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# Influence of the ionic strength on the amyloid fibrillogenesis of hen egg white lysozyme

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ABSTRACT

The study investigates the role of the electrostatic interactions in the fibrillation of the hen egg white lysozyme (HEWL). In order to achieve this aim the influence of the cations $Na^+$, $Mg^{2+}$ and $Al^{3+}$ on the amyloid fibril formation and amorphous aggregation was tested. The amyloids are formed in the solution without added salt but the Thioflavin T fluorescence gives the false-negative result. In these conditions, the HEWL fibrils are long and curvy. If the ionic strength of the solution is sufficiently high, the formed amyloids are shorter and fragmented. Our study shows that the addition of the aluminium salt promotes protein fibrillation. The amorphous aggregation dominates in the high concentration of electrolyte. The in vitro amyloid fibril formation seems to be regulated by universal mechanisms. The theories implemented in the polymer science or for colloidal solutions give the qualitative description of the aggregation phenomena. However, the specific interactions and the additional effects (e.g. fibril fragmentation) modulate the amyloidogenesis.


## 1. Introduction

Amyloids are the fibrillar aggregates of the protein associated with many serious diseases including but not limited to Alzheimer's and Parkinson's diseases [1-3]. In the present work, fibrillation of the lysozyme from the hen egg white (HEWL) has been investigated. The sequence of the amino-acids in the peptide chain of the HEWL is in 40% homologous to the human lysozyme and the tertiary structures of both enzymes are remarkably similar [4]. Deposition of the lysozyme in liver and kidneys is one of the symptoms of the human hereditary systemic amyloidosis [4].

However, the HEWL is often regarded not as a molecular target for treatment of serious illness but rather as a convenient model protein for basic research. This protein is stable, easy to handle and its structure and properties have been well characterised over the years.

The ability to form amyloids seems to be generic property of the polypeptide chain [5]. Thus, the data obtained for the hen egg white lysozyme could be interpreted in a wider context. The fact that the large number of the unrelated proteins and peptides undergo amyloidogenesis suggests that this process is largely universal, therefore it must be driven by an universal mechanism [6-9]. In the current work, we explore the hypothesis the that the electrostatic interactions might play crucial role in the formation of the amyloids.

The tendency of lysozyme to form fibrils depends on the pH of the solution [10]. In the optimal conditions (pH = 2), the HEWL is positively charged and for a single molecule the overall electric charge can reach +15e [11]. The colloidal solution of lysozyme is stabilized by the mutual repulsion of the macromolecules and the electrical double layer. The presence of electrolyte changes the potential of this barrier and in this way may affect the aggregation. Therefore, the research on the influence of the salt on the amyloidogenesis can lead to interesting observations about the mechanism of fibrillation.

The literature data provide partial information regarding the discussed subject. For example, the impact of the type and the concentration of salt on the fibrillation was tested for $\alpha$-synuclein [12], amyloid-$\beta$ peptide [13], islet amyloid polypeptide [14] and immunoglobulin light chain [15]. The special attention was paid to the effect of di- and trivalent ions including $Al^{3+}$, $Fe^{3+}$, $Cu^{2+}$, and $Zn^{2+}$ [12,13]. The aluminium ion is in the focal point of the research [16,17] due to its neurotoxicity [18].

The influence of electrolyte on the fibrillation of the hen egg white lysozyme has been also studied in several occasions. One of the first suggestions that the electrolyte is essential for the HEWL amyloidogenesis comes from the early paper of S.S. Wang [19]. In that work,

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the protein samples were incubated at elevated temperature in a solution containing the mixtures of NaCl and KCl (total concentration of both salts was around 140 mM). The authors concluded that the salt was necessary for the amyloid fibril formation, however, the impact of types of ions or the salt concentration on the discussed process were not examined.

The Hofmeister effect and the role of anions in the HEWL fibrillation were studied by Ponikova et al. [20]. In that work, the salts with the common cation ($Na^+$) and different anions were used. The samples also contained relatively high concentration of glycine buffer (70 mM). The obtained results showed that the amount of the formed fibrils was inversely proportional to the nucleation time (so called lag time). The authors postulated that the anions interacted directly with protein.

The mechanism of the lysozyme amyloidogenesis and its relation to the electrolyte concentration were investigated by Hill et al. [11]. The presented observations were based mainly on the results for the NaCl solution. The preliminary tests were made for the samples containing $MgCl_2$. The work suggests that the fibrillar assembly depends on the ionic strength of the solution and the process can take one of the three pathways. At lower concentration of NaCl (<150 mM) the monomers of the HEWL are directly incorporated into protofibrils. In the range of 150 mM > [NaCl] < 350 mM, the protein oligomers are primary building blocks. Above 350 mM of NaCl, the amorphous aggregation prevails. According to the authors, the specific ion-protein interactions are of lesser importance.

The studies on the lysozyme fibrillation have not been limited to the systems containing alkali halides or alkaline earth halides. Due to the presumed toxicity, the impact of $Cu^{2+}$ and $Zn^{2+}$ on amyloidogenesis has also been examined [21,22].

The main aim of current work is to investigate the process of the in-vitro amyloid fibril formation in the electrolyte solutions covering relatively wide ionic strength from 0.1 to 1.2 mol/dm$^3$. The influence of selected cations i.e. $Na^+$, $Mg^{2+}$ and $Al^{3+}$ on the early stage of the hen egg white lysozyme fibrillation was assessed.

As already mentioned, aluminium ions have adverse health effect. The exposure to $Al^{3+}$ is often regarded as a risk factor in the development of Alzheimer's disease [23] or Parkinson's disease [24]. The presence of aluminium was detected in the cores of senile plaques in brains of the Alzheimer's disease patients [25]. This element is also present in Lewy bodies in the substantia nigra in brains of the patients suffering from Parkinson's disease [26]. Therefore, it is worth investigating how this cation influences the fibrillation of other important protein i.e. lysozyme. It is also informative to compare the properties of $Al^{3+}$ with other cations at the equimolar concentration.

To simplify the analysis, the chloride anion was selected as a common counterion for all tested salts. This decision resulted from several reasons. The $Cl^-$ has small impact on thermal stability of the HEWL, even up to the concentration of 1 M [20,27]. The chloride anion is placed in the middle of the Hofmeister series, close to the borderline between ions classified as kosmotropes and chaotropes [14]. In contrast to the other anions, the fibrils formed in the presence of the $Cl^-$ have typical morphology and properties [20]. Based on these facts, it could be expected that the chloride salts are the most suitable for our studies.

The main conclusion arising from the current work is in agreement with the basic observation: the presence of salt is important for the HEWL fibrillar self-assembly. Additionally, present study provides deeper insight into the role of ionic strength of the solution and specific ion-protein interactions. At low concentration of salt the Thioflavin T fluorescence assay gives false-negative result despite the fact that the amyloids are detected with Atomic Force Microscopy. The high concentration of electrolyte leads to the formation of the amorphous aggregates.

## 2. Materials and methods

### 2.1. Solutions and fibrils preparation

Lysozyme from the chicken egg white, HEWL, (Fluka, Cat. No. 62971) was dissolved in the deionised water, then dialysed overnight against pure water and lyophilised. The crystalline powder was resuspended in the solution of HCl (pH = 2). The final concentration of the salts NaCl (POCH, purity 99.99%), $MgCl_2$ (Alfa Aesar, purity 98%), $AlCl_3$ (Alfa Aesar, purity 99%) in the solutions was equal 100 mM or 200 mM. If necessary, the pH of the solution was adjusted by the addition of the small amount of HCl. All samples were filtered through 0.1 μ m syringe filter (Minisart Cat. No. 16553). The concentration of the lysozyme in samples, determined by weight, was 25 mg/ml. For the comparison purposes, the analogue samples were prepared without addition of electrolytes. The samples were incubated with shaking (122–125 min$^{-1}$) at 60 °C in the thermostatic bath (GFL 1086) for 72 h. The non-incubated samples were stored in refrigerator at 4–6 °C.

### 2.2. Thioflavin T fluorescence (ThT) assay

The thioflavin T fluorescence (ThT) assay was performed according to the known procedure [28]. Briefly, Thioflavin T (Sigma-Aldrich Cat. No. T3516) was dissolved in PBS (10 mM phosphate buffer saline, 150 mM NaCl, pH = 7.0) to obtain 2.5 mM solution. The stock solution was filtered through 0.1 μ m syringe filter (Minisart Cat. No. 16553) and diluted with PBS. In the final solution, the ThT concentration was 9.7 μ M and the concentration of HEWL was equal 0.08 mg/ml. The fluorescence spectra were collected with the Jasco FP 8300 spectrofluorometer. The excitation wavelength was 440 nm, the emission spectra were recorded from 460 to 550 nm with the data interval of 0.5 nm and the scan speed 200 nm/min. The excitation bandwidth was set at 5 nm and the emission bandwidth was 10 nm. Photomultiplier voltage was set to 370 V. Six scans were averaged for every sample.

### 2.3. Atomic force microscopy (AFM)

The reaction mixtures were diluted with water to the concentration of 0.015 mg/ml. Twenty microlitres of this solution was placed on the freshly cleaved mica surface. After 5 min of the incubation, the sample was flushed two times with 50 μ L of the filtered deionised water. The disc was dried and stored in a desiccator over $P_2O_5$.

The AFM imagining was performed using the N-Tegra Aura microscope in a non-contact mode. Tip size was equal 10 nm, the frequency of the scanning was 0.8 Hz. The scans of size 90 × 90 μm, 10 × 10 μm and 5 × 5 μm (at 256 × 256 lines) were collected. The obtained data were analysed in the Gwyddion version 2.50.

### 2.4. Turbidimetric aggregation analysis

The incubated samples of the HEWL were diluted by the addition of water to reaction tube to the final concentration of the protein 8.3 mg/ml. The optical density (OD) was measured at 350 nm using the Thermo Evolution 300 spectrophotometer. The cell was equipped with the custom-made stirring accessory. For each sample, 10 scans were collected and then averaged.

### 2.5. Circular dichroism spectroscopy (CD)

The lysozyme solution diluted with water to the concentration 0.15 mg/ml was divided into two parts. One part was used for the determination of the HEWL concentration using UV–VIS spectroscopy, the second one was analysed using circular dichroism. The CD spectra were recorded in the ultra-violet region from 190 to