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Structural differences between native Hen egg white lysozyme and its fibrils under different environmental conditions

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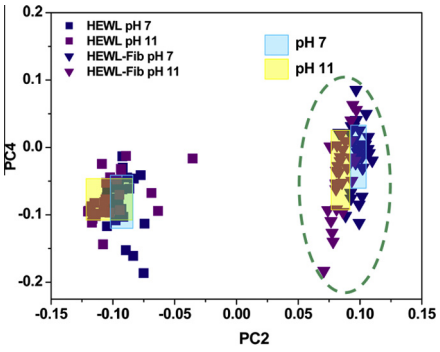
HIGHLIGHTS

- PCA of Raman data discriminates molecular structure of HEWL protein and fibrils.
- SD of scores in PCA plots determines variability in the structure within a species.
- Overall structure significantly differs for fibrils grown at different pH values.
- Overall structure of fibrils grown at different pH values in Cu(II) has similarity.
- Presence of Cu(II), inhibits the formation β-sheet component in HEWL fibrils.

GRAPHICAL ABSTRACT



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ABSTRACT

The difference in molecular structure of native HEWL and its fibrils, grown at a pH value near physiological pH 7.4 and at a pH value just above the pI, 10.7 in presence and absence of Cu(II) ions, is discussed. We focus on differences between the molecular structure of the native protein and fibrils using principal component analysis of their Raman spectra. The overlap areas of the scores of each species are used to quantify the difference in the structure of the native HEWL and fibrils in different environments. The overall molecular structures are significantly different for fibrils grown at two pH values. However, in presence of Cu(II) ions, the fibrils have similarities in their molecular structures at these pH environments. Spectral variation within each species, as obtained from the standard deviations of the scores in PCA plots, reveals the variability in the structure within a particular species.

Introduction

Molecular self-assembly of misfolded proteins into fibrils is believed to be the cause of many neurodegenerative diseases [1]. While such diseases are becoming quite common in today's world, the exact medical solution and early reliable diagnosis in effectively tackling its severity are still lacking. Self assembled protein fibrils, amyloid-β plaques, are known to induce apoptosis of the cells in the brain and stop normal functions of neurons [1]. For understanding the origin of fibril induced diseases, structural characterization of amyloid fibrils is crucial. A large number of experimental techniques are used to obtain the information on structure of the fibrils at the atomic/molecular level [2–4]. X-ray diffraction pattern of the protein could obtain an atomic and molecular-level information about the β-strand assembly of the microcrystals formed from polyamino acid chain sequence [5]. These chains are considered to have the same core structure of fibrils formed in case of proteins leading to amyloid formation. The amyloid fibrils are stabilized by hydrogen bonds involved in inter- and intra-molecular β-sheets of the fibrillar core [6]. In addition to the hydrogen bonding in the cross-β structure, Van der Waals and electrostatic

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forces between different domains of the protein play crucial roles in unfolding of the protein into fibril [7]. Vibrational spectroscopy provides a unique molecular perspective of the structural difference between native protein and its fibrils. Packing defect of the compact structure of polyglutamic acid amyloid fibril is established by vibrational circular dichroism (VCD) **[8]**. Infrared (IR) spectroscopic technique has been extensively used to study the structure of different globular proteins. The IR spectral profile of amide I band can be used to differentiate different types of helical and β-sheet structures in the protein **[9,10]**. Simulated IR spectra based on coupled oscillator model (one transition dipole oscillator is assigned to each peptide group) discusses the secondary structure of a large number of globular proteins **[9]**. Raman scattering is also used extensively to study the secondary structure of the proteins. In the literature, deep UV resonance Raman (UVRR) spectroscopy is used for structural characterization of the protein at all stages of fibrillation [11,12]. The simulation of deep UVRR spectra of amyloid fibrils $A\beta_{34-42}$ and $A\beta_{1-40}$, based on combined quantum and molecular mechanics, reveals effects of conformational fluctuations [13]. A recent report on simulated IR and Raman line profile shows that the spectral line of the latter is more sensitive to types of different components of the secondary structures of the protein. For example, the presence of parallel/anti-parallel β-sheet and uncorrelated inhomogeneity of a specific peptide group significantly influences Raman spectral profile [14,15].

Hen egg white lysozyme (HEWL) is a globular protein comprised of 129 amino acid residues. The protein has α-helix and *β*-sheet components with four disulfide bonds. Thermodynamic stability, folding mechanism and ability to form fibrils enable HEWL as a suitable model for studying in vitro fibrillation [16–19]. The structural characterization of HEWL fibrils is well documented in the literature [16–19]. The average length and diameter of a fibril is ∼5 μm and 4.0 nm [19]. The fibrils have inherent tendency to disintegrate under thermal fluctuations [7]. This disintegration contributes significantly to the growth kinetics of fibrils. We find several reports in the literature on structural modification of fibrils under different environmental conditions [19–26]. The nature and morphology of the fibrils depend on the pH, at which they are grown. Fibrils prepared in the acidic condition were found to be more stable with higher *β*-sheet content than when they are exposed to a solution of pH 7 [20,21]. In a recent report, temperature-dependent FTIR measurement suggested that amyloid fibril of HEWL with parallel β-sheet configuration is formed at elevated temperature, while oligomer with anti-parallel β-sheet configuration is formed at room temperature [22]. Two-step mechanism of the structural changes in HEWL during fibril formation is followed by two dimensional correlation UVRR spectroscopy [11,12]. There are fewer reports in the literature for HEWL fibrils grown at pH higher than its pI value, 10.7 [23]. In addition, the transition metal ions, specially Cu(II), play vital roles in pathological disorders [24,25]. For several proteins and polypeptides, Cu(II) ions are known to modulate the fibrillation pathway [26].

It is to be kept in mind that due to thermodynamics fluctuation, equilibrium dynamics, folding and unfolding of end groups, the structure of the fibrils formed from the unfolding of a protein are not unique. As fibrillation of a protein is a biological process and the phenomenon has medical importance, statistical measurements related to structural characteristics of native protein and its fibrils, carry special importance. Motivated by the large number of reports on molecular structure of HEWL fibrils under different environmental conditions, *we focus here on* the differences in structure of native protein and fibrils in the limit of efficacy and reliability of non-resonant Raman spectroscopy. We have compared the influence of Cu(II) ions and pH of the environment on the molecular structure of the native protein and its fibrils. The experiments are carried out at pH 7, a pH value close to the physiological pH 7.4, and also at pH 11, a pH value just above pI. Here, we have used Raman difference spectra and the well-established multivariate statistical technique–principal component analysis (PCA) for the quantitative discrimination between species.

Experimental and analysis details

Preparation of fibrillar sample

HEWL was purchased from Sigma Chemical Co. (St. Louis, USA). Copper sulfate pentahydrate ($CuSO_4$, $5H_2O$) and all other chemicals were obtained from SRL (India). HEWL was dissolved in double distilled water and the concentration of the solution was determined using a molar extinction coefficient of 37,646 M^{-1} cm^{-1} at 280 nm [27]. The concentration of the solution was 150 μM. Fibrils at pH 7 were grown by heating the aqueous solution of the protein (in the presence of 80% ethanol (v/v) and 20 mM phosphate buffer of pH 7 containing 20 mM NaCl at ∼ 60 °C) for 6 h. Fibrils at pH 11 were grown following the same route using phosphate buffer of pH 11, instead of 7. Fibrils in presence of Cu(II) ions were prepared in the same way keeping the protein and the metal ion ratio at 1:1 (150 μM). The solution was then incubated at room temperature for 1 day. Incubation of the protein results in formation of a gel-like fraction in the solution. The solution may contain partially unfolded protein, small fraction of native protein and fibrils [12,18]. The solution was then centrifuged at 6000 rpm for 30 min. to separate the fibrils.

Raman scattering

The samples under study were (i) native HEWL (A) HEWL fibrils (B) at pH 7; (ii) the same, (C) and (D), respectively, at pH 11; (iii) native HEWL in presence of Cu(II) (HEWL-Cu(II)) (E) and HEWL-Cu(II) fibrils (F) at pH 7 and (iv) the same, (G) and (H), respectively at pH 11. Thirty Raman spectra of each sample were averaged to obtain mean spectrum of a particular sample type. Native HEWL and fibrillar samples were subjected to Raman scattering keeping 180° scattering geometry using a micro-Raman spectrometer with 488 nm argon ion laser. The spectrometer is equipped with an optical microscope (Model BX 41, Make Olympus, Japan), single monochromator (Model TRIAX550, Make JY, Horiba, France), an edge filter, and a Peltier cooled CCD (1024 × 256 pixels) detector. The laser power on the samples was 5 mW. The data acquisition time for each Raman spectrum was 300 s. For Raman measurements, five drops of each sample were set to dry on a thoroughly cleaned aluminum foil at room temperature for 10 h. To determine the homogeneity of the spectral signal, Raman spectra were collected from at least six different spots of each drop using a 50× objective lens. Raman spectra of the buffer solutions were taken under identical experimental conditions. The laser irradiation under the given experimental conditions does not alter protein conformation. Over the reported spectral range, the buffer solutions do not exhibit any particular Raman band.

Straight line background subtracted Raman spectra over the spectral window between 1140 and 1760 cm^{-1} are presented in this study. Spectral normalization was carried out with respect to 1451 cm^{-1} CH_2 deformation peak height of the baseline-corrected spectrum. The fully processed data set was then analyzed with PCA using standard algorithms of MATLAB software (The MathWorks, Natick, MA).

Furthermore, the broad envelope of the amide I band of the mean spectrum of each species is deconvoluted with four Voigt functions for four main secondary structures (turn elements, α-helix, *β*-sheet and coil-like distribution) of the protein [14,15,28], keeping intensity, peak position of components as fitting parameters. The percentage amount of the integral intensity of the Raman mode, for each deconvoluted component, is estimated (total area of