Protectors of Oxidative Stress Inhibit Aβ (1-42) Aggregation *in vitro*

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Reactive oxygen species (ROS) have been investigated to have pivotal roles on amyloidogenecity of β-amyloid peptide (Aβ), the major component of senile plaques in Alzheimer's disease (AD) brain. Addition of radical scavengers is one of the on-going strategies for therapeutic treatment for AD patients. Hsp104 protein including two ATP binding sites from *Saccharomyces cerevisiae*, as a molecular chaperone, was known to function as a protector of ROS generation when exposed to oxidative stress in our previous study. This observation has led us to investigate Hsp104 protein as a molecular mediator of Aβ aggregation in this study. We have developed a new way of expression for Hsp104 protein using GST-fusion tag. As we expected, formation of Aβ aggregate was protected by wild type Hsp104 protein, but not by the two ATP-binding site mutant, based on Thioflavin-T fluorescence. Interestingly, Hsp104 protein was observed to keep Aβ from forming aggregates independent of ATP binding. On the other hand, disaggregation of Aβ aggregates by wild type Hsp104 was totally dependent on the presence of ATP. On the other hand, mutant Hsp104 with two ATP binding sites altered exhibited no inhibition. Another effective antioxidant, hydrazine analogs of curcumin were also effective in Aβ fibrilization as protectors against oxidative stress. Based on these observations we conclude that Hsp104 and curcumin derivatives, as protectors of oxidative stress, inhibit Aβ aggregation *in vitro* and can be candidates for therapeutic approaches in cure of some neurodegenerative diseases.

Key Words : Hsp104, Antioxidants, Curcumin, Aβ aggregation

Introduction

Several neurodegenerative diseases, such as Alzheimer's Disease (AD), Parkinson Disease (PD) and Huntington disease (HD), result from accumulation of damaged proteins induced by oxidative stress.[1] Protection of these misfolded proteins by some antioxidants have been investigated as a therapeutic strategy to prevent or treat these diseases.

β-amyloid peptide (Aβ) is a 42-43 amino acid peptide which is the major component of senile plaques in AD brain.[2] Reactive oxygen species (ROS) have been investigated to have pivotal roles in amyloidogenecity of Aβ fragments. It was reported that addition of radical scavengers such as ascorbic acid prevented aggregation process by metal-catalyzed oxidation, suggesting oxidative stress may be a cause of Aβ aggregate formation.[3] Once Aβ peptide aggregates into amyloid fibrils with a cross β-sheet conformation, the fibril became neurotoxic partly due to generation of its associated free radicals followed by lipid peroxidation and protein oxidation as a result of oxidative stress.[4]

A series of antioxidant derivatives, such as glutathione, other SH-containing antioxidants, vitamins, and polyphenolic compounds have been therefore designed and identified to scavenge free radicals generated from amyloid fibrils.[5] For example, vitamin E protects cells from cytotoxicity, but does not inhibit Aβ (1-42) fibril formation, suggesting that it functions through the scavenging of Aβ-associated free radicals.[6] In a separate study performed by Dhitavat, vitamin

E could quench Aβ-induced lipid peroxidation, but was not able to effectively quench ROS generated by pre-formed lipid peroxidation.[7] Recently another antioxidant, curcumin was identified as a protector of Aβ insult in rat and human[8] as well as a potent chemopreventive agent.[9] A low-dose curcumin (160 ppm) significantly reduced not only oxidative damages but also insoluble Aβ deposit when injected in an Alzheimer transgenic mouse.[10]

As molecular chaperones, small heat shock proteins (sHSPs) have been extensively studied for preventing aggregation of cellular proteins during various stresses.[11] αB-crystallin is colocalized in the plaques and prevented aggregation of Aβ protein.[12] Hsp27 inhibits amyloid formation up to 75% *in vitro*, but only reduces only by 6-36% if treated pre-formed amyloid.[13] A great deal of attention have been drawn to Hsp104 protein as a protector of oxidative stress due to impairment of mitochondrial integrity.[14] In our previous studies, we have observed that Hsp104 protein functions as a respiratory regulator in *S. cerevisiae*, judging from comparison data of the metabolic turnover rates in the TCA cycle for both wild type and 104 using ^{13}C-NMR spectroscopy.[15] Hsp104 helps mitochondria efficiently accomplish metabolic pathways within cells, in order not to produce excess ROS. Cell survival rates against H_2O_2 and menadione treatment have demonstrated that hsp104 protein keeps yeast cells from severe oxidative stress. ROS production was also diminished in the presence of functional Hsp104 protein (manuscript submitted). Importantly, the presence of two ATP binding sites plays significant role on rescuing cells from oxidative stress. Observation that Hsp104 protein functions as an ROS regulator led us to test it as a molecular

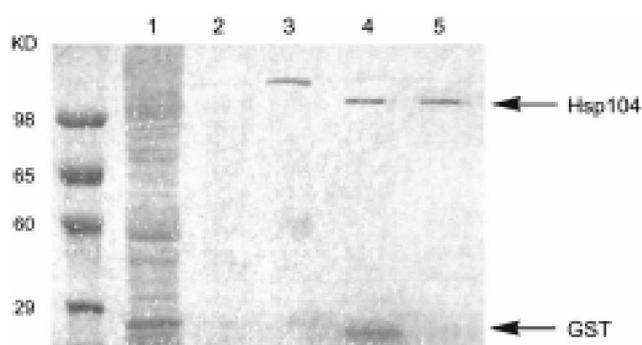*To whom correspondence should be addressed. Fax: +82-2-462-9954, e-mail: khlee@kunja.sejong.ac.kr

mediator of $A\beta$ fibril aggregation in this study. In order to easily purify Hsp104 protein into a homogeneity, we have developed a new way of expression system using GST-fusion tag. $A\beta$ fibril formation was monitored after coincubated with the two ATP-binding site mutant as well as wild type Hsp104 protein using Thioflavin-T fluorescence. As another effective antioxidant, hydrazine analogs of curcumin were also examined as protectors against oxidative stress and $A\beta$ fibrilization.

Results and Discussion

Purification of Hsp104 proteins by GST-fusion methods. Previously we have expressed the Hsp104 protein from A750 strain (BJ5457/p2UG104, pG-N795 obtained from S. Lindquist, MIT) by induction with deoxycorticosterone. It was subsequently purified by serial chromatographys using Affi-Gel Blue, DEAE and Superose 6 Gel Filtration column.[16] It turned out that the purified Hsp104 was not stable long enough to carry out the subsequent experiments, partly due to the presence of endogenous yeast proteases contaminated in the purified fraction. In order to overcome the drawbacks in using yeast system to express a protein, we attempted to express and purify the Hsp104 from bacteria (BL21) as a GST-fusion protein and each fraction was examined to measure the size of eluted protein in the silver stained 10% SDS-PAGE (Figure 1). Hsp104 protein fused with GST tag was estimated to have 132 kDa in the GST elution buffer fraction (lane 3). Upon treatment with thrombin, the GST tag was cleaved and found in 28 kD position (lane 4). Finally GST was removed by glutathione sepharose and only one band was shown at the corresponding position of Hsp104 protein (lane 5).
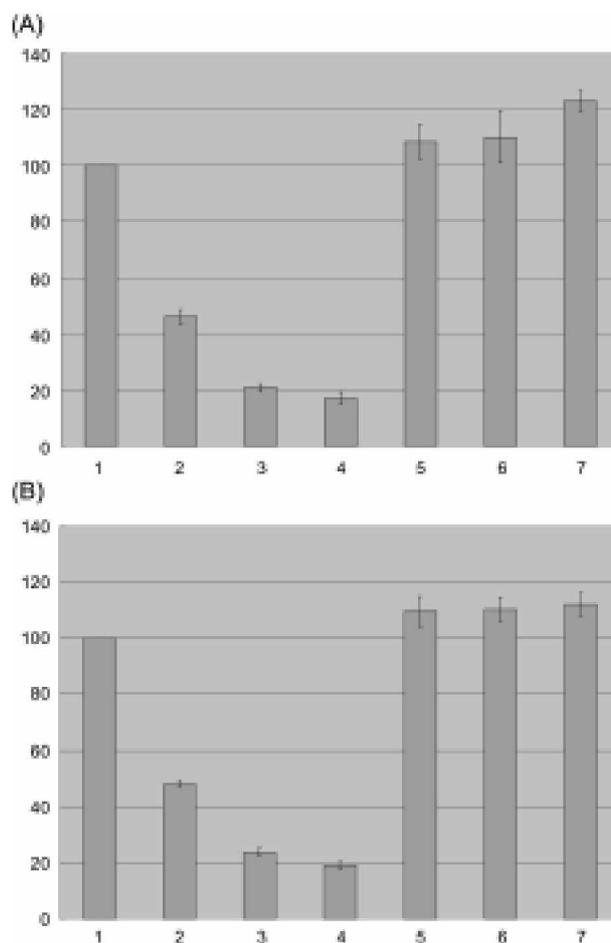
Protection of $A\beta$ aggregate formation by the purified Hsp104. Among heat shock proteins, αB-crystallin and hsp27 have been reported to effectively inhibit $A\beta$ aggregate formation *in vitro*.[12,13] Here we have tested the purified

Hsp104 protein as a protector of aggregation of $A\beta$ protein *in vitro* since it is a functional analog of mammalian Hsp27 in providing thermotolerance. More importantly, we have found that Hsp104 protein acts as a protector of membrane integrity, thereby reducing the amount of ROS generated due to metabolic imbalance between TCA cycle and oxidative phosphorylation pathway under stress conditions.[14] The presence of two ATP binding sites including K218 and K620 was absolutely required for antioxidant activity, as earlier demonstrated for other functions, such as providing thermotolerance and resolubilizing misaggregated proteins. We may therefore envision that the functional Hsp104 protein with two ATP binding sites can participate as an antioxidant to prevent A aggregate formation *in vitro*.

In order to verify if the purified Hsp104 acts as a protector of $A\beta$ aggregate formation via regulating oxidative stress, Congo red assays were initially performed in 96 well plates following 24 hr incubation and the amount of bound $A\beta$ was

Figure 1. Expression and purification of Hsp104 and Hsp104-pKT218/620 proteins by GST fusion systems. Hsp104/pGEX-4T3 was recombined and incubated with 1 mM IPTG to express a GST-fused Hsp104 protein. Each fraction from series of affinity chromatographys were examined in 10% SDS-PAGE in which samples were prepared from crude extract after induction (lane 1), unbound fraction after glutathion Sepharose 4B (lane 2), GST-fused Hsp104 (lane 3), thrombin treatment (lane 4) and the purified Hsp104 after glutathione sepharose 4B (lane 5).

Figure 2. Protection of $A\beta$ aggregate formation by purified wild type Hsp104 and ATP binding site mutant (Hsp104-pKT218/620). Aggregation of $A\beta$ was measured by ThT fluorescence with excitation at 450 nm and emission at 482 nm and reported as percentage of a control with $A\beta$ alone. Incubation conditions are $A\beta$ alone (lane 1), 0.2 μM Hsp104 (lane 2), 1.0 μM Hsp104 (lane 3), 2.0 μM Hsp104 (lane 4), 0.2 μM mutant (lane 5), 1.0 μM mutant (lane 6), 2.0 μM mutant (lane 7) in the presence of ATP (A) and in the absence of ATP (B).

calculated.[14] The degrees of protection by Hsp104 were comparable to those of Hsp27. In the present study, more sensitive thioflavin-T fluorescence assays were performed to measure the inhibition activity in $A\beta$ aggregate formation under the regulation of Hsp104 proteins. We made several interesting observations in which Hsp104 protein blocks aggregation of $A\beta$ peptide (at 100 μM) in a dose-dependent manner (lane 2, 3, and 4 in Figure 2A). If Hsp104 mutant with two ATP-binding site altered were incubated, no inhibition was observed (lane 5, 6 and 7 in Figure 2A). To our surprise, blocking of $A\beta$ aggregate formation by Hsp104 protein occurred regardless of the presence of 2mM ATP (Figure 2A and 2B).

ATP-dependent disaggregation of $A\beta$ aggregates by functional Hsp104. Lindquist and coworkers have reported that Hsp104 helped cells resolubilize certain proteins which was aggregated resulting from severe heat shocks.[17] Therefore we questioned whether functional Hsp104 protein can resolubilize $A\beta$ aggregates *in vitro*. 100 μM $A\beta$ was

allowed to form aggregates for 2 days and incubated at different concentrations of Hsp104 protein for further 24 hrs. It was observed that aggregate formation was reduced in a dose-dependent manner only in the presence of ATP (lane 2, 3, and 4 in Figure 3A). The degree of reduction was only 20-30%. On the other hand, $A\beta$ aggregate was not decreased with the ATP-binding mutant protein. When Hsp27 was used as a control, aggregate formation was diminished only within 10% range. It should be noted that disaggregation effect by Hsp27 was independent of the presence of ATP. Based on the results altogether, we were able to demonstrate the ATP-dependent disaggregation of A fibrils by functional Hsp104 in a concentration dependent manner. These findings will be applied to further elucidate the antioxidant effect of Hsp104 in other types of neurodegenerative diseases closely related with oxidative stress, such as PD with synuclein and HD with polyQ aggregates.

Effect of curcumin derivatives on protection of $A\beta$ misaggregation. Some antioxidant derivatives, such as vitamin E and N-acetylcystein (NAC) have been also investigated as candidates for effective treatment of ROS-mediated neurocytotoxicity. Recently, curcumin was identified as a protector of $A\beta$ insult *in vivo*.[8] It was reported that diketone moiety as well as the phenol ring structures of curcumin may be essential for the antioxidant activity by the structure-activity relationship studies.[18] In order to examine

(A)
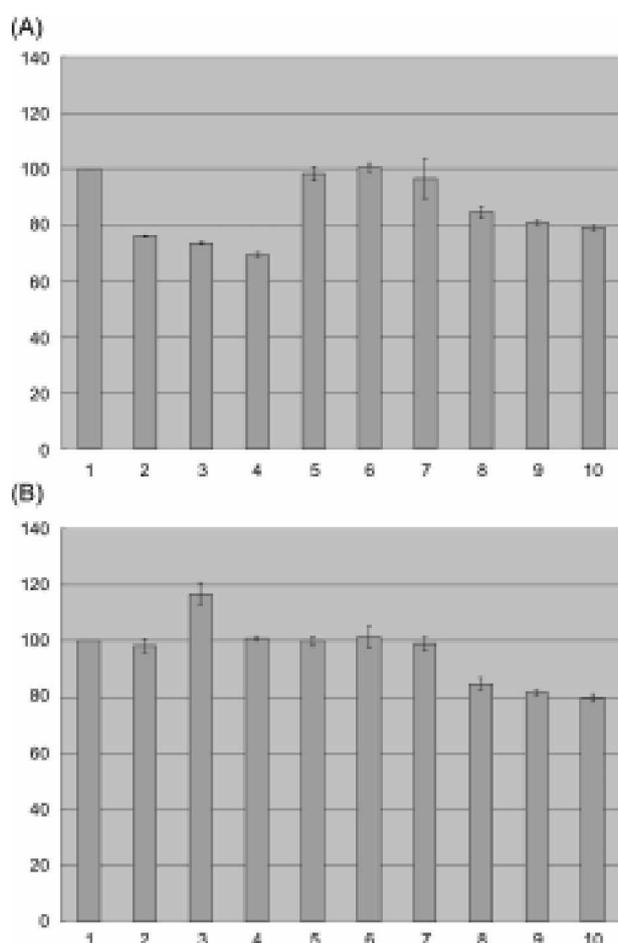
Figure 3. ATP-dependent disaggregation of $A\beta$ aggregates by wild type Hsp104 and ATP binding site mutant (Hsp104-pKT218/620). Disaggregation assays were performed with $A\beta$ alone (lane 1), 0.5 μM Hsp104 (lane 2), 3.0 μM Hsp104 (lane 3), 5.0 μM Hsp104 (lane 4), 0.5 μM mutant (lane 5), 3.0 μM mutant (lane 6), 5.0 μM mutant (lane 7), 0.5 μM Hsp27 (lane 8), 3.0 μM Hsp27 (lane 9), 5.0 μM Hsp27 (lane 10) in the presence of ATP (A) and in the absence of ATP (B).
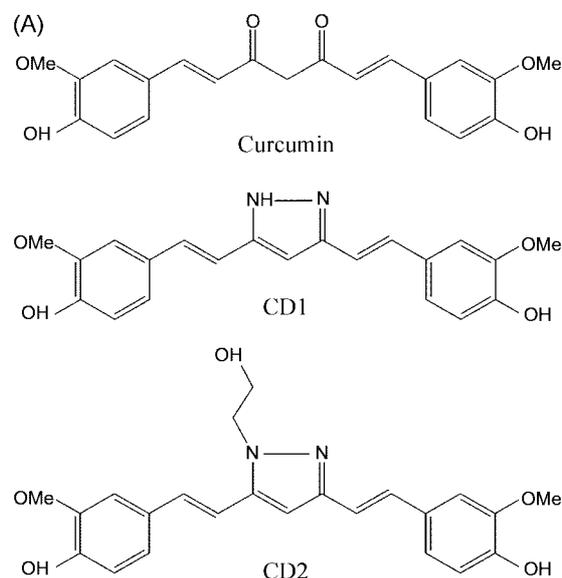
Figure 4. Curcumin derivatives as inhibitors of $A\beta$ aggregation. (A) Structures of curcumin, hydrazinocurcumin (CD1) and hydroxyethylhydrazinocurcumin (CD2). (B) Protection of oxidative stress was measured by cell survival rates in the presence of curcumin derivatives. The survival rate of Δhsp104 (lane 1) with no treatment was compared with that of 30min exposure of 1 mM H_2O_2 (lane 2), 1 mM H_2O_2+10 μM curcumin (lane 3), 1 mM H_2O_2+10 μM CD1 (lane 4) and 1 mM H_2O_2+10 μM CD2 (lane 5). (C) Inhibition of $A\beta$ aggregation was examined with $A\beta$ alone (lane 1), 0.1 μM curcumin (lane 2), 1.0 μM curcumin (lane 3), 10.0 μM curcumin (lane 4), 0.1 μM CD1 (lane 5), 1.0 μM CD1 (lane 6), 10.0 μM CD1 (lane 7), 0.1 μM CD2 (lane 8), 1.0 μM CD2 (lane 9), 10.0 μM CD2 (lane 10).
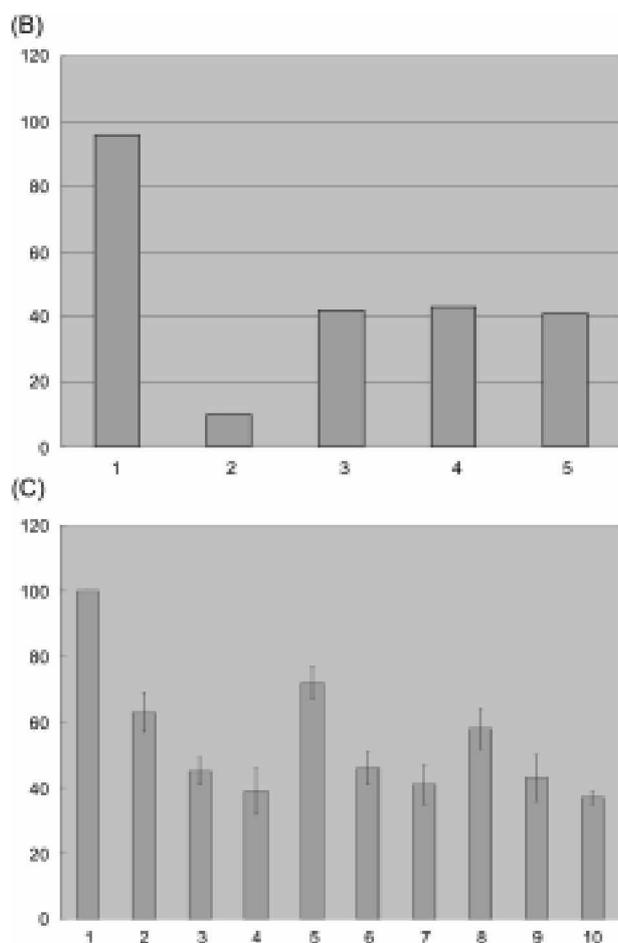
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(B)

(C)

Figure 4. Continued.

if the antioxidant function of curcumin and its derivatives confers the ability of Aβ inhibition *in vitro*, we have designed and synthesized curcumin derivatives. Since the diketone moiety is essential for the activity, we have cyclized the central groups by hydrazinolysis to yield hydrazinocurcumin (CD1) and hydroxyethylhydrazinocurcumin (CD2) (Figure 4A). We first incubated Δhsp104 strain, which we have found hypersensitive against oxidative stress (manuscript submitted), with 1 mM H$_2$O$_2$ and 10 μM curcumin and its derivatives. Among 6 compounds which we expected they would be, only two derivatives (CD1 and CD2) exhibited protection against oxidative stress as effective as curcumin itself (Figure 4B). With these three compounds, we measured the *in vitro* aggregation of Aβ protein by Thioflavin-T assay (Figure 4C). All of them showed 40-60% of protection in concentration dependent manners. These data suggest that curcumins and its hydrazino derivatives as antioxidants can prevent Aβ insult by direct blocking formation of Aβ aggregation. We are currently on investigation for a molecular mechanism of ROS protection *via* curcumin derivatives and their roles on Aβ aggregation.

Experimental Section

Materials. Yeast strains and plasmids used in this study were obtained from S. Lindquist laboratory (MIT). IPTG (isopropyl β-D-thiogalactopyanoside) was purchased from Gold biotechnology, glutathione sepharose 4B was obtained from Amersham. Thioflavin-T was purchased from Sigma. Aβ (1-42) was purchased from Biosource International and Hsp27 was obtained from Stressgen.

Construction of recombinant GST fusion system. The wild type copy of Hsp104 gene as well as the double mutated gene in the ATP binding sites were amplified by PCR from pYS104 and pKT218/620, respectively. The primers were designed to include extra BamH1 and Xho1 sites (the forwarding primer is 5'-CGCGGATCCTCAAC-TACACGTACCATAAAA-3' and the reversing primer is 5'-CCGCTCGAGTAAAAATCACACTATATAAA-3'). After treated with BamH1 and Xho1, the fragments were ligated with pGEX-4T3 vector (Pharmacia) and transformed into BL21. The corresponding hsp104 copies of 2.8 kb were amplified and confirmed by PCR from the recombinant plasmids (data not shown).

Expression and purification of Hsp104. Cells were grown into O. D. of 0.5-1.0, incubated with 1 mM IPTG and cultured for an additional 5hr. After collected by centrifugation, cells were sonicated in PBS and disrupted by sonication on ice. 1% Triton X-100 was added and centrifuged at 15,000 rpm for 20 min at 4 °C. The supernatant was loaded onto glutathione Sepharose 4B, washed by addition of 10 bed volumes of PBS. The GST-fused Hsp104 proteins were eluted by 10 mM glutathione elution buffer (10 mM glutathion in 50 mM Tris-HCl (pH 8.0)) and concentrated by dialysis and amicon filtration. The GST tag was cleaved off by thrombin digest and removed by glutathione sepharose 4B. Finally the purified Hsp104 proteins were obtained upon loading glutathione sepharose 4B followed by dialysis against 1 mM Tris (pH 8.0) and amicon filtration (3000 × g for 30 min at 4 °C). The purified wild type and mutant Hsp104 proteins were identified in 10% SDS-PAGE by silver staining.

Synthesis of curcumin derivatives. A synthetic derivative hydrazinocurcumin (CD1) was prepared as previously described using hydrazine in the presence of acetic acid.[19] A similar method was also applied to synthesize hydroxyethylhydrazinocurcumin (CD2) (manuscript in preparation). Each compound was dissolved at 1 mM in DMSO, and diluted to an appropriate concentration for the protection assay of aggregate formation.

Survival against oxidative stress. Yeast strain W304 (Δhsp104) was cultured in YPA (1% Bacto-yeast extract, 2% Bacto-peptone, 1% potassium acetate) to O.D.$_{600nm}$ of 0.25. Cells were collected and resuspended in phosphate buffer with 10 μM curcumin derivatives. Cells were exposed to 1mM H$_2$O$_2$ and incubated for 30 min. Aliquots were taken at each time point and plated on YPD plates. After 2 days of incubation at 30 °C, survived cells were counted and reported as a percentage of negative control which was cultured without any treatment.

Measurement of aggregate formation by ThT fluorescence. Amyloid fibril formation was measured by fluoro-

metric methods described in Naiki *et al.*[20] Immediately before the experiment, a 1 mg/mL stock solution of Aβ (1-42) was prepared in distilled deionized water. A solution of 100 μM Aβ (1-42) peptide was incubated in dark at 37 °C in PBS (100 mM NaCl, 10 mM NaH$_2$PO$_4$, pH 7.4) with or without different concentration of Hsp104 protein. In the disaggregation experiments, Aβ (1-42) peptide was aggregated for 48 hrs prior to subsequent incubation with Hsp104 protein. After 24 hr of incubation, 4 μM Thioflavin-T was added and fluorescence intensity was measured with excitation at 450 nm and emission at 482 nm. The date from three identical samples in separate experiments were averaged to obtain the final value.

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