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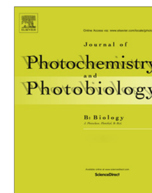

Crowded milieu prevents fibrillation of hen egg white lysozyme with retention of enzymatic activity

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ABSTRACT

Molten globule state plays a crucial role in the amyloidogenesis of several proteins. Hen egg white lysozyme (HEWL) acquires a molten globule state at alkaline pH (12.75). Our study reveals a significant inhibitory effect of high molecular weight polyethylene glycols (PEG) (PEG 20000 and PEG 35000) against alkali-salt mediated fibrillation of HEWL. Native state of HEWL is stabilized in the presence of PEGs accompanied by a decrease in the β-sheet content. Enzymatic activity of HEWL is mostly retained in the presence of polyethylene glycols. The comparable hydrodynamic radius (R_h) of PEG 20000 and native HEWL is central reason to the greater inhibitory potency of PEG 20000 against HEWL fibrillation.

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1. Introduction

Molten globule state of proteins is known to play a critical role in the aggregation pathway of various disease related proteins [1–5], which has motivated numerous *in vitro* researches for understanding the facet of fibrillation process. It is defined as a partially unfolded state which contains exposed hydrophobic surface, a small number of secondary structure elements and fluctuating tertiary structure [6]. It is known to be the key intermediate in protein folding process [7]. Series of proteins are able to form molten globule state under various conditions [8–16].

In vitro amyloid formation is usually carried out in very dilute solutions [17] unlike a cellular environment that is crowded and much more complex. Cells are enriched by an abundance of solutes which occupy ~10–40% of the total fluid volume [18–21] that is being neglected during *in vitro* fibrillation studies. It has been noted that ~20–30% of cell cytoplasm is occupied with proteins, RNA, membranes, polysaccharides, and several organelles [22]. Most of these solutes exert nonspecific interactions but still affect protein mediated biological reactions where excluded volume effect plays a crucial role [23]. Macromolecular crowding imparts a significant effect on protein self-assembly and the comparative stability of native and unfolded states of proteins [24–26]. The principal aspects of macromolecular crowding are (i) the excluded volume effect predominates at a lower concentration range of the crowding agent facilitating protein association, which is surpassed by viscosity effects at higher concentration resulting in a decrease in the rate of association, (ii) the nature and type of interactions of the crowding agents with the concerned protein, (iii) the accelerating and/retarding effects of other factors in dilute solution that still remains in the crowded milieu [27]. Studies have shown a differential role of crowding agents depending on the type of protein and conditions applied [28–30]. Crowding increases the stability of the native state of globular proteins [31]. It has been noted that chaperones can exert improved activity under crowded conditions [32]. Therefore, it becomes necessary to get an insight in the effect of crowding on the aggregation propensity of proteins *in vitro*.

Hen egg white lysozyme (HEWL), a globular protein consisting of 129 amino acid residues, possesses exclusive structural stability, unique folding mechanism and thermodynamic properties [33–36]. HEWL undergoes self association under the influence of different external factors such as change in pH, temperature and in the presence of several additives [37–43]. Human lysozyme, a structural homologue of HEWL, is responsible for nonneuropathic hereditary systemic amyloidosis disease [44]. Resemblance in fibrillar species formed *in vitro* in case of human lysozyme with those obtained from patients and also their similarity with the HEWL fibrils has enabled HEWL to become a suitable model template [35,45]. Moreover, the membrane activity of HEWL fibrils and ability of HEWL oligomers/fibrils to cause cell death via different apoptotic/necrotic pathways have enhanced the importance of HEWL as a model system [46,47].

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Mixed macromolecular crowding agents such as bovine serum albumin (BSA) and Ficoll 70 inhibit amyloid formation of HEWL exhibiting a chaperone activity where they stabilize the compact native state of protein [30]. Human lysozyme variant tends to form amyloid in crowded milieu due to lower stability of its native state [48–50]. However, HEWL can restrict amyloid formation in the crowded environment due to the enormous stability of its native state [30]. Recent report has shown participation of molten globule like state during the thermal unfolding of human lysozyme under amyloidogenic condition [10]. HEWL is known to acquire a molten globule like state at alkaline pH [51] and is also capable of forming amyloid fibrils at alkaline pH (pH > 12) [52,53]. Presence of salt (KCl) facilitates the hydrophobic exposure of HEWL [54]. HEWL fibrillation occurs also in the presence of tertiary butanol (20%) at alkaline pH (12.7) [42]. Earlier investigations have revealed differential effects of polyethylene glycols (PEG) in the aggregation pathway of proteins [55–60]. From this laboratory, we have reported an interesting phenomenon of absolute reduction of Cu(II) during fibrillation process of HEWL at pH 7 [61]. Recently we have also shown that alkali-salt mediated fibrillation of HEWL which occurs via formation of a molten globule state, is prevented in the presence of green tea polyphenols [62]. In the present article, we have targeted alkali-salt mediated fibrillation of HEWL to explore whether PEGs can restrain fibrillation process of HEWL. We have monitored the effect of high molecular weight PEGs (PEG 20000, PEG 35000) of varying concentration (50 g/l to 250 g/l for each) on the fibrillation propensity of HEWL under alkaline-salt mediated condition. Aggregation process of HEWL was investigated using different spectroscopic and microscopic techniques. Our findings add interesting information regarding the effect of crowding on the fibrillation process of HEWL.

2. Materials and methods

2.1. Materials

Hen egg white lysozyme (HEWL), Thioflavin T (ThT), 8-anilino-1-naphthalenesulfonic acid (ANS), and *Micrococcus lysodeikticus* were purchased from Sigma Chemical Co. (St. Louis, USA) and used as received. Polyethylene glycol 20000 (20PEG) (SRL), Polyethylene glycol 35000 (35PEG) (Fluka) and all other chemicals were obtained from SRL (India).

2.2. Preparation of fibrillar solutions

HEWL stock solution was prepared using a molar extinction coefficient of 37,646 M^{-1} cm^{-1} at 280 nm [63]. Protein solution (150 μM) was incubated at pH 12.75 (maintained using 200 mM KCl/NaOH) at ~37 °C for 6 h followed by incubation at ambient temperature in the absence and presence of PEGs with varying concentrations (50 g/l to 250 g/l). EUTECH pH meter (pH 510) was used to monitor the required pH measurements. In each case, 20 mM of pH 12.75 solutions was used for dilution to acquire the desired concentration in the final working solution.

2.3. Thioflavin T (ThT) fluorescence

ThT fluorescence intensity increases markedly upon binding with amyloid fibrils enabling it as a suitable probe to monitor aggregation pathway of proteins [64,65]. The ThT intensity of each solution was monitored after incubation at ~37 °C for 6 h. For each set, a mixture of 27 μl of protein solution and 10 μl of ThT (1 mM) was incubated for 2 min and scanned in a Horiba Jobin Yvon Fluoromax 4 spectrofluorimeter. Final protein and dye concentrations were maintained at 2 μM and 5 μM respectively. Excitation and emission maxima were kept at 450 nm and 485 nm respectively. Slit width and integration time were fixed at 5 nm and 0.3 s. Each spectrum was corrected with respect to the corresponding blank.

2.4. ANS binding assay

To detect the exposure of hydrophobic regions, ANS binding assay was performed. An aliquot of 27 μl from each set was withdrawn after incubation at ~37 °C for 6 h followed by addition of 4 μl of ANS (stock concentration 5 mM) to achieve final protein and dye concentration of 2 μM and 10 μM respectively. Samples were incubated in dark for 1 h at ambient temperature and scanned between 400 and 600 nm keeping excitation maxima at 370 nm in a Horiba Jobin Yvon Fluoromax 4 spectrofluorimeter. Slit width and integration times were fixed at 5 nm and 0.2 s respectively. Each spectrum was corrected with respect to the corresponding blank.

2.5. Turbidity assay

Formation of larger protein aggregates was monitored using turbidity assay. From each set, an aliquot of 15 μl was withdrawn and scanned in a Shimadzu 2450 UV–vis spectrophotometer at 350 nm keeping the final protein concentration of 5 μM using quartz cuvette of 1 cm path length. Light scattering caused by aggregated particles results an increase in the absorbance value at 350 nm [66].

2.6. Circular dichroism (CD) spectroscopy

Far–UV circular dichroism spectra were acquired in a Jasco–810 spectrophotometer using quartz cuvette of 0.1 cm path length. To acquire CD spectra aliquot from each set was withdrawn after an incubation of 6 h and scanned between 190 and 240 nm at 25 °C at a scan rate of 50 nm/min. In each case, final protein concentration was 20 μM. Protein secondary structure content was estimated using an online server, DICHROWEB [67].

2.7. Fourier transform infrared spectroscopy (FTIR)

HEWL samples were subjected to the FTIR measurements and spectra were accumulated at room temperature using a Thermo Nicolet 6700 FTIR spectrometer equipped with a zinc selenide (ZnSe) attenuated total reflectance (ATR) accessory, a deuterated triglycine sulfate (DTGS) detector and a KBr beam splitter. An aliquot of 200 μl was withdrawn from each HEWL solution for FTIR measurements. An average of 256 scans was recorded in the region of 4000–400 cm^{-1} with a 4 cm^{-1} resolution for each spectrum. Control spectra were acquired under the same experimental conditions and subtracted from the respective experimental spectra. The region of the amide I band obtained was subjected to second derivative followed by Gaussian curve fitting to determine the total area and area corresponding to each secondary structure component in the 1700–1600 cm^{-1} region. Secondary structure parameters were estimated following the method described earlier [68]. In each case, the fitted Gaussian band corresponds to a secondary structural component according to the frequency of its maximum: α-helix content (~1648–1660 cm^{-1}), β-sheet (1610–1640 cm^{-1}), random coil (1640–1648 cm^{-1}), turn (1665–1680 cm^{-1}) and β-antiparallel (1680–1692 cm^{-1}) [68]. These analyses were performed using Origin 7.5 software.

2.8. Enzymatic activity assay of HEWL solutions

The enzymatic activity of native and different HEWL fibrillar solutions has been measured following the standard procedure

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