# 3
# Protein –Protein Interaction during *in vitro* Amyloid Formation

Inherent ability of various proteins to self-associate and to form highly arranged supramolecular structures is a fundamental process in biology. Both collagen and amyloidogenic proteins are known to form insoluble higher order structures from their soluble native states. Most of the proteins coexist in different microenvironments or sub cellular compartments in the body and perform vital life processes such as protein-protein interactions and formation of protein complexes. Triple helical collagen molecules are known to undergo a fibril formation process that makes collagen fibrils. These collagen fibrils are vital to both structural and functional properties of the ECM (extra cellular matrix) (Kielty and Grant 2003). On the other side, the conversion of normal globular proteins/peptides into higher order amyloid aggregates is known to cause several health complications such as Alzheimer's disease, Parkinson's disease and Huntington's disease (Zerovnik 2002; Kielty and Grant 2003; Sipe 2005). It is well known that the aggregation of a specific protein is usually linked with its related amyloid disease. For example, aggregation of alpha synuclein in Parkinson's disease, aggregation of Aβ peptides in Alzheimer's disease and aggregation of huntingtin in Huntington's disease. However, it is unclear that whether such aggregation process of a specific protein would affect the functionality of other proteins that are present in its surrounding. Interestingly, few medical investigations have indicated the coexistence of two different amyloid-linked diseases in individual patient (Moss, Mastri et al. 1988; Tada, Coon et al. 2012; Takei, Oguchi et al. 2013). Hence, the question of how the aggregation process of a particular protein would influence the aggregation properties of other proteins present in its vicinity is very important.

Reports on the relationship between structural proteins such as collagen and amyloidogenic proteins are rarely available in literature. However, few studies reported that collagen and collagen model peptides can inhibit amyloid formation of proteins (Kiuchi, Isobe et al. 2002; Kakuyama, Söderberg et al. 2005; Cheng, Dubal et al. 2009; Parmar, Nunes et al. 2012). However, the underlying principle of the protective effect of collagen against amyloid formation is not clearly known. Hence, it is important to understand collagen-amyloid relationship which may help us both in fundamental and applied research.

## 3.1 INTERACTION BETWEEN GLOBULAR PROTEINS DURING AMYLOID FORMATION

In some recent studies, coexistence of two amyloid-linked diseases in the same individual has been reported. Such reports attract many researches to study this fundamental observation. In a recent study coexistence of Huntington's disease and amyotrophic lateral sclerosis (ALS) was observed in the same patient (Tada, Coon et al. 2012). Some reports have also revealed the coexistence of both Alzheimer's disease (AD) and Huntington's disease (HD) in patients (Moss, Mastri et al. 1988). In yet another recent immunohistochemical investigation the coaggregation of α-synuclein and Cu/Zn superoxide dismutase protein has been reported (Takei, Oguchi et al. 2013). Moreover, research investigations at cellular level have also revealed the potential interactions between two different amyloidogenic proteins (Lauren, Gimbel et al. 2009; Kar, Arduini et al. 2014). For example, Lauren et al. have shown the possible interactions between cellular prion proteins and amyloid-β oligomers (Lauren, Gimbel et al. 2009). In

another study it was shown that D-polyglutamine amyloids can effectively drive the amyloid formation of cellular L-polyglutamine proteins leading to cell death (Kar, Arduini et al. 2014). Reports on co-existence of two amyloid-linked diseases are rarely available in literature and the mechanistic understanding of coaggregation among different globular proteins is largely unknown. Hence, fundamental knowledge of the interactions between an aggregating protein and the proteins located in its surrounding offers new opportunities, not only to understand the mechanism of amyloid formation but also to shed light on the critical role of amyloid-linked higher order entities such as inclusions, plaques and lewy bodies.

Here in this study, some investigations had been performed to understand the occurrence of coaggregation and cross-seeding between different globular proteins in *in vitro* system. Serum albumin (BSA), lysozyme, insulin and cytochrome *c* proteins were taken as convenient model systems to examine the occurrence of coaggregation and cross-seeding among them. In this *in vitro* investigation, two issues have been addressed: (1) to understand the occurrence of coaggregation among proteins under aggregating conditions by mixing their monomers (see Annexure A); and (2) to examine the occurrence of cross-seeding by mixing the amyloid fibrils of a particular protein with the monomers of other proteins. A combination of different biophysical techniques were used here, including Thioflavin T assays, and the obtained results reflect the evidence of rapid coaggregation between proteins and the surprising cross-seeding efficiencies of individual amyloid fibrils.

### 3.1.1 Occurrence of Coaggregation Between Soluble Protein Monomers

Temperature induced amyloid formation of BSA, lysozyme and insulin were carried out by incubating the protein monomer samples near to their melting temperature ($T_m$). Here, the aggregation was performed at ~70 $^{o}$C at pH 7.4 in PBS and its kinetics were recorded at regular intervals by taking the ThT signals (see Annexure A). The typical kinetic curves comprised of a lag phase, a growth phage and a saturation phase were observed in individual reactions of BSA (Figure 3.1,■), insulin (Figure 3.1, ▲) and lysozyme (Figure 3.1,●). The fibrils obtained from individual aggregation reactions showed typical amyloid-like morphology, as evident from TEM images (Figure 3.2a to 3.2c). Coaggregation studies were first conducted for four combinations of mixed monomers, namely: lysozyme+BSA, lysozyme+insulin, BSA+insulin and BSA+lysozyme+insulin. These four combinations of protein monomers showed faster aggregation kinetics than the kinetics of individual aggregation reactions (Figure 3.1a to 3.1c,◆ and 3.1d,■). No distinct lag phase was observed for these coaggregation reactions. Further TEM images of the fibrils show typical morphology as seen for normal amyloid fibrils, as shown in Figure 3.2d and 3.2e.

To further confirm the occurrence of coaggregation, the aggregation kinetics of individual proteins was examined at a concentration which was relatively higher than the concentration of any of these coaggregation reactions. Results clearly indicate that the individual amyloid formation kinetics is much slower (Figure 3.3a) than the kinetics of the reactions comprising of mixed protein monomers. For example, in the case of BSA-lysozyme mixed monomer sample (~2.9 μM BSA and ~12.5 μM of lysozyme), the aggregation shows a faster kinetics without a lag-phase (Figure 3.3a, ▲), whereas the aggregation profile of only lysozyme (~48.9 μM, Figure 3.3a,●), or only BSA (~10.5 μM, Figure 3.3a,■), underwent much slower kinetics with a prolonged lag phase of ~6 h.

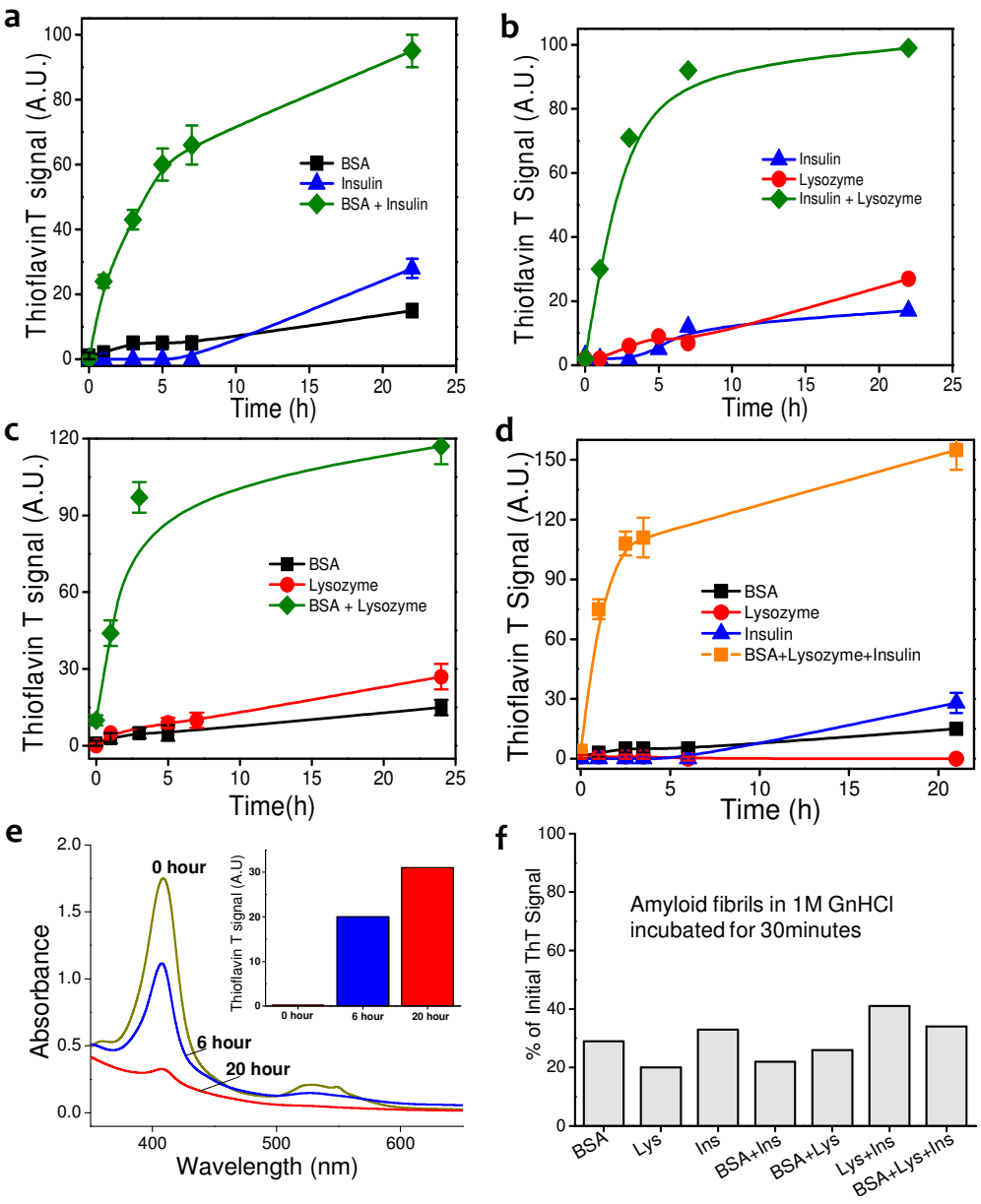


**Figure 3.1 :** Coaggregation studies of mixed monomers of different globular proteins in PBS at ~70°C. **(a)** BSA and Insulin coaggregation: (■) BSA at ~4.9 μM; (▲) insulin at ~45.4 μM; (◆) BSA (~2.9 μM) + insulin (~ 27.5 μM). **(b)** Insulin and lysozyme coaggregation: (●) Lysozyme at ~27.9 μM; (▲) Insulin at ~45.3 μM; (◆) Insulin (~27.5 μM) + lysozyme (~12.5μM). **(c)** BSA and lysozyme coaggregation: (●) Lysozyme at ~25.9 μM; (■) BSA at ~ 4.9 μM; (◆) BSA (~2.9 μM) + lysozyme (~12.5 μM). **(d)**BSA, lysozyme and Insulin coaggregation: (■) BSA at~4.9 μM; (▲) Insulin at ~40.2 μM; (●) Lysozyme at ~18.9 μM; (■) BSA (~1.9 μM) + lysozyme (~8.4 μM) + insulin (~18.4 μM). **(e)** Coaggregation of mixed monomers of BSA and cytochrome *c*. Visualization of the absorbance profile of cytochrome *c* monomers at different time points: 0 hour (olive curve), 6 hours (blue curve) and 20 hours (red curve). Inset shows the progress of amyloid formation by Thioflavin T readings. **(f)** Stabilities of the amyloid fibrils against chemical denaturant, GnHCl.

To gain more insights into the occurrence of coaggregation, a mixture of proteins was prepared where BSA was mixed with visibly detectable protein cytochrome *c*. The aggregation kinetics of the sample was followed by monitoring the loss of monomer concentration of cytochrome *c* (see Annexure A). Since the absorbance profile of cytochrome *c* gives distinct

peaks at ~550 nm and ~410 nm, (Babul and Stellwagen 1972) its presence can be easily detected when it is mixed with BSA monomers. The results (inset of Figure 3.1e) show a rapid coaggregation between BSA and cytochrome *c* into amyloid fibrils. The decrease in the absorbance value at 550 nm with increasing Thioflavin T signal magnitude during the process of aggregation further supports the occurrence of coaggregation between BSA and cytochrome *c* (Figure 3.1e). Additional experiments show that these two proteins undergo aggregation with a delayed lag time when incubated as individual reactions (Figure 3.3b). This observation further supports the occurrence of co-aggregation.

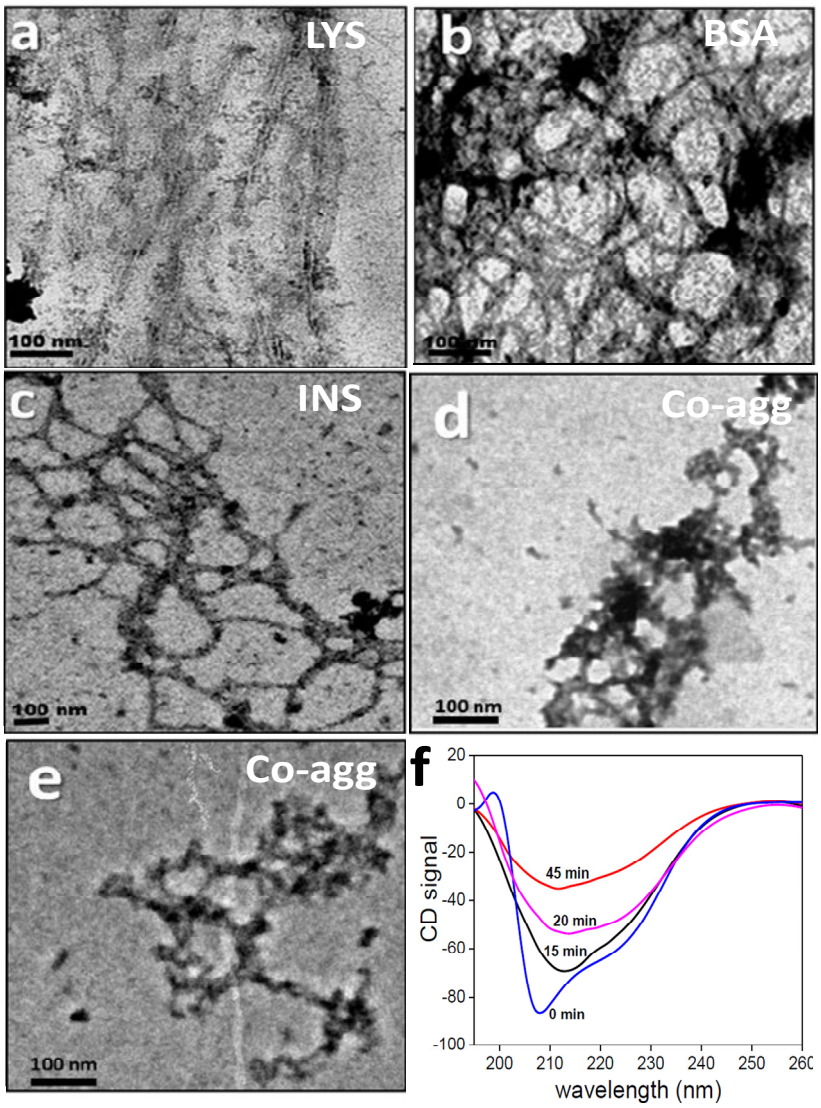


**Figure 3.2 :** Structural and conformational analysis of mature coaggregates of proteins. Electron micrographs of amyloid fibrils: (a) Lysozyme; (b) BSA; (c) Insulin; (d) and (e) are the mature fibrils obtained from coaggregation of BSA + Insulin + Lysozyme sample. Scale bar, ~ 100 nm. (f) Circular dichroism spectra of coaggregation of mixed monomers of BSA, lysozyme and cytochrome c at different time points. Due to precipitation of aggressive coaggregation later time points could not be obtained.

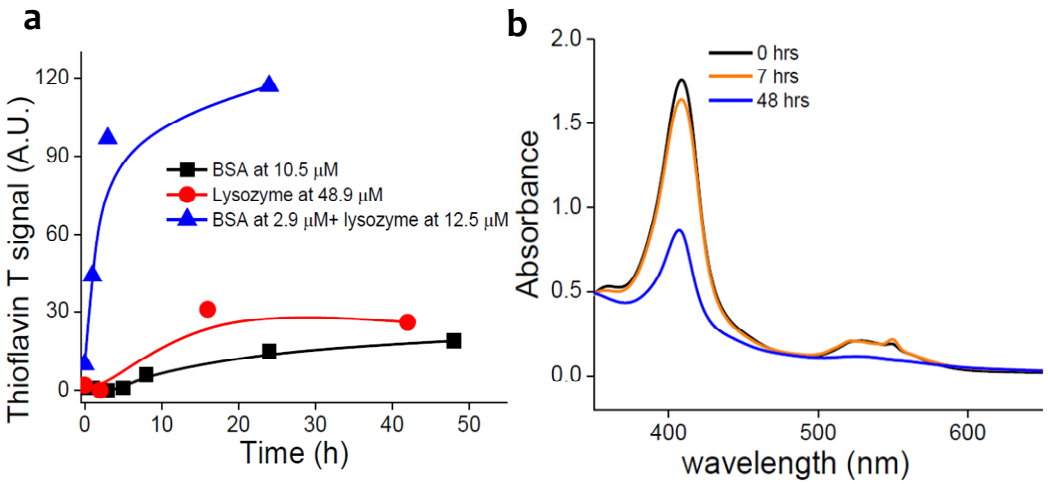


**Figure 3.3 :** Control aggregation experiments of globular proteins at ~70 °C at pH7.4. (a) Aggregation kinetics of individual reactions of BSA at ~10.5 μM (■), lysozyme at ~48.9 μM (●) and BSA (~2.9 μM) + lysozyme (~12.5 μM) mixed sample (▲). (b) Change in cytochrome c concentration as a function of time. Cytochrome C (at 24.53 μM), ThT measurements at different time points of cytochrome c sample are as follows: 0hrs = 0, 7 hrs = 0, and 48 hrs = 7. This aggregation kinetics of cytochrome c alone was taken as control experiment for both the experiments mentioned in Figure 3.1e and Figure 3.4d.

### 3.1.2 Conformational Changes Observed During Co-Aggregation of Proteins

Circular dichroism (CD) experiments were conducted to characterize the change in structural conformation of the fibrils generated through coaggregation reactions. For the reaction which starts with the mixed monomers of BSA+insulin+lysozyme, a gradual change in the nature of the CD curve was observed (Figure 3.2f). Such conformational changes indicate the gradual formation of β-structures during the progress of the amyloid formation (Figure 3.1d, ■ and 3.2f). To further understand the nature of the coaggregated fibrils, stability of these fibrils were measured against a chemical denaturant. Comparison of Thioflavin T signal of the fibril suspensions in the absence and in the presence of GnHCl (1M, incubated for 30 minutes) was shown in Figure 3.1f. This result does not show any significant differences in the stabilities of the fibril samples.

### 3.1.3 Occurrence of Cross-Seeding Among Proteins During Amyloid Formation.

Because the coaggregation reactions are observed to be so rapid without any lag phases, there is a possibility that once the aggregation promoting amyloid nuclei or oligomers are formed, they may have the potential (as seeds) to recruit the monomers of other proteins present in the sample mimicking a self-seeded aggregation process. To understand this issue of cross-seeding, preformed amyloid fibrils (or seeds) of individual proteins were incubated initially with the monomers of different proteins in separate vials and monitored the kinetics of each of these aggregation reactions by recording Thioflavin T signals. Data shown in Figure 3.4 clearly indicate that amyloid fibrils of these proteins can efficiently initiate aggregation of the monomer samples of each other leading to the occurrence of cross-seeding. Furthermore, the efficacy of cross seeding is found to be similar to the self-seeding abilities of each of these proteins (Figure 3.4a to 3.4c). Also a decrease in the monomer concentration of cytochrome *c* was observed during the progress of amyloid formation (Figure 3.4d and inset). These data are highly consistent with the coaggregation data. It is also possible that both coaggregation and cross-seeding can occur at the same time leading to a faster aggregation process.

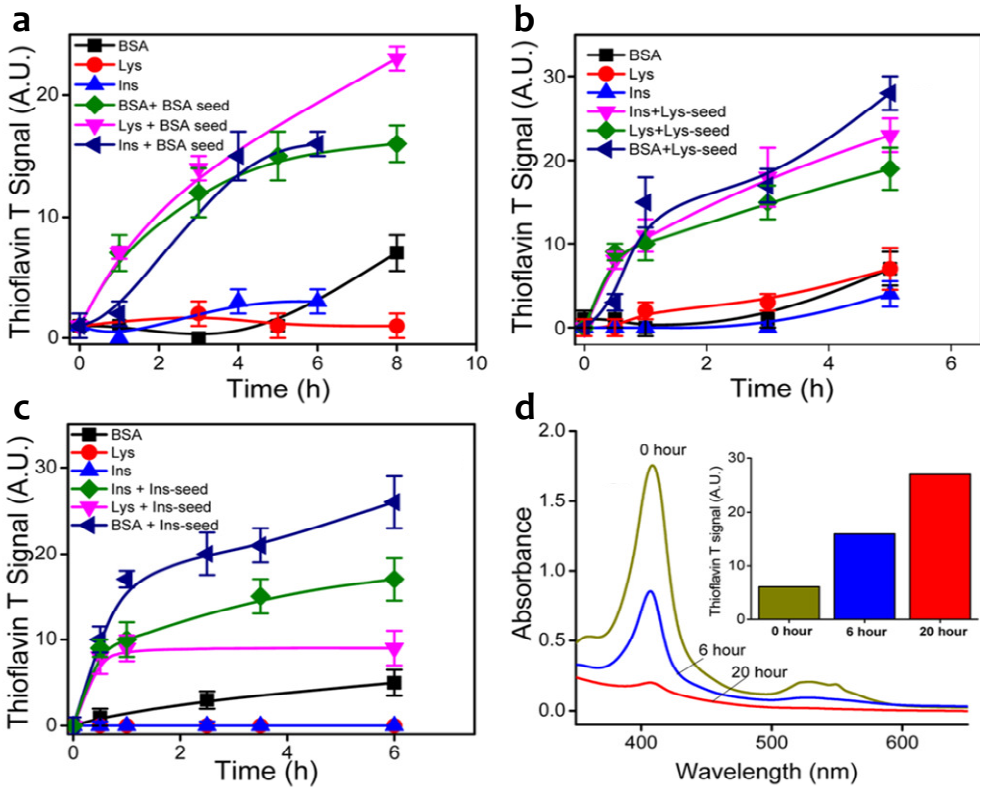


**Figure 3.4 :** Cross-seeding of proteins during amyloid formation **(a)** BSA at ~7.5 μM (■); lysozyme at ~ 27.9 μM (●); Insulin at ~52.3 μM (▲); BSA + BSA seed (◆); lysozyme + BSA seed (▼); Insulin + BSA seed (◀). **(b)** BSA at ~6.0 μM (■); lysozyme at ~27.9 μM (●); Insulin at ~52.3 μM (▲); BSA + lysozyme seed (◀); lysozyme + lysozyme seed (◆); Insulin + lysozyme seed (▼). **(c)** BSA at ~4.5 μM (■); lysozyme at ~18.9 μM (●); Insulin at ~40.2 μM (▲); BSA + Insulin seed (◀); lysozyme + Insulin seed (▼); Insulin + Insulin seed (◆). **(d)** Aggregation of cytochrome c monomers (at ~24.5 μM) in the presence of BSA amyloids (seeds). Inset shows the Thioflavin T readings.

### 3.1.4 Absence of Coaggregation and Cross-seeding Under Physiological Conditions of Buffer and Temperature

It is well known that the population of temperature induced partially folded species play a critical role in the amyloid formation of proteins (Litvinovich, Brew et al. 1998; Fandrich, Fletcher et al. 2001; Chiti and Dobson 2009). One of the reasons behind the higher propensities of intermediate states to undergo aggregation is their solvent-exposed hydrophobic moieties which promote intermolecular interactions (Chiti and Dobson 2009). In this investigation, both aggressive coaggregation and cross-seeding was observed between different proteins at 70°C. Because this temperature value is just below the $T_m$ values (Table 3.1) (Babul and Stellwagen 1972; Matsuura, Powers et al. 1993; Shih and Kirsch 1995; Kang and Singh 2003; Huus, Havelund et al. 2005; Sathya Devi, Chidi et al. 2009; Ciolkowski, Pałecz et al. 2012; Lisi, Png et al. 2014) of the studied proteins in physiological buffer conditions, it is predicted that they would contain higher levels of their aggregation prone intermediate species. To confirm this hypothesis, cross-seeding experiments were carried out between lysozyme, BSA and insulin at 37°C where these proteins are expected to remain mostly in their native states. Results as shown in Figure 3.5 did not confirm the occurrence of aggregation, coaggregation and cross-seeding. Even after 3 days of incubation, no aggregation was observed in both self-seeded and cross-seeded reactions (Figure 3.5). Hence, the non-occurrence of cross-seeding at 37°C suggests the

critical role of partially folded protein species for coaggregation. It is also possible that at higher concentration they may undergo coaggregation process because individually proteins are known to form aggregate at higher concentrations under physiological conditions (Apetri, Maiti et al. 2006).

**Table 3.1 :** Transition temperature values (Tm) of the studied globular proteins in physiological buffer conditions: BSA, Lysozyme, Insulin and Cytochrome *c*

| Proteins | $T_m$ | References |
|---|---|---|
| **BSA** | ~70 $^0$C | Sathya Devi et al. Spectroscopy. **2009;** 23: 265–270. |
| | ~67 $^0$C | Kang et al. International Journal of Pharmaceutics. **2003**; 260:149–156. |
| **Lysozyme*** | ~73 $^0$C | Ciolkowski et al. Colloids and Surfaces B: Biointerfaces. **2012**; 95: 103– 108. |
| | ~74 $^0$C | Shih et al. Protein Science. **1995;** 4:2050-2062. |
| **Insulin** | ~70 $^0$C | Huus et al. Biochemistry **2005;** 44: 11171-11177. |
| | ~66 $^0$C | Matsuura et al. J. Am. Chem. SOC. **1993**; 115:1261-1264. |
| | ~67 $^0$C | Lisi et al. Biochemistry **2014**; 53: 3576−3584. |
| **Cyt C** | ~84 $^0$C | Sun et al. Biomacromolecules **2012**; 13: 3736−3746. |
| | ~67 $^0$C | William et al. Biophys. J. Biophysical Society. **1991;** 59: 48-54. |

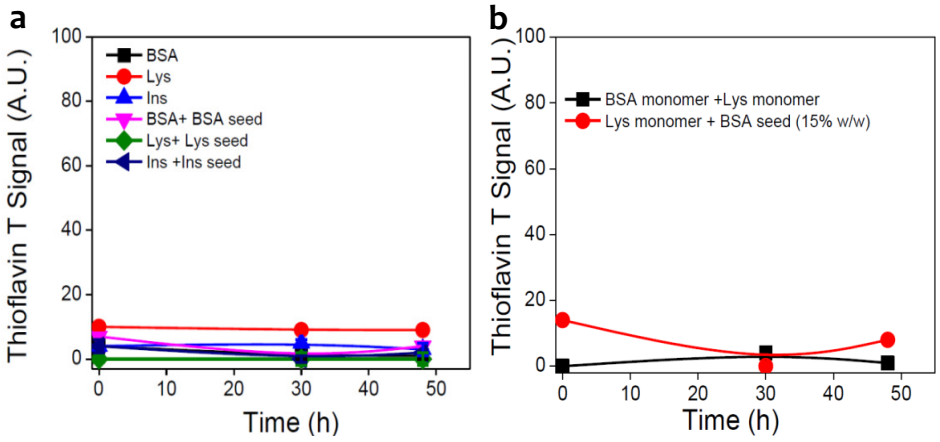


**Figure 3.5 :** Coaggregation and cross seeding experiments between lysozyme, BSA and Insulin at 37°C in PBS. **(a)** BSA only at 10.5µM (■); Lysozyme only at 34.9µM (●) and Insulin only at 104.7µM (▲); BSA+ BSA seed (▼); Lysozyme + lysozyme seed (◆); Insulin +insulin seed (◀). **(b)** BSA monomers (10.5µM) + Lysozyme monomer (34.9µM) mixed sample (■); Lysozyme monomers (34.9µM) + BSA seeds (●). The seed concentration was ~15% weight/weight. All these data are average values of at least two independent experiments. Thioflavin T readings were also measured for these samples after two weeks; however no change of Thioflavin T signal was detected.

### 3.1.5 Sequence Similarity Analysis Among Globular Proteins

Recently, the coaggregation of two differently labeled polyQ sequences was reported using mixed-isotope infrared spectroscopy (Buchanan, Carr et al. 2014). A previous of aggregation of multidomain protein constructs indicated that a >70% of sequence identity would highly favor aggregation whereas a <30-40% of identity will not promote aggregation

(Wright, Teichmann et al. 2005). Therefore, bioinformatics analysis among BSA, lysozyme, insulin and cytochrome *c* were conducted and it was observed that no significant similarity between their sequences were taken place (see Table 3.2). Hence, it was clear that the coaggregation and cross-seeding seems to be more dependent on temperature-induced aggregation prone intermediates rather than their sequence identities.

**Table 3.2 :** Sequence analysis of the proteins.

| S. No. | Query | Subject | Sequence Similarity regions predicted by pBLAST | Alignment Score (Bits) | E-value |
|---|---|---|---|---|---|
| 1 | BSA (4F5S) | Lysozyme (193L) | Query 4 KSEIAHRFKDLGEEHFKGLVL 24<br>+ E+A K G ++++ G L<br>Sbjct 5 RCELAAAMKRHGLDNYRGYSL 25<br>Query 252 CADDRADLAKYICD 265<br>C D R + + +C+<br>Sbjct 64 CNDGRTPGSRNLCN 77<br>Query 188 VLTSSARQRLRCASIQKFGERALKAWSVAR 217<br>+L+S + CA + AW R<br>Sbjct 83 LLSSDITASVNCAKKIVSDGNGMNAWVAWR 112 | 15.4 | 1.5 |
| | | Cytochrome C (1HRC) | Query 522 IKKQTALVELLKHKPKATEE 541<br>IKK+ T +L+ + KAT E<br>Sbjct 86 IKKKTEREDLIAYLKKATNE 105<br>Query 65 SLHTLFG 71<br>+LH LFG<br>Sbjct 32 NLHGLFG 38 | 18.9 | 0.081 |
| | | Insulin Chain A (4I5Z) | Query 554 VDKCCAA 560<br>V++CCA+<br>Sbjct 3 VEQCCAS 9<br>Query 356 LEECCA 361<br>+E+CCA<br>Sbjct 3 VEQCCA 8 | 14.6 | 0.13 |
| 2 | Insulin Chain A (4I5Z) | BSA (4F5S) | Query 4 EQCCA 8<br>E+CCA<br>Sbjct 357 EECCA 361<br>Query 10 VCSLYQ 15<br>VC YQ<br>Sbjct 314 VCKNYQ 319<br>Query 17 EN 18<br>EN<br>Sbjct 299 EN 300 | 18 | 0.023 |
| 3 | Insulin Chain B (4I5Z) | BSA (4F5S) | Query 24 FFYTP 28<br>+FY P<br>Sbjct 147 YFYAP 151<br>Query 28 PKA 30<br>PKA<br>Sbjct 223 PKA 225<br>Query 10 HLV- EALYLV-----C---------- GER GF 24<br>HLV E L+ C GE GF<br>Sbjct 378 HLVDEPQNLIKQNCDQFEKLGEYGF 402<br>Query 17 LV 18<br>LV<br>Sbjct 233 LV 234<br>Query 17 LV 18<br>LV<br>Sbjct 574 LV 575 | 15.1 | 0.38 |
| 4 | Lysozyme (193L) | Insulin (4I5Z) | No similarity observered | NIL | NIL |

## 3.2. PREVENTION OF AMYLOID FORMATION BY COLLAGEN

There are no direct evidences in literature that describe collagen-amyloid relationship. However, some studies have revealed the protective nature of collagen and collagen peptides against amyloid formation of proteins (Kiuchi, Isobe et al. 2002; Kakuyama, Söderberg et al. 2005; Cheng, Dubal et al. 2009; Parmar, Nunes et al. 2012). It has been shown that type IV collagen can inhibit Aβ amyloid formation (Kiuchi, Isobe et al. 2002) and type VI collagen can prevent Aβ neurotoxicity by making interactions with Aβ oligomers (Cheng, Dubal et al. 2009). In another work, molecular interactions between CLAC (collagenous Alzheimer amyloid plaque component; which is a type XXV collagen) and Aβ fragments have been reported to reduce Aβ fibril elongation (Kakuyama, Söderberg et al. 2005).

Therefore to gain knowledge on collagen–amyloid relationship, in the current research work, the effect of type I collagen on lysozyme amyloid formation was studied. Type I collagen is the most abundant form of collagen in the body. Lysozyme is a widely studied globular protein which is generally considered as a model protein to study amyloid formation of proteins under *in vitro* conditions (Merlini and Bellotti 2005). Hence, type I collagen and lysozyme were selected as a convenient model systems to understand collagen-amyloid relation.

### 3.2.1. Effect of Collagen on Amyloid Formation of Lysozyme

Here, lysozyme amyloid formation was carried out at an elevated temperature of ~58 $^{o}$C and at pH 2.0 by following the established protocol (Morozova-Roche, Zurdo et al. 2000; Kar and Kishore 2007). ThT assay was employed to monitor the aggregation kinetics of the lysozyme samples, where ThT readings of the samples were recorded at regular interval of time. In Figure 3.6, it was observed that the control reaction of lysozyme (in the absence of collagen) followed sigmoidal aggregation kinetics having a distinct lag phase, an exponential phase and a stationary phase. Lag phase of the control reaction was around 100 h which further confirmed the formation of amyloid formation (Figure 3.6, ■).

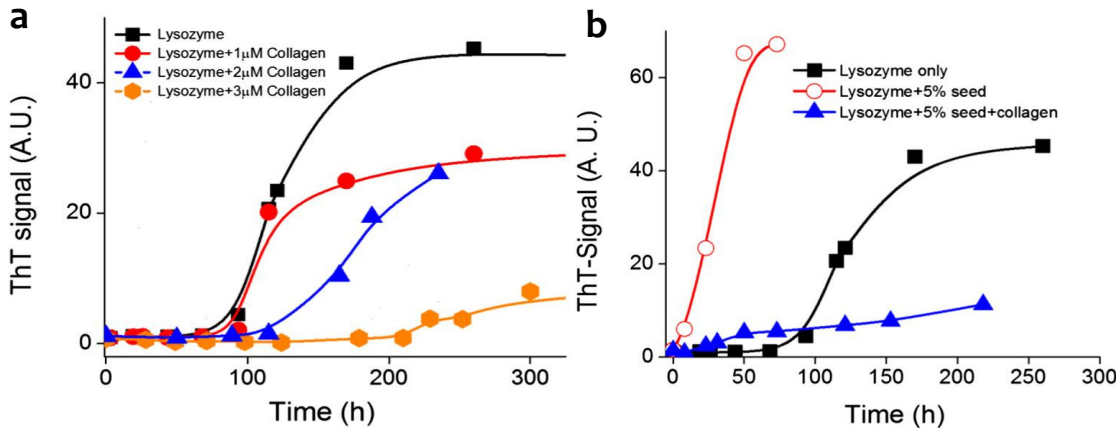


**Figure 3.6 :** Amyloid formation of lysozyme in presence of collagen. (a) Inhibition of spontaneous aggregation lysozyme by type I collagen. Lysozyme aggregation at 10 mg.ml$^{-1}$ was studied in presence of different concentrations of type I collagen: lysozyme+0 μM collagen (■), lysozyme +1 μM collagen (●), lysozyme +2 μM collagen (▲) and lysozyme +3 μM collagen (⬢). (b) Inhibition of seed-induced amyloid formation of lysozyme in presence of collagen: lysozyme+0 μM collagen (■), lysozyme + 5% (w/w) seeds (○), lysozyme + 5% (w/w) seeds + 3 μM collagen (▲). Seed is the preformed lysozyme fibrils. All the data showed here are the average values of at least three independent measurements.

All the obtained results on aggregation studies signify the inhibition of both spontaneous and seed-induced aggregation of lysozyme in presence of collagen. In spontaneous aggregation reactions inhibition effect of collagen was studied in dose dependent manner at

three different molar ratios of lysozyme:collagen, likewise, 1000:1, 350:1, and 250:1 (Figure 3.6a, ●▲⬢). It was also observed that with increasing concentration of collagen the lag time of amyloid formation was gradually delayed. The maximum inhibition was at highest molar ratio of lysozyme:collagen, that is at 250:1, where lag time was recorded around 210 h, after that slight aggregation was observed. It was also observed that even after 700 h only 10% of the total soluble lysozyme was underwent aggregation. This result was further confirmed by calculating the concentration of soluble protein before and after aggregation.

### 3.2.2. Inhibition of Seeded Elongation of Lysozyme Amyloid Formation

Since type I collagen was strongly inhibiting lysozyme amyloid formation, as a next step, its effect on a seeded aggregation reaction of lysozyme was explored. Preformed lysozyme amyloid fibrils (or seeds), 5% (weight/weight), when added to monomers of lysozyme solution, a rapid aggregation was observed without any lag phase (Figure 3.6b, ○). However, in the presence of collagen (at 250:1 molar ratio of lysozyme:collagen) a strong inhibition effect was observed where the lag time was delayed to 30 h after which a slight rise in the Thioflavin-T signal was observed (Figure 3.6b, ▲). Again the aggregation inhibition was confirmed by determining the concentration of the soluble protein at regular intervals. Suppression of seeded aggregation reaction supports the inhibition ability of type I collagen against lysozyme amyloid formation. It may be the reason that the unfolded collagen chains are either interacting with lysozyme monomers and lysozyme fibrils (seeds) or to both these species preventing the onset of the aggregation process.

### 3.2.3. Effect of Collagen on Lysozyme Activity

Since strong inhibition of lysozyme amyloid formation was observed in the presence of collagen, the effect of collagen molecules on the biological activity of lysozyme was explored. Lysozyme activity assays were carried out following established protocol in previous work (Kar and Kishore 2007). At pH 6.5 optimal catalytic activity of lysozyme was observed and in presence of collagen slight changes were noticed in the activity that were not significantly different (Figure 3.7b, lower panel). Next to see the effect of collagen on lysozyme activity at low pH was carried out, where lysozyme loses its activity due to change in its structural confirmation. At pH 2.0 only 10% of the optimal activity of lysozyme (observed at pH 6.5) was observed and on addition of collagen to the sample did not alter the catalytic activity of lysozyme (Figure 3.7b, upper panel). Observed results suggest that the interaction between collagen chain and lysozyme does not promote regaining of the active site cleft.

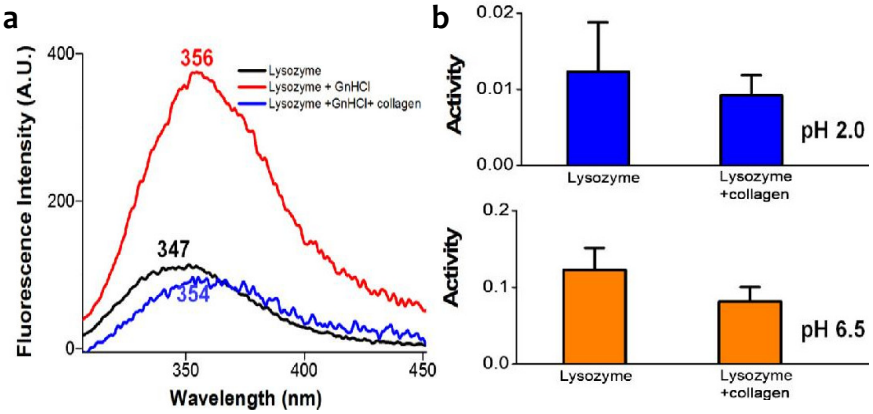


**Figure 3.7 :** (a) Intrinsic tryptophan fluorescence spectra of lysozyme: lysozyme only (–), lysozyme + 5 M GnHCl (–), lysozyme + collagen + 5 M GnHCl (–). (b) Biological activity of lysozyme in the presence of collagen. Catalytic activity of lysozyme both in the presence and absence of collagen was performed at pH 2.0 (upper panel) and at pH 6.5 (lower panel). All the measurements were obtained at 25 °C and the ratio of lysozyme to collagen was maintained at 100:1. Each plot shown from both fluorescence and activity measurements represents the average value of two to three independent measurements.

### 3.2.4. Effect of Collagen on Conformational Stability of Lysozyme

To understand the effect of collagen on the conformational properties of lysozyme both CD and intrinsic tryptophan fluorescence measurements were performed. CD spectra of lysozyme monomers before aggregation showed a typical signature seen for lysozyme's native structure (Figure 3.8a, —) and after completion of the aggregation process the CD spectra indicated the presence of only β-structured species (Figure 3.8a, —). These results confirmed the conversion of soluble lysozyme monomers into cross- β amyloid fibrils. Next, CD measurement of the lysozyme sample were carried out from an inhibited reaction (lysozyme/collage molar ratio = 250:1). In an inhibited reaction (in the presence of collagen) the CD spectra of lysozyme (Figure 3.8a, —) looked almost identical to the native lysozyme CD spectra (Figure 3.8a, —). This indicated that the presence of collagen can promote the retention of the native structure of lysozyme.

To further understand the effect of collagen on thermal stability of lysozyme, thermal denaturation experiments of lysozyme in the presence of collagen were conducted. Thermal melting of lysozyme was recorded by monitoring the CD signal at 207 nm. The results obtained on thermal denaturation of lysozyme clearly indicate that in the presence of collagen the $T_m$ of lysozyme was increased from 62.3 °C to 67.4 °C. Such an increase of $T_m$ values suggests the gain of conformational stability of lysozyme in the presence of collagen.

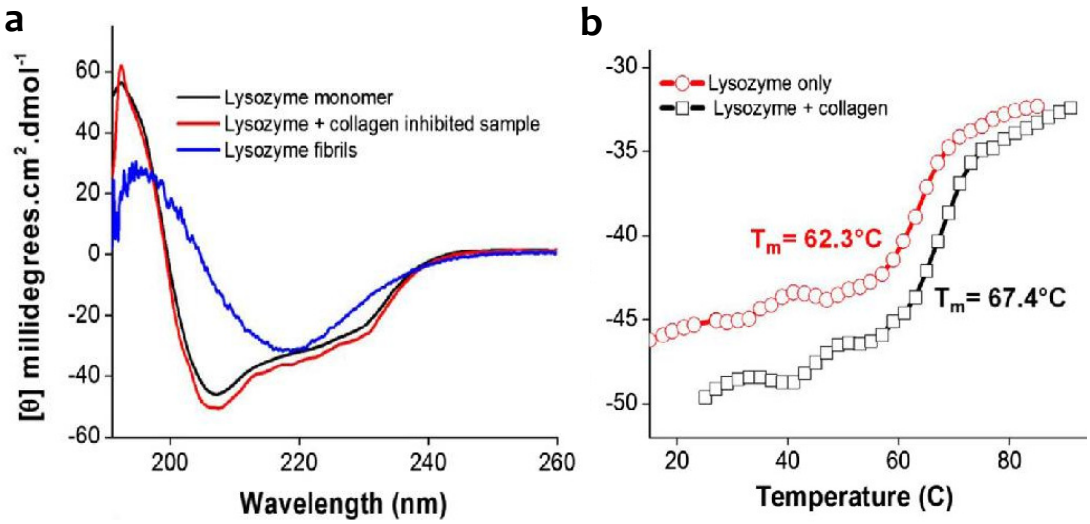


**Figure 3.8 :** Effect of collagen on lysozyme conformation and stability by circular dichroism (CD). (a) CD spectra of lysozyme samples: lysozyme monomers before aggregation (—), lysozyme amyloid fibrils after aggregation (—), inhibited lysozyme sample in the presence of collagen (—). (b) Thermal unfolding of hen egg white lysozyme in the absence (○) and the presence (□) of type I collagen. Temperature scan rate for the thermal unfolding experiments was maintained at 2°C/min. The value of transition temperature ($T_m$) of lysozyme was 62.3 °C and in the presence of collagen the $T_m$ was increased to 67.4 °C.

To check the refolding capacity of type I collagen on a chemically denatured lysozyme molecule, experiments were performed with 5 M GnHCl treated lysozyme. Chemically denatured lysozyme showed a red shift of 9 nm as the emission maxima ($\lambda_{max}$) changed from 347 nm to 356.5 nm with enhanced fluorescence intensity (Figure 3.7a, —). However, in the presence of collagen (lysozyme:collagen molar ratio = 250:1) a substantial decrease in the intensity (accompanied by a slight blue shift of 2 nm) of $\lambda_{max}$ was observed (Figure 3.7a, —). These results clearly proved the ability of collagen to alter the conformation of the lysozyme while inhibiting the protein's aggregation. Both CD and fluorescence data indicated that in the presence of collagen lysozyme gained its conformational stability.

## 3.3. DISCUSSION

The first study of this chapter reveals the occurrence of both coaggregation and cross-seeding among globular proteins under *in vitro* conditions. The obtained results prove that both coaggregation and cross-seeding occurs only at 70°C but not at 37°C. At physiological temperature conditions proteins are known to be in their native states whereas at higher temperature (near to their $T_m$) they attain partially unfolded conformations. Hence, it is possible that the driving force for coaggregation and cross-seeding of proteins is more dependent on the temperature induced aggregation prone intermediate species rather than their native state. Further, it was also observed that the aggregation process of any individual protein sample (fundamentally, species of 100% sequence identity) shows slower kinetics than that of any mixed protein samples (Figure 3.3a,●,■). This again suggests that coaggregation is less dependent on sequence identities of the proteins. These investigations indicate two important clues: i) there may be some conserved regions within each protein's sequence to hinder its self-association into amyloids; and ii) it is also possible that there is a net gain of additional intermolecular interactions during coaggregation of two different proteins. Further, increase in the ThT fluorescence and decrease in the monomeric protein concentration of BSA and cytochrome *c* sample (Figures 3.1e and 3.2d) also confirmed the occurrence of coaggregation. However, these methods are not sufficiently sensitive to characterize the structural properties of coaggregated fibrils and individual fibrils. It is possible that amyloid type fibrils are formed by coaggregation in which both proteins in a sample interact via backbone H-bonds in single amyloid cross-β architecture. It is also possible that binding of one species with another stabilizes fibers or intermediate species and enhances the aggregation kinetics. For example, αβ-crystalline binds to fibers of Aβ peptide, without inserting into the framework of cross-β structure (Shammas, Waudby et al. 2011).

In the second study of this chapter, it was observed that type I collagen has the potential to prevent amyloid formation of lysozyme. It is believed that increase in the population of the partially folded species of lysozyme is a major factor for the onset of its amyloid formation (Morozova-Roche, Zurdo et al. 2000). Intermolecular association between such partially folded species is believed to be the cause for the amyloid formation. One of the reasons behind the inhibition effect of collagen could be due to the interaction between the collagen chains and the partially folded species of lysozyme. There is a possibility that collagen–lysozyme interaction would stabilize the partially folded intermediate species and alter their conformation into a more native like structure. This assumption was supported by the CD data where a 5 $^{o}$C increase of the transition temperature of lysozyme was observed in the presence of collagen (Figure 3.8b). Further, fluorescence data (Figure 3.7a) also showed that a chemically denatured lysozyme could regain its lost conformational stability in the presence of collagen. The molecular structure of collagen has a characteristic triple helical conformation, consisting of three polyproline II like chains supercoiled around a common axis by interchain hydrogen bond (Ramachandran and Kartha 1955; Rich and Crick 1961; Brodsky and Persikov 2005). The type I collagen (from Rat Tail Tendon) molecule which is used in the current study consists of three chains, two α1 (1453 residues each) chains and one α2 chain (1372 residues). Since the transition temperature of type I collagen is about 42 $^{o}$C (Jokinen, Dadu et al. 2004), all the triple-helical molecules at 58 $^{o}$C are expected to exist as unfolded individual chains. Therefore in this study the collagen– lysozyme interaction leading to inhibition effect is between the unfolded chains of the collagen and the partially folded lysozyme species. It is also possible that the exposed residues of partially folded lysozyme and the unfolded chains of collagen may interact with each other. One of the key factors for amyloid formation is that the condition under which aggregation happens should favor the intermolecular interactions including hydrogen bonding (Chiti and Dobson 2009). Interaction between an unfolded collagen chain and a partially folded lysozyme could be possible through their exposed hydrophobic and aromatic residues. One of such critical interactions may include aromatic–proline interaction since collagen chains have a

high content of the imino acids (proline and hydroxyproline). Analysis of the amino acid sequence of the collagen chains (Figure 3.9a and Figure 3.9b) indicates the presence of large number of proline residues and a substantial number of aromatic and hydrophobic residues. Thus, aromatic–proline interactions as well as hydrophobic interactions between collagen chain and the amylodogenic segment of lysozyme might be critical to the inhibition mechanism. To gain more insight into the collagen–lysozyme interaction, the effect of lysozyme was studied on collagen fibril formation at 37 °C in PBS. Results indicate no effect of lysozyme on the onset of collagen fibril formation (Figure 3.9c). Since at 37 °C lysozyme would retain its native conformation and collagen molecules would exist as intact triple helices, a lysozyme–collagen interaction seems to be less favorable.

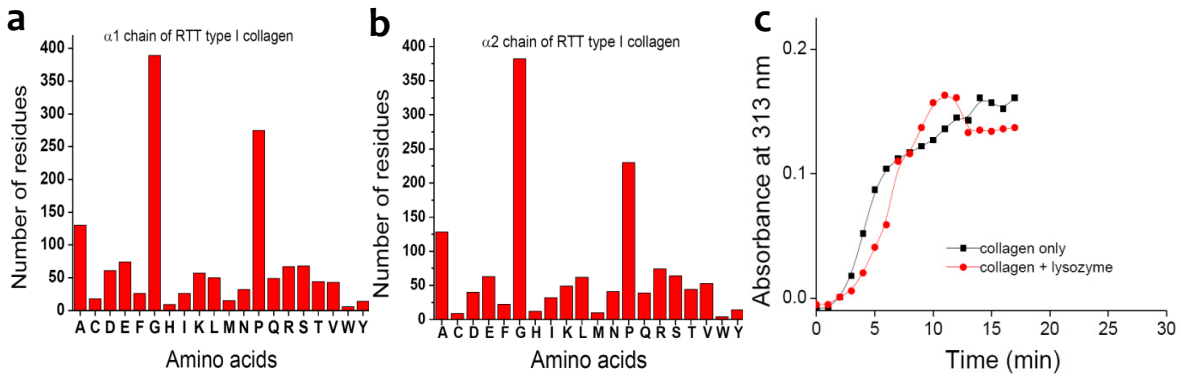


**Figure 3.9 :** (a) Amino acid composition of α1 chain of type I collagen. (b) Amino acid composition of α2 chain of type I collagen. (c) Effect of lysozyme on collagen fibril formation at pH 7.3, 37 °C. Type I collagen (from Rat Tail Tendon) (~0.2mg/ml) was dissolved in 5% acetic acid solution and an appropriate volume of 10X PBS was added to adjust the sample buffer to pH ~7.3. The process of fibril formation was monitored by recording the change in the optical density of the sample at 313 nm using a spectrophotometer. Details of the symbols are as follows: collagen only (■), collagen + lysozyme (●). The molar ratio of collagen to lysozyme was 1:100.

## 3.4 CONCLUSION

In conclusion, the first study provides evidence of rapid coaggregation and cross-seeding between different globular proteins during amyloid fibrils. The onset of coaggregation was found to be more dependent on the amyloid prone intermediate species of the participating proteins (Figure 3.10). These results may have direct relevance to the mechanism of co-existence of two amyloid-linked diseases in individual patients. Moreover, since amyloid-linked hallmarks such as plaques and inclusions are poorly understood, these new findings on coaggregation and cross seeding may help in greater understanding of amyloid formation and its associated diseases.

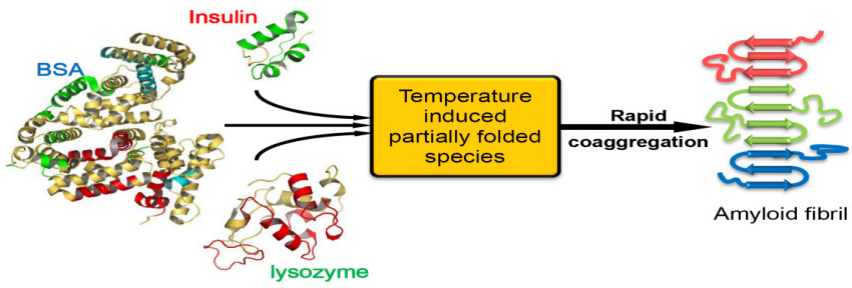


**Figure 3.10 :** Schematic representation of rapid coaggregation among globular proteins during amyloid formation.

From the second study it was observed that type I collagen is capable of inhibiting the amyloid formation of lysozyme. Based on these results, it is predicted that the intermolecular interaction between unfolded chains of collagen and partially folded aggregation-prone lysozyme species is more favorable and it could be one of the reasons behind the inhibition effect (Figure 3.11). Though further experiments are required to generalize the antiamyloid activity of collagen on other amyloidogenic proteins, these results may have implications for collagen based therapeutics against amyloid-linked neurodegenerative diseases.

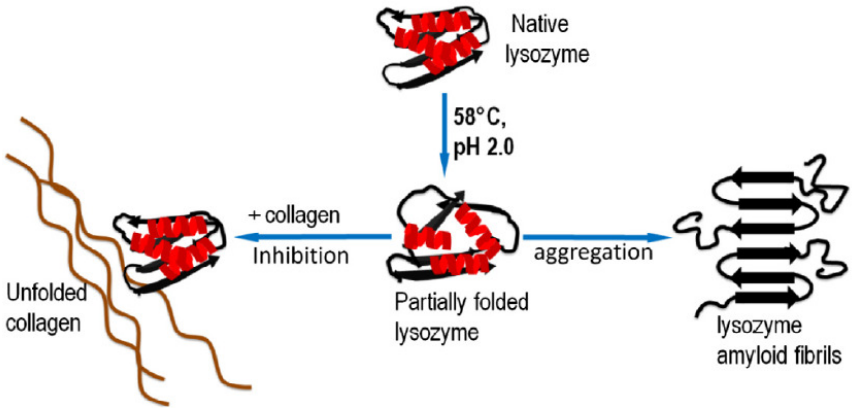


**Figure 3.11 :** Schematic representation of inhibition of lysozyme amyloid formation by type I collagen.

...