International Journal of Research in Pharmacy and Biosciences
Volume 6, Issue 1, 2019, PP 17-20
ISSN 2394-5885 (Print) & ISSN 2394-5893 (Online)

Inhibition of Amyloid Fibrillation of Hen Egg White Lysozyme by Oxidizing Agent

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ABSTRACT

Amyloid fibrillation is protein aggregation phenomenon relevant to some devastating human diseases such as Alzheimer's disease and Parkinson's disease. This study aims to understand the inhibition effect of oxidizing agent on amyloid fibrillation. Hen egg white lysozyme was used a model protein and Fenton's reagent was used as a model oxidizing agent. Thioflavin T assay was used as an amyloid-specific technique to quantify amyloid fibrillation and atomic force microscopy was used to tackle the morphology changes. Our results indicated that Fenton's reagent was able to efficiently inhibit amyloid fibrillation. These preliminary results implicated that a molecule with oxidizing capability may serve as potential amyloid inhibitors.

Keywords: *Amyloid, fibrillation, lysozyme, Fenton's reagent*

INTRODUCTION

Amyloid fibrillation is a unique protein aggregation phenomenon. Under appropriate condition, protein monomers can self-assemble with each other to form a type of fibrillar aggregate. The aggregate consists of a pair of beta-sheets. The elongation of the beta-sheet along one direction makes the aggregate appear like a fibril. Such fibril is referred to as amyloid fibril. [1-3] Deposition of amyloid fibrils in tissue and organs in human body will lead to some devastating human disorders such as Alzheimer's disease and Parkinson's disease[4]. Searching for amyloid inhibitors or modulators is no doubt very beneficial to the prevention and treatment of amyloid-related diseases.

In this work, we would like to explore the inhibition effect of oxidizing agent on the amyloid fibrillation using hen egg white lysozyme as a model protein and Fenton's reagent as a model oxidizing agent. Hen egg white lysozyme is used here as a model system and it is a widely used model system in amyloid research.[5-9] Fenton's reagent is well known oxidizing agent. Two detection techniques, thioflavin T (ThT) assay and atomic force microscopy (AFM), were used in this work. Amyloid fibrillation requires a delicate interaction between protein side chains. Our hypothesis is that if the side chains of the protein could be modified with oxidizing agent, amyloid fibrillation will be modulated or inhibited. The preliminary results in this work indicate that our strategy to inhibit amyloid fibrillation is successful.

MATERIALS AND METHODS

Materials

Hen egg white lysozyme was obtained from Amresco. Thioflavin T was purchased from Acros. Hydrochloric acid (HCl), sodium chloride (NaCl), hydrogen peroxide (H_2O_2), and ferrous chloride ($FeCl_2$) were purchased from local vendors. The chemicals were used without further treatment. Distilled water with 18.2 MΩ·cm resistivity was used throughout the work.

Sample Preparations

Amyloid fibril of lysozyme was prepared with the incubation method in a thermal incubator which can accurately control the temperature of the incubation solution. The following incubation solution was used: 10 mg/mL lysozyme, 8 mg/ml NaCl, pH=2, and 65 °C. Before incubation, the solution was first centrifuged with 10000 rpm and then passed through a 0.22 um filter to filter out some particles. To test the effects of fenton's reagent, the incubation solution will contain H_2O_2 and Fe^{2+} at different concentration. Fenton's reagent was also used to treat the pre-generated amyloid fibrils to see its disassembly effect. Fenton's reagent was made by mixing the stock solutions

of 4M H_2O_2 and $FeCl_2$ at different ratios.

ThT Assay

ThT assay was use to quantify the amount of generated amyloid fibrils. The assay was performed with a Hitachi F-7000 fluorescence spectrophotometer using an excitation wavelength of 450 nm and a detection wavelength of 485 nm. The concentration of ThT solution is 10 µM. The buffer is 20 mM phosphate buffer at pH=6.3. During incubation, aliquots of incubation solutions were taken out of the incubation vial at selected time points and were subjected to the assay. For each measurement, 10 µl of incubation solution was added into 1000 µl of ThT solution in a quartz cuvette.

Atomic Force Microscopy (AFM) Measurement

The AFM measurements were performed on mica surface using NT-MDT AFM system using tapping mode. Typically, a drop of incubation solution was first diluted and then deposited onto mica surface. The surface was then rinsed with water and air-dried for AFM measurement. The AFM probe was purchased from NT-MDT. The image was processed with Nova software.

Results and Discussion

The following are the results of the inhibition effect of Fenton's reagent on lysozyme amyloid fibrillation investigated by ThT assay. ThT assay is a specific dye to amyloid fibril and can quantify amyloid fibril during incubation. It is also widely sued to evaluator the amyloid inhibitor. An observed decrease of ThT intensity due to the effect of a chemical will indicate an inhibitory effect.

Fenton's reagent is a mixture of H_2O_2 and Fe^{2+} where Fe^{2+} serves as a catalyst. Through the following reactions (1) and (2), H_2O_2 generates two radicals which are strong oxidizing agents.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + OH^- \quad (1)$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO\bullet + H^+ \quad (2)$$

Figure 1 shows the effect of Fenton's reagents on the amyloid fibrillation of lysozyme and Table 1 lists the experimental details of Figure 1. As we can see from the trend of ThT intensity change under the influence of Fenton's reagent, with the presence of Fenton's reagent, ThT intensity decreased significantly as compared with the control. For example, after eight-day incubation, the control sample (sample #1) showed ThT intensity of 2067, yet this number decreased to 1123 for sample #2, 622.7 for sample #3, and 472.4 for sample #4. Furthermore, the results showed that the ratio between H_2O_2 and Fe^{2+} was an important factor to affect the inhibitory performance of Fenton's reagent. From sample #2 to sample #4, we had increased H_2O_2 to Fe^{2+} ratio and we also had corresponding ThT intensity decrease. This means higher H_2O_2 to Fe^{2+} ratio was better to the inhibitory effect of Fenton's reagent.

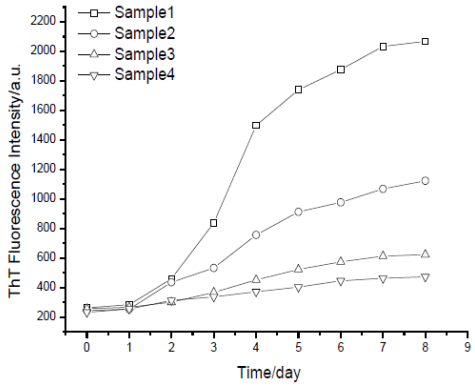

Figure1. *Effects of different concentrations of Fenton's reagent on lysozyme amyloid fibrillation by ThT assay*

Table1. *Effects of different concentrations of Fenton's reagent on lysozyme amyloid fibrillation by ThT assay*

Incubation Time(Days)	Sample #1	Sample #2	Sample #3	Sample #4
0	262.7	243.5	256.4	232.4
1	283.7	253.7	266.3	254.7
2	459.7	435.7	300.7	312.4
3	837.8	532.7	366.9	336.7
4	1498	756.1	451.7	371.4
5	1739	912.7	522.8	402.7
6	1876	976.4	573.8	445.6
7	2034	1068	612.7	462.7
8	2067	1123	622.7	472.4

Note: *Sample #1: control; Sample #2: 2.5µL H_2O_2 + 5µL Fe^{2+}; Sample #3: 5µL H_2O_2 + 5µL Fe^{2+}; Sample #4:*

10μL H_2O_2 + 5μL Fe^{2+}.

The above inhibitory experiment was performed by adding Fenton's reagent into the incubation solution at time=0. This set of experiments tested the effect of Fenton's reagent on the amyloid fibrillation process. One may wonder whether Fenton's reagent could disassemble the pre-formed amyloid fibrils. To this end, we designed the following experiment. After 8-day incubation, we added Fenton's reagent to these preformed amyloid fibrils. As we can see in Table 2 and Figure 2, Fenton's reagent could causes a pronounced ThT intensity decrease on the preformed amyloid fibrils. This result suggests that Fenton's reagent could disassemble the preformed amyloid fibrils. This actually illustrates the nature of the inhibitory effect of Fenton's reagent.

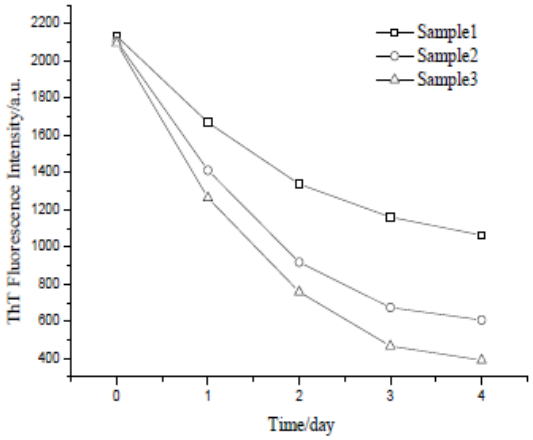

Figure2. *Effects of different concentrations of Fenton's reagent on preformed lysozyme amyloid fibrils by ThT assay*

Table2. *Effects of different concentrations of Fenton's reagent on preformed lysozyme amyloid fibrils by ThT assay*

Incubation Time (Days)	Sample #1	Sample #2	Sample #3
0	2134	2107	2096
1	1669	1412	1264
2	1338	917.6	757.6
3	1160.3	673.8	466.6
4	1062.5	606.5	390.4

Note: *Sample #1:2.5μL H_2O_2+5μL Fe^{2+}; Sample #2:5μL H_2O_2+5μL Fe^{2+}; Sample #3:10μL H_2O_2+5μL Fe^{2+}.*

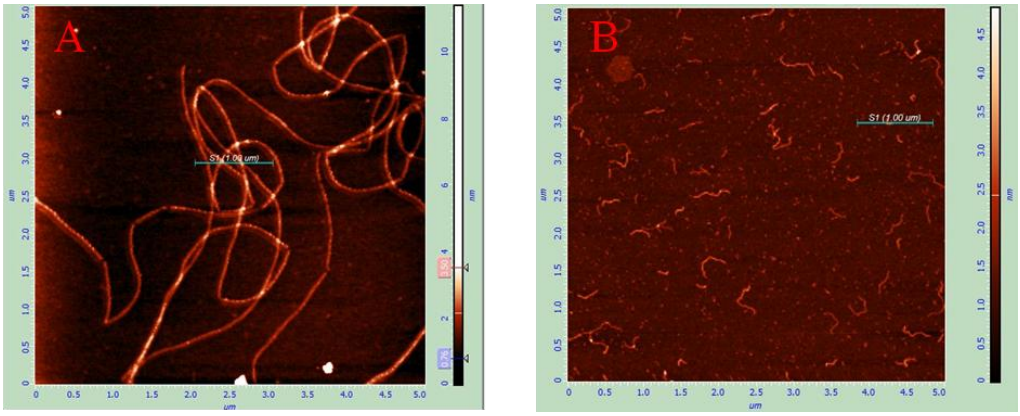

Figure3. *Effect of Fenton's reagent on the morphology of lysozyme amyloid fibril.A: control; B: with Fenton's reagent.*

We also used AFM to tackle the morphological changes of lysozyme amyloid fibrils. As shown in Figure 3, the control sample (in the absence of Fenton's reagent) display abundant long and thick fibrillary aggregates on mica surface. By contrast, the sample in the presence of Fenton's reagent only show some thin and short fibrillary aggregates. Therefore, the AFM study supports the argument that Fenton's reagent has an inhibitory effect on amyloid fibrillation of

lysozyme.

Conclusion

Amyloid fibrillation is relevant to various devastating human diseases such as Alzheimer's disease and Parkinson's disease. Searching for amyloid inhibitors is no doubt very beneficial for the treatment of these amyloid-related diseases. Here, we investigated the inhibition effect of oxidizing agent such as Fenton's on amyloid fibrillation. Hen egg white lysozyme was used a model protein and Fenton's reagent was used as a model oxidizing agent. ThT assay and AFM morphology studies both indicated that Fenton's reagent was able to efficiently inhibit amyloid fibrillation. These preliminary results implicated that a molecule with oxidizing capability may serve as potential amyloid inhibitors.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgement

Authors would like to acknowledge the financial support (hbu2018ss20) from Hebei University.

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Citation: *Lujuan Yang, Wenbin Zhao, Yang Yu, Gang Ma. "Inhibition of Amyloid Fibrillation of Hen Egg White Lysozyme by Oxidizing Agent", International Journal of Research in Pharmacy and Biosciences, vol. 6, no. 1, pp. 17-20 2019.*