	Eosin Y	Aβ	1.62	[72]

and the morphology was totally fibrillar. However, the TEM image of β-lg in the presence of FA showed that the aggregation was reversed to a different extent. When the β-lg:FA molar ratio was 1:1, no significant morphological change of fibrillar structure was observed and only intensity of aggregation was reduced (Figure 6B), but when the amount of FA was increased, the fibrillation of β-lg decreased. The amyloid fibrillation decreased significantly when the β-lg:FA molar ratios were 1:5 and 1:10 respectively (Figure 6, C&D). These findings clearly suggest that at 1:5 and 1:10 β-lg:FA molar ratios, FA showed strong protective effect on β-lg aggregation. Therefore, our *in vitro* observations demonstrate that FA has potent anti-fibrilogenic activity on bovine β-lg aggregation.

3.10 *Competitive protein aggregation inhibitory effect of FA*

To check the potency of FA as a protein aggregation inhibitor, we compared it with other reported compounds' inhibitory effect. The FA is an intermediate polyphenolic compound of curcumin, a very well-known natural antioxidant and a biologically active compound. The advantage of FA over curcumin is its relative higher solubility compared to curcumin. Like curcumin, FA also shows anti-amyloidogenic activities. The difference in the aggregation inhibition efficiency of FA and other small molecules like curcumin may be due to a different mode of binding with the corresponding proteins.[71–73] The literature survey indicated that FA is effective to inhibit aggregation of a small β-sheeted protein Aβ (containing 42 amino acid residues) in 1:4 (Aβ:FA) molar ratio.[73] Our aim for this study was to check its inhibitory effect on a β-sheet predominant large protein, like β-lg (contaning162 amino acid residues), and we observed satisfactory results. However, in comparison with other Aβ aggregation inhibitors, FA showed better result and also proved its efficacy as a potent inhibitor of aggregation of a large protein like β-lg which can be shown in the Table 2.

3.11 *Docking results*

Docking study predicts position, microenvironment and non-covalent forces involved in a small molecule-protein binding.[74,75] Here, we can speculate these features for FA–β-lg interactions with the help of docking. The result allows the perception that the FA molecule is docked on the β-lg surface near N-terminal residue (Figure 7a). Ionic interactions found

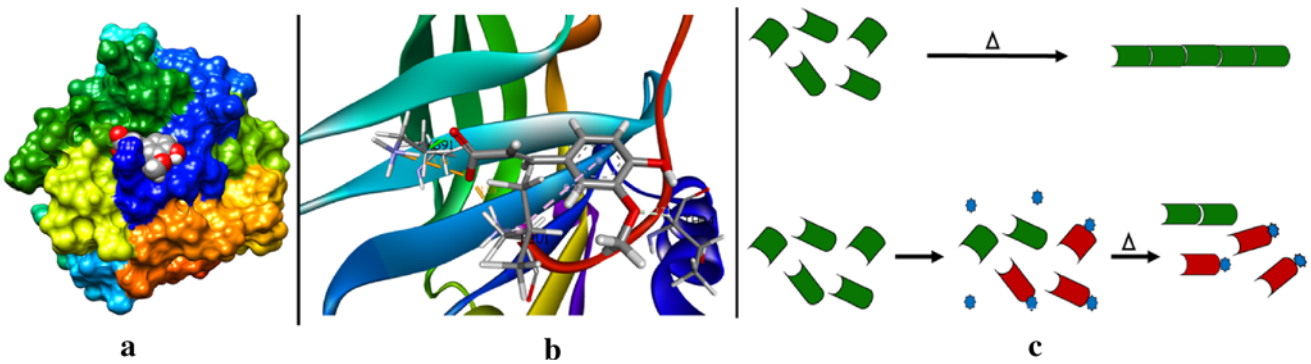

Figure 7. Docking pose of (a) bind site of FA on β-lg surface, (b) binding modes. (c) Probable mechanism of inhibition of aggregation.

between N-terminal -NH_3 of Lue1 and Lys-91 amino acid with carboxylic group of FA, separately. The -NH_3 group of Lys-91 also forms a hydrogen bonding with carboxylic group of FA. One π-alkyl and oxygen lone pair-HC interactions also found between FA and Lue1 and Thr4 residue, respectively (Figure 7b). To understand the probable protein aggregation inhibition mechanism of FA, the docking study is of immense importance. The protein molecules have a different conformation, but some of them can induce the aggregation process through joining protein with similar conformation, one by one. This type of aggregations leads to fibril formations. Here, the FA molecule forms ionic and hydrogen bonding interactions (strongest among all non-covalent interactions) which can stabilize the conformation of protein where it binds. Therefore, such binding can lock a protein conformation and provide less scope to achieve the protein conformer responsible for aggregation. It may be a probable mechanism of inhibition to form large β-lg aggregates through FA–β-lg interactions (Figure 7c).

4. Conclusion

In this study, we examined the inhibitory effect of a phenolic acid, FA, on heat-induced fibrillation of bovine β-lactoglobulin by utilizing different biophysical methods. It has been observed that FA can strongly inhibit the thermal β-lactoglobulin fibrillation in a 10-fold molar excess over protein concentration. The inhibitory potency of FA *in vitro* fibril formation was concentration-dependent. At higher concentrations, FA prevents the conformation alteration of the protein from β-sheet → α-helical structure. All spectroscopic result confirmed that FA protected the native form of β-lg, thereby inhibited the conformational alteration required for its amyloid fibril formation under thermal condition. Moreover, results from molecular docking study suggested that phenyl, hydroxyl and carboxylic groups in FA contribute towards its inhibitory action against β-lg amyloid fibrillation. Thus, from our findings, it may be reasonable to consider that FA could prevent the progress of amyloid fibril formation.

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