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Research article

Polyols (Glycerol and Ethylene glycol) mediated amorphous aggregate inhibition and secondary structure restoration of metalloproteinase-conalbumin (ovotransferrin)

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ABSTRACT

Under physical or chemical stress, proteins tend to form aggregates either highly ordered (amyloid) or unordered (amorphous) causing many pathological disorders in human and loss of proteins functionality in both laboratory conditions and industries during production and storage at commercial level. We investigated the effect of increasing temperature on Conalbumin (CA) and induced aggregation at 65 °C. The enhanced Thioflavin T (ThT) and ANS (1-anilinonaphtalene 8-sulfonic acid) fluorescence intensity, show no shift on Congo red binding, additionally, transmission and scanning electron microscopy (TEM) (SEM) reveal amorphous morphology of the aggregate. Our investigation clearly demonstrated that polyols namely Glycerol (GL) and Ethylene glycol (EG) are so staunch to inhibit amorphous aggregates *via* restoring secondary conformation. Addition of polyols (15% GL and 35% EG) significantly decrease the turbidity, Rayleigh scattering ThT and ANS fluorescence intensity. The dynamic light scattering (DLS) data show that hydrodynamic radii (R_h) of the aggregates is ~20 times higher than native CA while nearly similar for GL and EG protected CA due to condensation of core size with little difference.

1. Introduction

The intricate mode and processes involved in the induction and inhibition of protein aggregation bear great interest in the field of biotechnology, therapeutics, food and pharmaceutical industries. In destabilizing conditions, proteins tend to form aggregates either amorphous or amyloids as a consequence of the achieved intermediate states by unfolding or misfolding. Such conformational aberrations are highly linked with many pathological disorders [1,2]. The hydrophobic and hydrophilic forces established between solvent and protein molecules possess a principle impact on the conformation stabilization of proteins.

The product of proteins unfolding or misfolding lead to the exposure of hydrophobic patches which offers to interact through and leads to the formation of firm complex *via* transformation of soluble monomeric entities to large insoluble ones [3]. The establishment of novel interactions in the protein molecule occurs due to enhanced exposure of thiol groups or hydrophobic patches to thermally induced aggregation [4]. The cataract disease for instance is a result of aggregation of a γD-crystalline protein which has been categorized as amorphous in nature [5], similarly Fanconi syndrome [6], light-chain deposition disease (LCDD), and myeloma nephropathy [7]. While, Alzheimer's, Parkinson's, spongiform encephalopathies, and type II diabetes mellitus [1,2,8] are amyloid aggregates. In the production of bacterial recombinant proteins, the formed inclusion bodies are also classified as amorphous aggregates. It is an outcome of the accumulation of excessively produced polypeptides followed by the unavailability of sufficient chaperons to govern the folding process to rescue from aggregation and restoration of nascent peptides [9,10]. In a similar vein in liquor industry, the amorphous aggregate formation takes place in the form of protein haze during the course of white wine production [11]. Thermally induced aggregation is one of the most studied factors where several proteins have been reported to have undergone the process of aggregate formation at elevated temperature but the effective temperature and duration of incubation fluctuates from protein to protein. For instance, a slight increase in the temperature (~37 °C) can cause aggregation of the Apo form of muscle glycogen phosphorylase b

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(apoPhb) of rabbit origin [12], while Aquaporin 0 (AQP0) aggregates at 60 °C [13], bovine hemoglobin aggregates at ~70 °C [14], recombinant human interferon alpha2b (rhIFNα2b) aggregates at 50 °C [15] while SARS-associated corona virus (SARS-CoV) protein aggregates at most extreme condition *i.e.* boiling temperature [16]. Thermal and cetyltrimethylammonium bromide [14], NaCl [17], acidic pH [18], metal [19], atheronal-A, atheronal-B, cholesterol [20], Rosin modified surfactant, (QMRAE) [21], non-fluorinated and fluorinated cosolvents [22] are few examples of physical as well as chemical inducer where protein unambiguously forms amorphous aggregate. While, cationic surfactant like CTAB induce amyloid fibrils in stem bromelain [23].

Oligomers of Aβ responsible for the synaptic dysfunction, upon their interaction with fragment of prion protein N-terminal (PrPN1), they modify the conformation which finally result in amorphous aggregate formation [24].

From a therapeutic perspective, it is quite obligatory to design and generate idea which can combat the aggregation inducing factors and protect the proteins *in vivo* as well as *in vitro*. In such trend, that dual function has been displayed by quercetin for the conformation conservation of insulin, which inhibits the fibril formation and also shows disorganizing ability by amyloid fibril (ordered structure) conversion into amorphous aggregates (unordered structure) [25]. In this series, clofazimine inhibits as well as destabilize the fibrils of hen egg white lysozyme [26]. Moreover, Aspartate-β-semi aldehyde dehydrogenase forms aggregates, *via* soluble to insoluble polymerization as function of concentration and time. Lumry–Eyring with nucleated polymerization (LENP) model revealed that increasing concentrations of glycerol inhibits the formation of insoluble, high molecular weight aggregates by delaying the polymerization [27]. The weak polarity-reducing property of ethylene glycol curtails the interaction between hydrocarbon spacer arm mediated nonspecific interaction of N^6-(6-Aminohexyl)-AMP and functional hydrophobic sites present on the pig heart lactate dehydrogenase. The low concentration of ethylene glycol achieves high retrieval of enzymatic activity along with increased yield of enzyme during chromatographic elution within the concentration range of 20–30% (v/v) [28]. Fat as additive used for prion, fetches the more trifling condition for its inactivation, while, glycerol provides stability to the prion 27–30 or prion rods during heat treatment by shielding the peptide backbone [29]. An earlier study on reversing the impact on mutant archetype of Familial Creutzfeldt–Jakob disease (CJD) H187R, has confirmed that glycerol effectively diminishes the PrP187R accumulation in lysosomes of transfected cells followed by transportation up to the cell surface [30].

Furthermore, the osmolytes are known to affect the kinetics and lengthen the lag phase of amyloid but this effect does not solely depend on the viscosity of cosolutes. Despite of viscosity difference between sorbitol and glycerol, both can enhance the lag phase by more than two folds but triethylene glycol (viscosity similar to glycerol) and polyethylene glycol (PEG-400) (with highest viscosity), shorten the lag phase [31].

Conalbumin (ovotransferrin, metalloproteinase) is a glycoprotein made up single polypeptide chain; it is comprised of 686 amino acid residues, holding high affinity sites for iron binding. The two iron binding sites are distributed in comparable tertiary structure of each lobes "N" (1–332) and "C" (342–686) [32]. Ovotransferrin exhibits killer activities against a wide range of microorganisms [33,34]. Further, immunomodulation [35], anti-oxidative [36] and anti-carcinogenicity [37] are the other significant biological activities associated with ovotransferrin. The primary goal of the current study is to induce aggregation in CA by thermal treatment and characterization the type of aggregates. Further, we checked the protective and anti-aggregation property of glycerol (GL) and ethylene glycol (EG) at 65 °C. To the best of our knowledge, no previous studies reported the GL and EG mediated inhibition of thermally induced amorphous aggregates by a multi-technique approach.

2. Materials and methods

2.1. Materials

Iron-free conalbumin from chicken egg white (C 0755), Thioflavin T (T 3516), ANS (A 10288) and Congo red (CR) were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Extrapure glycerol (072929) and ethylene glycol (05291) was purchased from SISCO research laboratories Pvt. Limited, Mumbai, India. All other reagents and buffer compounds used were of analytical grade.

2.2. Concentration determination of protein

Protein stock was prepared in 20 mM sodium phosphate buffer pH 7.0, and concentration was measured by using the extinction coefficient at $E^{1\%}_{280nm} = 12.0$ on Perkin Elmer (Lambda 25) double beam spectrophotometer attached with peltier temperature programmer (PTP-1). A filtered buffer through a 0.45 μm Millipore Millex-HV PVDF filter was used throughout the study.

2.3. Turbidity measurements

Turbidity measurements were performed on a Perkin Elmer UV–vis spectrometer model lambda 25 in a cuvette of 1 cm path length. All the measurements of protein sample with increasing concentration (v/v) of polyols were monitored by measuring absorbance at 350 nm at room temperature. Conalbumin (5 μM) was incubated for 60 min in both the conditions, for thermal range 25–85 °C and on increasing concentrations of GL and EG at 65 °C.

2.4. Rayleigh light scattering (RLS) measurements

Rayleigh scattering measurements were carried on a Shimadzu spectrophotometer RF-5301 PC at 25 ± 0.1 °C with a 1 cm path length cell. The protein samples incubated with increasing concentrations of polyols (GL and EG) were excited at 350 nm and spectra were recorded over the wavelength range of 300–400 nm. The data were plotted at 350/350 nm. Conalbumin (5 μM) was incubated for 60 min in both the conditions, for thermal range 25–85 °C and on increasing concentration of GL and EG at 65 °C.

2.5. Tryptophan fluorescence measurements

Fluorescence measurements were performed on a Shimadzu spectrophotometer RF-5301 PC, equipped with a constant temperature holder attached to a Neslab RTE-110 water bath with an accuracy of ±0.1 °C. The fluorescence spectra were measured at 25 ± 0.1 °C with a 1 cm path length cell at excitation/emission slit width 5–5 nm. To collect the fluorescence spectra, 5 μM of protein was excited at 295 nm and emission spectra were recorded in the range of 300–400 nm.

2.6. Circular dichroic measurements

CD measurements were carried out on a Jasco spectropolarimeter (J-815) equipped with a Peltier-type temperature controller (PTC-424S/15). The instrument calibration was performed with d-10-camphorsulphonic acid. Spectra were collected in a cell of 1 mm path length with scan speed of 100 nm/min and response time of 1 s for all of the measurements. Each spectrum was the average of 2 scans. The protein concentration was kept 5 μM for far- UV CD.