Thermostability of lysozyme amyloid fibrils

N M Melnikova¹, M I Sulatsky², Yu D Diordienko¹ and A I Sulatskaya¹

¹ Laboratory of Structural Dynamics, Stability and Folding of Proteins, Institute of Cytology, Russian Academy of Sciences, 4 Tikhoretsky avenue, 194064 Saint Petersburg, Russia
² Laboratory of Cell Morphology, Russian Academy of Sciences, 4 Tikhoretsky avenue, 194064 Saint Petersburg, Russia

melnikova07nm@gmail.com

Abstract. Ordered protein aggregates, amyloid fibrils, are a marker of many serious diseases, such as Alzheimer’s, Parkinson’s, prion diseases, etc. At present, special attention is paid to the study of external influences that can affect the structure and stability of mature amyloid fibrils, which may be in demand in the development of approaches to the therapy of amyloidosis, as well as in the creation of new high-strength materials on the basis of these protein aggregates. An external factor, the influence of which on fibrils was studied in this work, was temperature denaturation. It was shown that heating lysozyme amyloid fibrils to 60 °C does not lead to their degradation, but leads to a reversible increase in the intramolecular mobility of amyloid-forming proteins, but does not change their morphology. At the same time, boiling of lysozyme amyloids leads to their irreversible degradation, which occurs at least 5 days after exposure: fibrils that form larger clusters change their secondary structure, and fibrils with a lesser degree of clustering are divided into separate fibers. Obtained data about the factors that change the stability and structure of amyloids can be applied in biotechnology for creating new high-strength nanomaterials on their basis.

1. Introduction

Highly ordered protein aggregates, amyloid fibrils, are a marker of many serious diseases, such as Alzheimer’s, Parkinson’s, prion diseases, and others [1, 2]. At the same time, physiological amyloid fibrils in bacteria, unicellular eukaryotes, fungi, plants, insects and mammals were found, which are necessary for the normal functioning of cells and tissues, which emphasizes the biological significance of amyloid formation [3, 4]. Due to the widespread occurrence of amyloid fibrils, as well as the variety of their functions (mechanical protection and modification of the cell surface; biotic or abiotic surface adhesion; cellular internalization; ensuring resistance to various external influences; biosynthesis of pigments, control of homeostasis, storage and the release of hormones, signal transduction, etc. [5, 6]) the study of amyloids is a very urgent task. The relevance of amyloid fibrils study is also due to their unique mechanical properties, such as high strength and extensibility, which makes them an attractive object for the creation of new nanostructures and nanomaterials [7]. The aim of this work was to analyze the resistance of amyloid fibrils to high temperatures. The object of the study was model amyloid fibrils formed from the globular protein lysozyme with different morphology.

2. Experimental techniques

Fibrillogenesis of lysozyme was carried out using two different protocols, making it possible to obtain aggregates with different morphologies [8]. According to one of these protocols, lysozyme was
incubated in 20% acetic acid in the presence of 100 mM NaCl (pH 2) at 37 °C. According to the second protocol, the protein was incubated in 0.05 M KH2PO4-NaOH buffer in the presence of 3 M GdnHCl (pH 7) at 57 °C. Both samples were incubated with constant stirring for 2 days. Then the fibrils were transferred to distilled water. The prepared amyloid fibrils were studied by the absorption and fluorescence spectroscopy, method of circular dichroism and electron microscopy. In addition, using a specially developed approach based on the samples preparation by equilibrium microdialysis [9, 10, and 11], the interaction with amyloids of a specific fluorescent probe thioflavin T (ThT) was studied. The recorded fluorescence intensity of the samples was corrected on the primary inner filter effect according to a specially elaborated protocol [12].

3. Results
According to electron microscopy data, amyloid fibrils prepared at pH 2 predominantly are thin fibers, having a tendency to form clots. At the same time, in the buffer solution with pH 7 shorter fibrils with a significantly higher degree of clustering are formed (Figure 1).

Figure 1. Visualization of lysozyme amyloid fibrils prepared at pH 2 (a) and pH 7 (b) and transferred to water. The scale bar is 1 μm.

First of all, we investigated the stability of the prepared amyloid fibrils when changing temperature in the range from 0 to 60 °C. The fluorescence spectra of the sample (free and bound to fibrils ThT) and reference (free ThT) solutions obtained by the equilibrium microdialysis were recorded when they were heated from 0 to 60 °C. It turned out that heating the prepared samples leads to a decrease in the fluorescence intensity of ThT bound to fibrils. In order to check whether this decrease is reversible, we cooled the samples to 0 °C. The curve characterizing the heating of the samples almost completely coincided with the curve characterizing their cooling for both samples (Figure 2), which made it possible to conclude that the processes occurring in the samples when they are heated to 60 °C are reversible.

In order to understand what exactly is responsible for the decrease in the ThT fluorescence intensity with heating the sample, we analyzed images of amyloid fibrils obtained by electron microscopy before and after external exposure. We found no changes in the structure or degradation of amyloids after their heating to 60 °C (Figures 3, 4).

It can be assumed that an increase in the sample temperature increases the probability of the ThT molecule transition to the conformation with an angle between the benzothiazole and aminobenzene rings equal to 90 °C, which, in turn, leads to nonradiative deactivation of the excited state of the dye and a decrease in the quantum yield of its fluorescence. This can occur due to a reversible change in the intramolecular mobility of side radicals of amyloid-forming proteins and fragments of the ThT molecule relative to each other, without changing the structure or degradation of amyloid fibrils. Thus, obtained results indicate that lysozyme amyloid fibrils prepared under different conditions are stable.
when heated to 60 °C, which confirms the idea of a high resistance of amyloids to some extreme external influences.

**Figure 2.** Corrected on the primary inner filter effect the fluorescence intensity of ThT (at 480 nm, maximum of fluorescence spectrum) bound to lysozyme amyloid fibrils, prepared at pH 2 (a) and pH 7 (b). Changes in fluorescence intensity are shown with increasing (0-60 °C) and decreasing (60-0 °C) temperature.

**Figure 3.** Visualization of lysozyme amyloid fibrils prepared at pH 2, before (a) and after (b) heating to 60 °C. The scale bar is 0.25 μm.

**Figure 4.** Visualization of lysozyme amyloid fibrils prepared at pH 7, before (a) and after (b) heating to 60 °C. The scale bar is 1 μm.

Then, we used even more extreme environmental conditions and analyzed whether boiling affects the structure and stability of lysozyme amyloid fibrils. Registration of the intrinsic tryptophan
fluorescence spectra of amyloid fibril samples prepared under different conditions made it possible to conclude that changes in the sample with lysozyme amyloid fibrils continue for at least 5 days after their boiling and are irreversible (Figure 5).

**Figure 5.** Spectra of intrinsic tryptophan fluorescence of lysozyme amyloid fibrils prepared at pH 2 (a) and pH 7 (b) on the first, third and fifth days after boiling.

Electron microscopy data of the samples a week after boiling indicate that boiling lysozyme amyloid fibrils, prepared at pH 2, leads to their partial degradation: declustering of not numerous fibrils clots into the separate thin filaments (Figure 6) and to a slight change in their secondary structure, which was evaluated by the recorded circular dichroism (CD) spectra of the samples in the far UV region (Figure 7 a).

**Figure 6.** Visualization of lysozyme amyloid fibrils prepared at pH 2, before (a) and after (b) boiling. The scale bar is 0.25 μm.

**Figure 7.** CD spectra of lysozyme amyloid fibrils prepared at pH 2 (a) and pH 7 (b), before and after boiling.
The boiling of lysozyme amyloid fibrils prepared at pH 7 also led to their degradation, which, however, was more manifested in a change in the secondary structure of amyloid-forming proteins (Figure 7 b), but practically did not lead to declusterization of fibrillar clots (Figure 8).

Figure 8. Visualization of lysozyme amyloid fibrils prepared at pH 7, before (a) and after (b) boiling. The scale bar is 1 μm.

4. Conclusion
Based on the obtained results, it was concluded that heating lysozyme amyloid fibrils with different morphology up to 60 °C causes only a reversible increase in the intramolecular mobility of amyloid-forming proteins. At the same time, boiling of lysozyme amyloids leads to their irreversible degradation: amyloids that form larger clusters change their secondary structure, while fibrils with a lesser degree of clustering are divided into separate fibers. New data about the factors that influence the stability of amyloids can be applied in biotechnology for creating new high-strength nanomaterials on their basis.

Acknowledgments
This work was supported by the Russian Science Foundation (project № 18-74-10100).

References
[1] Iadanza M G, Jackson M P, Hewitt E W, Ranson N A and Radford S E 2018 Nat. Rev. Mol. Cell. Biol. 19 755-73
[2] Eisenberg D and Jucker M 2012 Cell 148 1188–1203
[3] Chiti F and Dobson C M 2006 Annual Rev. Biochem. 75 333-66
[4] Fersht A 1998 Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding vol 42, ed W H Freeman and Company (New York: W H Freeman) p 631
[5] Fowler D M, Koulov A V, Balch W E and Kelly J W 2007 Trends Biochem. Sci. 32 217-24
[6] Pham C L L, Kwan A H and Sunde M 2014 Essays Biochem. 56 207–19
[7] Cherny I and Gazit E 2008 Angewandte Chemie International Edition 47 4062-69
[8] Sulatskaya A I, Rodina N P, Povarova O I, Kuznetsova I M and Turoverov K K 2017 Journ. Of Mol. Struct. 1140 52-8
[9] Kuznetsova I M, Sulatskaya A I, Uversky V N and Turoverov K K 2012 Mol. Neurobiol. 45 488-49
[10] Sulatskaya A I, Kuznetsova I M and Turoverov K K 2011 The Journal of phys. chemistry B. 115 11519-24
[11] Kuznetsova I M, Sulatskaya A I and Uversky V N, Turoverov K K 2012 Plos One 7(2) 30724
[12] Fonin A V, Sulatskaya A I, Kuznetsova I M and Turoverov K K 2014 Plos One 9(7) 103878