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ORIGINAL ARTICLE

L-Arginine reduces thioflavin T fluorescence but not fibrillation of bovine serum albumin

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Abstract This work examines the effects of L-arginine (L-Arg) on the aggregation and amyloid fibrillation of bovine serum albumin (BSA). We demonstrate that L-Arg dose-dependently reduces thioflavin T (ThT) fluorescence of BSA within the L-Arg concentration range used (0–1.4 M). However, as revealed by electron microscopy, size exclusion chromatography, and dynamic light scattering results, L-Arg does not prevent amyloid-like fibril formation by BSA. We conclude that L-Arg competes against ThT for binding sites on BSA amyloid-like fibrils, leading to biased results in ThT fluorescence measurements. Moreover, the use of ThT fluorescence assay to screen for potential inhibitors against amyloid fibrillation can give misleading results.

Keywords Amyloid fibril · Aggregate · Arginine · ThT fluorescence · Bovine serum albumin

Introduction

Protein aggregates can be generally categorized into two groups: amorphous aggregates with no long-range order and amyloid fibrils (or amyloid-like fibrils) with highly ordered structure. Amyloid fibrils can be characterized by several tinctorial and physicochemical properties in common: exhibition of β-sheet-rich conformation, fibrillar morphology, strong binding with amyloid specific dyes, protease-resistance, and insolubility in most solvents (Dobson 2004; Lansbury 1999; Ross and Poirier 2004; Serpell et al. 2000; Uversky and Fink 2004; Wang and Good 2005).

Aggregation of proteins is a nuisance in the applications of biopharmaceutical industry where it can interfere with the production and characterization of therapeutic proteins/peptides (De Bernardez Clark 2001; Morozova-Roche and Malisauskas 2007; Singh and Panda 2005). On the other hand, the deposition of amyloid fibrils has been associated with several debilitating degenerative diseases including hemodialysis amyloidosis, type II diabetes, Parkinson disease, and Alzheimer's disease (Chiti and Dobson 2006; Ross and Poirier 2004; Uversky and Fink 2004; Wang and Good 2005). Moreover, recent findings suggest that amyloid fibrils can be derived from proteins that are irrelevant to any amyloid disease under certain conditions in vitro, thus suggesting that the tendency to form amyloid fibrils could be a merely generic property of any polypeptide chain (Chiti and Dobson 2009; Ferrao-Gonzales et al. 2000; Lai et al. 1996; Shtilerman et al. 2002; Wang 2005).

As of now, no medicines or therapeutics have been developed to cure people that suffer from amyloid-associated diseases. Evidence has suggested that the inhibition or reduction of fibrillation and the destabilization of the existing fibrils are possible preventive strategies against amyloid diseases, and substantial efforts are underway to seek molecules that are capable of restraining the formation of fibrillar species (Estrada and Soto 2007; Gazova et al. 2008; Porat et al. 2006).

L-arginine (L-Arg) is an amino acid with a pI of about 10.8 and possesses the most basic side chain with similarity to the denaturing agent guanidine. L-Arg has been widely employed as a suppressor of protein aggregation, an enhancer of protein renaturation, and an inhibitor of non-specific protein adsorption in biopharmaceutical industry and protein folding research (Ishibashi et al. 2005). It has

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been reported that aggregation suppression by L-Arg occurred with the partially unfolded protein intermediates (Arakawa et al. 2006; Reddy et al. 2005). Furthermore, the suppression of protein aggregation by L-Arg might be attributed to the deceleration of protein–protein association reactions or the interactions between the guanidinium group of L-Arg and the tryptophan side chains (Baynes et al. 2005; Tsumoto et al. 2004).

Bovine serum albumin (BSA) is a 583-residue protein with well-characterized structural information. Due to its availability in large quantities, extensively studied folding pathways, and amyloid-like fibril-forming propensity in particular (Holm et al. 2007; Pearce et al. 2007; Wei et al. 2009), BSA has been chosen as a model protein for investigating amyloid fibrillation. Here, we examine the influence of L-Arg on the in vitro amyloid-like fibril formation of BSA. Our results reveal that L-Arg does not prevent the formation of fibrils derived from BSA but does decrease the intensity of thioflavin T (ThT) fluorescence emission. Our findings suggest that special attention should be paid to avoid the misleading results when searching for lead fibrillation inhibitors using ThT fluorescence assay (Hudson et al. 2009).

Materials and methods

Chemicals

BSA and L-Arg were obtained from Sigma (USA). Hydrochloric acid (HCl) was obtained from Nacalai Tesque, Inc (Japan). All other chemicals, unless otherwise specified, were purchased from Sigma (USA).

In vitro BSA fibrillation

To induce amyloid-like fibril structures, BSA samples (2 g/L) dissolved in Tris–HCl buffer solution (20 and 1.54 mM NaN_3, pH 7.4) and then incubated in a water bath incubator at 65°C during the course of incubation according to previous study (Holm et al. 2007; Pearce et al. 2007; Wei et al. 2009).

Bicinchoninic acid (BCA) protein assay

The concentrations of BSA samples were determined according to the method using BCA protein assay (Stoscheck 1990).

ThT binding assay

With its characteristic enhanced fluorescence upon binding to amyloid, ThT dye has gained widespread use for detecting amyloid through fluorescence microscopy (Goldsbury et al. 2000; LeVine 1993; Naiki et al. 1989). Stock solution of ThT was prepared in de-ionized water, and the concentration was determined spectrophotometrically using its molar extinction coefficient at 416 nm of 26,600 M^{-1} cm^{-1} (Darghal et al. 2006). Samples co-incubated without or with L-Arg were diluted 25-fold with 10 μM ThT in Tris-buffer (final BSA concentration = 0.08 g/L, final ThT concentration = 9.6 μM) and fluorescence intensities were recorded at 480 nm with excitation at 445 nm at room temperature via Cary Eclipse Fluorescence Spectrophotometer (Varian, USA).

Transmission electron microscopy (TEM)

BSA samples were placed on a carbon-stabilized, formvar-coated grid. Grids were negatively stained with 1% (w/v) aqueous phosphotungstenic acid and then examined and photographed in a Hitachi, H-7650 transmission electron microscope with a Gantan model 782 CCD Camera (Tokyo, Japan) at an accelerating voltage of 75 kV.

Dynamic light scattering (DLS)

DLS experiments were used to characterize the size of BSA samples. Samples were poured into small-volume (4 mL) disposable cuvettes with a 1 cm light path. DLS measurements were carried out using a Zetasizer 3000 ZS (Malvern Instruments, UK) with the appropriate settings of viscosity and refractive index at 0.89 centipoises and 1.59, respectively. Samples were illuminated with a laser with a wavelength of 633 nm. The DLS intensities of samples at a 173° angle in kilo counts per second were collected.

Size exclusion chromatography (SEC)

The BSA sample solution incubated at 65°C after 48 h with or without L-Arg was passed through a Vivaspin 6 centrifugal concentrator filter unit with nominal molecular weight limit (NMWL) of 100 kDa (Sartorius, USA) to obtain the monomeric fraction. 1 mL of the filtrate was then injected into a Superdex 75 column (Amersham Biotech) previously equilibrated with buffer solution (20 mM Tris-buffer with 1.54 mM NaN_3, pH 7.4) and eluted with 0.5 mL/min mobile phase flow rate at room temperature. The corresponding chromatogram was recorded with the detector setting of wavelength at 280 nm.

Right angle light scattering

The scattered light intensities (at a fixed angle of 90°) were detected in the BSA samples on a Cary Eclipse

Fluorescence Spectrophotometer (Varian, USA) at equal excitation and emission wavelengths of 450 nm.

Circular dichroism spectroscopy

Circular dichroism (CD) spectra of BSA samples were recorded on a JASCO J-815 spectrometer (Jasco, Japan) at 25°C using a bandwidth of 2.0 nm, a step interval of 0.1 nm, and an averaging time of 2 s. A 2 mm quartz cell was used for far-UV (190–260 nm) measurements. Three scans each of duplicate samples were measured and averaged. The results were plotted as ellipticity (mdeg) versus wavelength (nm).

Fluorescence titration experiments

Different procedures of fluorescence titration experiments were described as follows. *Procedure A* (a) ThT-fibrillar BSA solution titrated with L-Arg: 12.5 mL of 0.08 g/L fibril-containing BSA solutions with various concentrations of ThT (10, 20, or 40 μM) were prepared. A stock solution of 2.5 M L-Arg in buffer solution (titrant) was added in increments of 100 μL to the ThT-fibrillar BSA solution and the fluorescence intensity (denoted by F, excitation and emission wavelengths are 445 and 480 nm, respectively) of the resultant mixture was measured after each addition. As a control, the pure buffer solution was used as the titrant and the corresponding fluorescence intensity was measured (denoted by F_0). The ratio between F and F_0 was defined as the relative fluorescence intensity. (b) L-Arg-fibrillar BSA solution titrated with ThT: 12 mL solutions of 0.5 g/L BSA containing various concentrations of L-Arg (0 or 0.25 M) were prepared. A stock solution of 100 μM ThT in buffer solution was added in increments of 200 μL to the L-Arg-BSA solution and the fluorescence intensity (denoted by F, excitation and emission wavelengths are 445 and 480 nm, respectively) of the resultant mixture was measured. As a control, the pure buffer solution was used as the titrant and the corresponding fluorescence intensity was measured (denoted by F_0). The ratio between F and F_0 was defined as the relative fluorescence intensity. *Procedure B* (a) ThT-fibrillar BSA solution titrated with L-Arg: 0.08 g/L fibril-containing BSA solutions with various concentrations of ThT (10, 20, or 40 μM) were prepared. An aliquot of the previous ThT-fibrillar BSA solution with various concentrations of L-Arg was titrated into the ThT-fibrillar BSA solution (final L-Arg concentration, 0–0.35 M) and the fluorescence intensity of the resultant mixture (F) was measured by setting the excitation and emission wavelengths at 445 and 480 nm, respectively. F_0 was used to denote the fluorescence intensity for the case of BSA with 0 M Arg at each ThT concentration. The ratio between F and F_0 was defined as the relative fluorescence intensity at each L-Arg concentration. (b) L-Arg-fibrillar BSA solution titrated with ThT: solutions of 0.08 g/L BSA containing various concentrations of L-Arg (0, 0.05, 0.15, or 0.25 M) were prepared. An aliquot of the previous L-Arg-fibrillar BSA solution with various concentrations of ThT was titrated into the ThT-fibrillar BSA solution (final ThT concentration: 5, 10, 20, 30, or 40 μM) and the fluorescence intensity of the resultant mixture (F) was measured by setting the excitation and emission wavelengths at 445 and 480 nm, respectively. F_0 was used to denote the fluorescence intensity for the case of BSA with 0 M L-Arg at each ThT concentration. The ratio between F and F_0 was defined as the relative fluorescence intensity at each ThT concentration.

Results and discussion

Effect of L-Arg on the ThT fluorescence intensity of BSA samples

The all-α helix multi-domain globular protein BSA was reportedly to have the propensity for fibrillation at higher temperatures (Holm et al. 2007; Pearce et al. 2007; Wei et al. 2009). With BSA used as a model system, the effect of L-Arg on protein fibrillation was first examined by ThT fluorescence assay. The concentrations of L-Arg used herein are 0.2, 0.6, 1.0 and 1.4 M, which are typical in industrial in vitro renaturation processes. As depicted in Fig. 1, the ThT fluorescence emission of BSA by itself increases dramatically in the first 25 h, and then reaches the fluorescence plateau after ~43 h. However, the addition of L-Arg dose-dependently attenuated the ThT fluorescence intensity of BSA within the L-Arg concentration range (0–1.4 M) used. For example, 0.2, 0.6, 1.0, or 1.4 M L-Arg reduced the stationary fluorescence intensity to approximately ~93, ~79, ~68, or ~47% of its control value, respectively (the percentage value = the percentage of the maximum ThT fluorescence intensity = the stationary ThT fluorescence intensity of BSA with L-Arg/the stationary ThT fluorescence intensity of BSA by itself). The lag period was not involved in the BSA aggregation process with or without L-Arg, which is consistent with the finding observed in the earlier work (Holm et al. 2007). The reduction in ThT fluorescence by the additive such as L-Arg could possibly be attributed to any of the following: (1) the bona fide prevention of fibrillation, (2) a simple spectroscopic quenching artifact from the additive, or (3) the influence of L-Arg on the binding of ThT to BSA fibrils. No fluorescence was detected for L-Arg alone under the assay conditions and all BSA samples (with or without L-Arg) showed similar fluorescence spectra at the beginning of fibril formation (incubation time $t = 0$) (spectra not

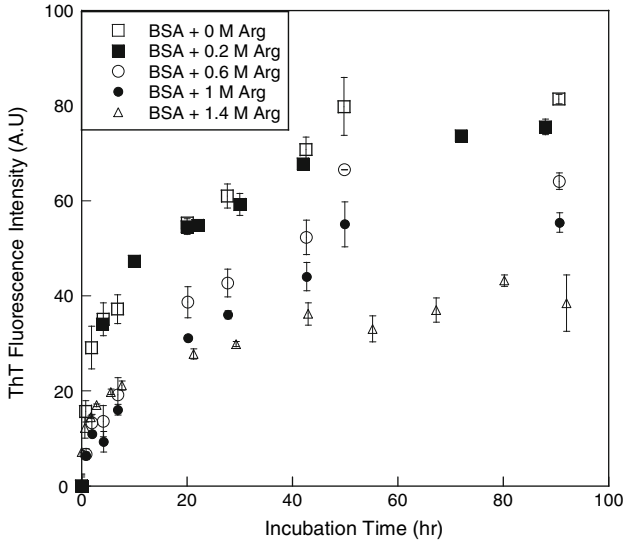

Fig. 1 The effect of L-Arg on the ThT fluorescence of BSA as a function of time. Each point represents the mean of at least four independent measurements

shown), which ruled out the possibility of spectroscopic quenching artifact due to the addition of L-Arg. In addition, we have performed control experiments, in which L-Arg alone was added to buffer containing ThT, and confirmed that, within the range of the concentrations used, no contribution of L-Arg itself to the reduction or quenching of ThT fluorescence intensity was detected. Also, a three-dimensional fluorescence spectrometric scanning on buffer solutions containing either ThT or L-Arg was conducted by measuring the emission intensity at 0.5 nm intervals from excitation wavelengths of 400–500 nm and obtained emission wavelengths of 400–600 nm. We found that there was no overlap between the emission spectra of ThT and L-Arg solutions in the range of 400–600 nm. Therefore, we could conclude that there was no interference between L-Arg and ThT, and that L-Arg itself did not contribute to the quenching of ThT fluorescence intensity. Assuming that increased ThT fluorescence is correlated to augmented fibril formation, our ThT fluorescence results would strongly suggest that L-Arg either has an inhibitory potency

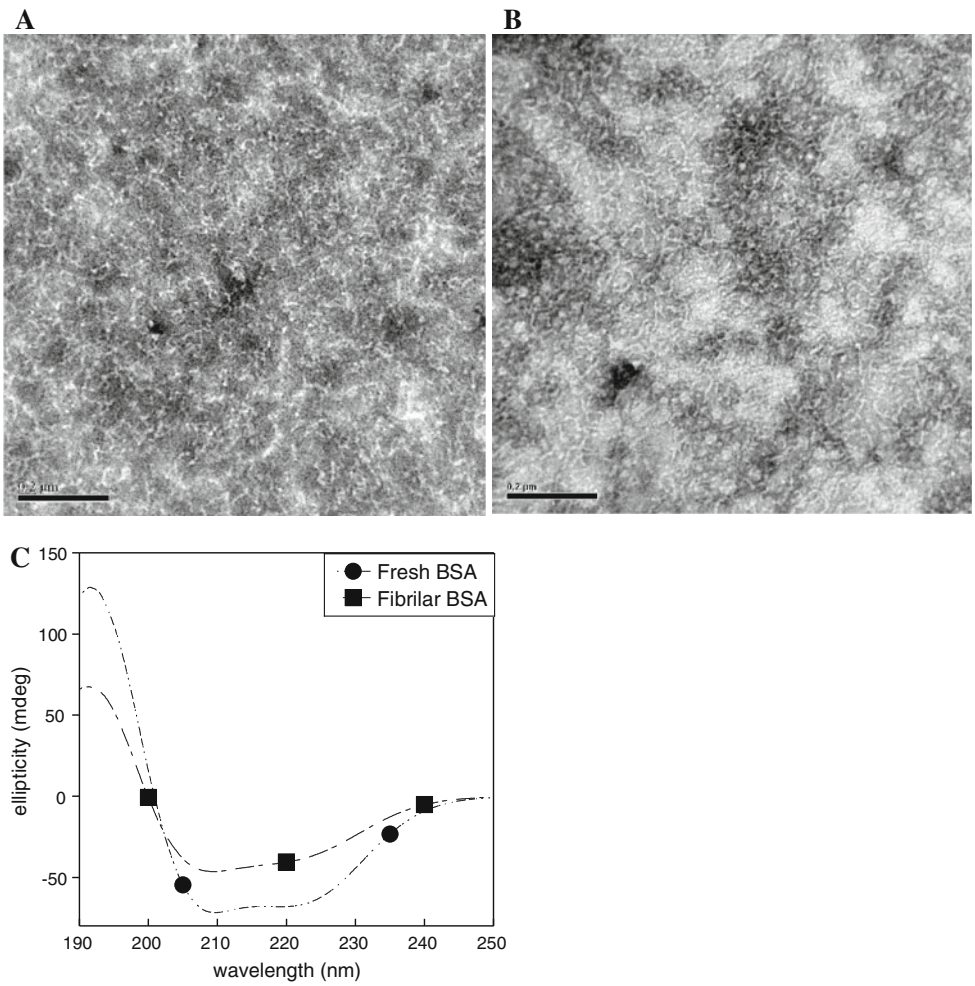

Fig. 2 **a** Electron micrograph of the negatively stained BSA sample without L-Arg after 90 h of incubation (*bar* 0.2 μm). **b** Electron micrograph of the negatively stained BSA sample with 1.0 M L-Arg after 90 h of incubation (*bar* 0.2 μm). **c** Representative far-UV CD spectra of BSA by itself incubated at 65°C were collected at 0 and 48 h of incubation

against BSA fibrillation or has an impact on the ThT binding to BSA fibrils.

Effect of L-Arg on the morphological and structural features of BSA samples

It would be inappropriate to conclude that the observed reduction of ThT fluorescence arose from bona fide preventive effect of L-Arg against fibrillation before checking the TEM images. Presented in Fig. 2 are the representative micrographs of BSA samples with 0 and 1.0 M L-Arg, respectively. It is evident that the control (BSA by itself) and L-Arg-containing BSA sample exhibited fibrillar species (Fig. 2a, b). Also, as shown in Fig. 2a, b, these amyloid-like fibrils were found to be the dominant species in BSA samples solution upon prolonged incubation at 65°C. The TEM imaging revealed that the amyloid-like BSA fibrils assembled during our in vitro experiments were observed to be ~2–5 nm in diameter and ~100 nm in length. In addition, these fibrillar species displayed a flexible and curly filamentous morphology, which was also seen in other proteins (Aso et al. 2007; Cardoso et al. 2002; Yamaguchi et al. 2004). We also performed circular dichroism (CD) measurements to gain insights into the secondary structural feature of amyloid-like BSA fibrils. As revealed in our CD results (see Fig. 2c), the initial far-UV CD spectrum of BSA by itself possesses minima at ~210 and 222 nm, indicating that BSA retains a predominant helical conformation (α-helix, 66%; β-sheet, 6%; turn, 9%; random, 19%) in its native state. However, upon incubation at 65°C, pH 7.4 for 48 h, a considerable reduction in the α-helical content (from 65 to 40%) was accompanied by increases in unordered structure (from 19 to 31%, ~1.6-fold increase) and β-sheet content (from 6 to 14%, ~2.3-fold increase) in the BSA sample solution containing amyloid-like fibrils. A similar behavior of secondary structural change has been reported in other amyloid-like fibril-forming proteins (Holm et al. 2007; Pearce et al. 2007). Since the presence of L-Arg interfered with the CD measurement, we were not able to obtain the far-UV CD spectrum of the BSA sample with 1.0 M L-Arg.

Effect of L-Arg on the size distribution/characteristic dimension of BSA samples

We next monitored the size distribution of species in the BSA samples using DLS. Representative size distributions for the samples of BSA with and without 1.0 M L-Arg at 14 h of incubation as well as the native BSA were retrieved from the analysis of autocorrelation data collected at 173° scattering angle. As shown in Fig. 3, uni-modal distributions were observed in all BSA samples. The size range of fresh BSA sample was found to be ~3–11 nm and the 14-h

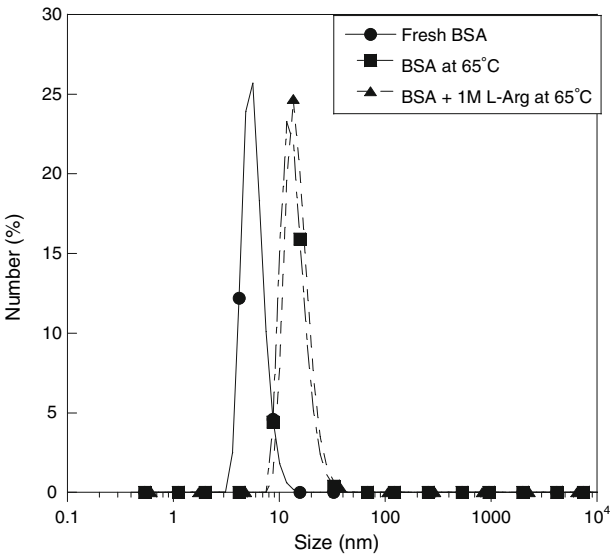

Fig. 3 Representative dynamic light scattering derived size distributions for BSA or BSA with 1.0 M L-Arg at 0 h of incubation, BSA at 14 h of incubation, and BSA with 1.0 M L-Arg at 14 h of incubation

incubation resulted in the observed upward shift in the size distribution (~8–24 nm). This increase in size may derive from the formation of aggregated species (e.g., amyloid fibrils) in solution. Moreover, the observed size distributions at 14 h were not statistically different between BSA and BSA–L-Arg mixture, suggesting that L-Arg fails to prevent BSA from forming aggregated species (e.g., amyloid-like fibrils), which is consistent with the preceding TEM results.

Quantitative comparison between BSA samples with and without L-Arg

Our preceding TEM observations confirmed the formation of amyloid-like fibrils in the samples of BSA with and without L-Arg. A quantitative comparison between the BSA samples with and without L-Arg was performed using size exclusion chromatography (SEC). We demonstrate in Fig. 4 that almost no difference in the chromatogram was detected between the BSA samples with and without 1.0 M L-Arg upon 48 h incubation. Moreover, compared to the native BSA, significantly reduced amount of BSA was observed in the 48-h incubated BSA sample in the absence or the presence of 1.0 M L-Arg. By measuring the amount of unconverted native BSA that remained in the supernatants after centrifugation of the incubated BSA samples (with or without L-Arg) via a filter unit with nominal molecular weight limit (NMWL) of 100 kDa, the conversion fraction of native BSA to larger BSA species after 48 h incubation was calculated to be ~98.7%, suggesting that mostly larger BSA species (>100 kDa, dimer or larger

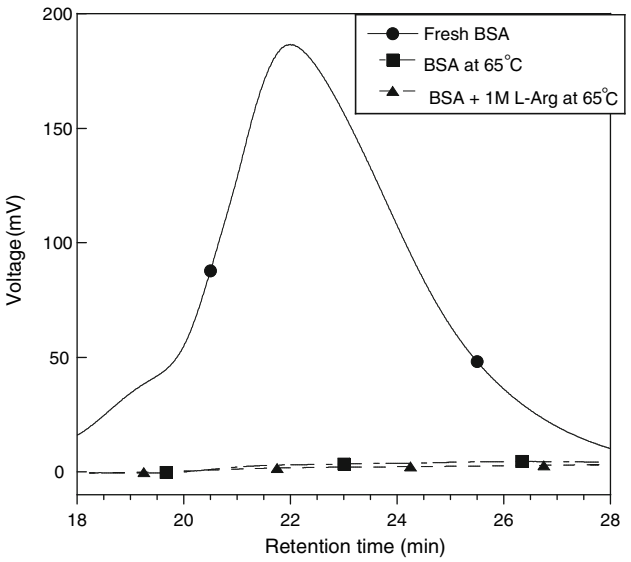

Fig. 4 The size exclusion chromatograms of fresh BSA and monomeric fractions of BSA samples with and without 1.0 M L-Arg. The BSA sample incubated at 65°C after 48 h with or without 1.0 M L-Arg was passed through a filter unit with nominal molecular weight limit of 100 kDa and the filtrate was collected as the monomeric fraction of BSA sample

species) were present in the 48-h incubated BSA sample regardless of the presence of L-Arg.

Right angle light scattering measurements (both excitation and emission at 450 nm) were also used to provide information regarding the size of the aggregates or the extent of aggregation in BSA samples. BSA samples with 1.0 M L-Arg incubated at 65°C for at least 48 h evidently showed a comparable scattered light intensity level relative to that of the control (BSA by itself) (data not shown), indicating that similar sizes of particles were present in both BSA samples with and without L-Arg. Moreover, we have performed control experiments, in which BSA in the presence or absence of 1.0 M L-Arg was incubated at 85°C and pH 7.4. Findings from our laboratory and others indicated that the large amorphous (non-amyloid-like) BSA aggregated species were formed in the solution (Holm et al. 2007; Militello et al. 2003; Vetri et al. 2007) and L-Arg was able to significantly reduce the level of amorphous aggregates (data not shown). We also found that, after the same incubation period, the scattered light intensity observed in the BSA sample (with or without L-Arg) prepared at 65°C was appreciably lower than that of BSA sample at 85°C (without L-Arg) (data not shown). Our preceding results suggested that large amorphous non-amyloid-like materials were less likely to be present in BSA samples prepared at 65°C. Based on our ThT fluorescence of the control, TEM, and right angle light scattering results, we are able to conclude that the amyloid-like fibrils were the dominating species in BSA samples with and without L-Arg. In addition, in line with the DLS (e.g., distribution) and SEC (e.g., quantity) results, it is possible to suggest that both BSA samples yielded comparable amounts of amyloid-like fibrillar species. According to our previous findings, we could conclude that L-Arg does not exert a bona fide preventive effect on the fibrillation of BSA.

Fluorescence titration experiments

Based on our preceding findings, we could hypothesize that the ThT fluorescence reduction may be ascribed to the competition between ThT and L-Arg for binding sites on BSA fibrils. To that end, we performed a series of fluorescence titration experiments. Presented in Fig. 5a, b (also shown below) are the results from the fluorescence titration experiments following the procedures A and B, respectively. We show in Fig. 5a [by procedure A(a)] that, regardless of the ThT concentration used, the relative fluorescence intensity decreased monotonically with increasing concentration of added L-Arg and leveled off as the quantity of L-Arg reached above $\sim$0.15 M and was able to displace $\sim$42% of the bound ThT [the percentage of displacement = 100% $\times$ (fluorescence intensity of ThT-fibrillar BSA solution titrated with the buffer solution − fluorescence intensity of the ThT-fibrillar BSA solution titrated with the L-Arg stock solution)/(fluorescence intensity of the ThT-fibrillar BSA solution titrated with the buffer solution)]. A similar trend was observed in the effect of the concentration of L-Arg on the relative fluorescence intensity using titration procedure B(a) (see Fig. 5b). As seen in the insets of Fig. 5a [by procedure A(b)] and Fig. 5b [by procedure B(b)], while the fluorescence signals rose with increasing ThT concentration in both the fibrillar BSA solution and the L-Arg-fibrillar BSA solution, the L-Arg-containing BSA fibril solution clearly exhibited a significantly lower fluorescence intensity than that of its counterpart. A close inspection of our fluorescence titration data revealed that the value of relative fluorescence intensity calculated from the data of the inset in Fig. 5a [by procedure A(b)] (e.g., [ThT] = 10 μM, $F/F_0 = \sim 0.36$) does not correspond with the results presented in Fig. 5a [by procedure A(a)] (e.g., [ThT] = 10 μM, $F/F_0 = \sim 0.58$). On the contrary, in Fig. 5b the data obtained by procedures B(a) and B(b) gave a similar plateau value of the relative fluorescence intensity ($\sim$0.46), suggesting that the results from both cases are in good agreement. While further investigation is warranted to determine the reasoning behind the differences in the results among the titration procedures [the order of limiting values: procedure A(a) > procedure B(a) or (b) > procedure A(b)], we speculate that the aforementioned discrepancy might have arisen from the differences in the affinity

of protein complex (e.g., ThT-fibrillar BSA or L-Arg-fibrillar BSA) to the titrants (L-Arg or ThT) (Biancalana et al. 2009). However, despite the fact that the nature of binding affinity of ThT or L-Arg to BSA fibrils remains incomplete, several points could be made from our fluorescence titration findings: (1) all titration results indicate that the presence of L-Arg caused the reduction in ThT fluorescence emission. (2) It is evident from our findings that L-Arg was not able to replace all the bound ThT, ruling out the possibility that a simple competition for BSA binding sites exists between L-Arg and ThT (see Fig. 5a, b).

L-Arg has been widely used in biopharmaceutical industry and protein folding research due to its capabilities of assisting refolding of denatured proteins, solubilizing proteins from inclusion bodies, and suppressing/reducing aggregation of proteins, thus leading to an increasing in renaturation yields (Ghosh et al. 2009; Ishibashi et al. 2005; Lyutova et al. 2007; Reddy et al. 2005). A number of studies suggested that L-Arg exhibits an inhibitory potency against protein fibrils. Gibson et al. indicated that the fibrillation of bovine insulin could be markedly inhibited by L-Arg via ThT fluorescence assay (Gibson and Murphy 2006). As evidenced by ANS fluorescence assay, L-Arg was found to attenuate the fibril formation by the N-terminal domain of nuclear poly-A binding protein PABPN1 (Lodderstedt et al. 2008).

An elevation in ThT fluorescence signal has been considered as an important indicator for the presence of amyloid fibrils associated with β-sheet structure (LeVine 1999). ThT exhibits only weak fluorescence in an aqueous environment (free ThT). Conversely, ThT undergoes dramatic changes in its fluorescence properties, including a shift in the emission wavelength and an increase in the quantum yield, upon binding to amyloid fibrils (Groenning et al. 2007; Naiki and Gejyo 1999). While ThT binding assay is widely used for the screening of fibrillation inhibitors (LeVine 1993), the modalities underlying fibril-induced ThT fluorescence remain poorly understood. Several recent reports have nonetheless proposed different mechanisms concerning the interaction between ThT and amyloid (Biancalana et al. 2009; Groenning et al. 2007; Khurana et al. 2005; Krebs et al. 2005). Khurana et al. proposed that the enhancement of fluorescence emission results from the interaction of amyloid fibril and ThT micelle with $\sim$3 nm in diameter (Khurana et al. 2005). Others have suggested that ThT fluorescence increase induced by fibril comes from the excimer formation which is ascribed to the binding of ThT dimer in the central pore of the fibril or along the fibril (Groenning et al. 2007). The widely accepted hypothesis is that ThT molecule would intercalate itself into the grooves between solvent-exposed side chains of the amyloid fibril, running parallel to the fibril axis (Biancalana et al. 2009; Krebs et al. 2005).

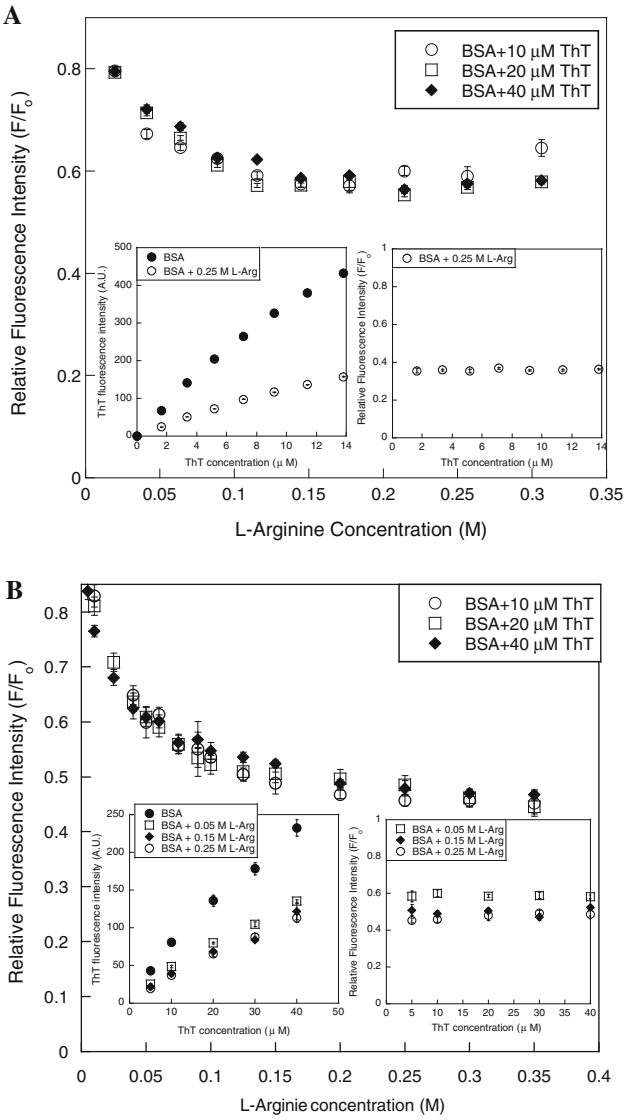

Fig. 5 Results of fluorimetric titration experiments. The relative fluorescence intensity of BSA fibril containing ThT (10, 20, or 40 μM) as a function of L-Arg concentration was monitored. The *left inset* shows the response curves plotting the fluorescence intensity of BSA fibril solution with varying concentration of L-Arg against the ThT concentration. The *right inset* shows the relative fluorescence intensity of BSA fibril solution containing varying concentration of L-Arg against the ThT concentration. The results of the titration experiments following the procedures A and B described in the "Materials and methods" section are demonstrated in **a** and **b**, respectively

In conclusion, our results revealed that L-Arg was not able to inhibit or attenuate amyloid fibrillation by BSA. Moreover, we have demonstrated that the ThT fluorescence emission associated with BSA fibrils was interfered with L-Arg thus giving rise to false negatives in ThT binding assay. While further research is needed to decipher the

nature of binding of L-Arg to amyloid fibrils, given the facts that L-Arg interferes with ThT fluorescence response and carries the guanidine group, it is likely that the interaction between L-Arg and BSA fibrils involves the hydrophobic force. In agreement with others (Hudson et al. 2009), findings from our current work also highlight the point that ThT fluorescence assay should be used with caution when quantifying amyloid fibrillation in the presence of exogenous additives.

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