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Ascorbic acid inhibits human insulin aggregation and protects against amyloid induced cytotoxicity

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ABSTRACT

Protein aggregation into oligomers and fibrils are associated with many human pathophysiologies. Compounds that modulate protein aggregation and interact with preformed fibrils and convert them to less toxic species, expect to serve as promising drug candidates and aid to the drug development efforts against aggregation diseases. In present study, the kinetics of amyloid fibril formation by human insulin (HI) and the anti-amyloidogenic activity of ascorbic acid (AA) were investigated by employing various spectroscopic, imaging and computational approaches. We demonstrate that ascorbic acid significantly inhibits the fibrillation of HI in a dose-dependent manner. Interestingly ascorbic acid destabilise the preformed amyloid fibrils and protects human neuroblastoma cell line (SH- SY5Y) against amyloid induced cytotoxicity. The present data signifies the role of ascorbic acid that can serve as potential molecule in preventing human insulin aggregation and associated pathophysiologies.

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1. Introduction

Protein aggregation has received extensive attention in biology, medicine and biophysics because of its implication in several human diseases [1]. It is currently known that more than 20 human diseases such as Alzheimer's, Parkinson's, Huntington's and type II diabetes are associated with protein aggregation [2]. Even though the aggregating proteins associated with amyloid diseases are varied in terms of their sizes, primary, secondary and tertiary structures, but their corresponding amyloid aggregates display common features [3]. Characteristic amyloid fibrils shows dye binding property, fibrillar morphology, cross-β structure, and cytotoxicity, suggesting a common mechanism of amyloid formation [4–6].

Insulin is a 51 amino acid residue peptide hormone that exists as an equilibrium mixture of mono-, di-, tetra- and hexa-mer in solution [7]. It is composed of two peptide chains (A and B), linked by two disulfide bonds [8]. Insulin is a key hormone regulating glucose metabolism in the human body and is widely used as a drug for diabetes treatment [9]. In pancreas insulin is stored as a hexamer in presence of zinc ion and once it diffuses into the blood the hexameric form dissociates into physiologically active monomers [10]. Insulin monomers are less stable than the hexameric form and tend to form amyloid aggregates. Insulin is able to form amyloid at the site of injection in case of insulin dependent diabetic patient resulting in a pathological condition known as injection amyloidosis. Insulin under suitable *in-vitro* conditions such as low pH and high temperature forms typical amyloid fibrils and long being used as a model protein to study amyloid formation [11,12].

Ascorbate is a vital antioxidant molecule in the brain, it helps in a number of other important functions including participating as a co-factor in several enzymatic reactions [13]. Ascorbate is transported into the brain and neurons via the Sodium-dependent Vitamin C Transporter-2 (SVCT2), which causes accumulation of ascorbate within cells against a concentration gradient. The oxidised form of ascorbate is dehydroascorbic acid which is transported via glucose transporters of the GLUT family [14]. Once dehydroascorbic acid in cells, it is rapidly reduced to ascorbate.

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Ascorbate is anticipated to be a neuromodulator of glutamatergic, dopaminergic, cholinergic and GABAergic transmission and related behaviours [15]. Neurodegenerative diseases typically involve high levels of oxidative stress and thus ascorbate has been posited to have potential therapeutic roles against Alzheimer's, Parkinson's and Huntington's disease [16]. Yallampalli et al. investigated the role of ascorbic acid in beta amyloid induced calcium increase and its protective effect in PC 12 cells [17]. Huang et al. reported that ascorbic acid prevents apoptosis and cell death in SH- SY5Y neuroblastoma cells induced by β-amyloid [18]. Some studies reported that lower blood ascorbic acid correlated with cognitive impairment. It is therefore critical to determine how ascorbic acid modulates amyloid formation.

Several studies have focussed on the anti-aggregation mechanism of polyphenols, nanoparticles, synthetic compounds and vitamins due to their direct involvement in reducing oxidative stress [19–24].

In the present work, we have explored the anti-amyloidogenic behavior of ascorbic acid against insulin aggregation using various biophysical, imaging, cell cytotoxicity and computational biology methods. The anti-aggregation potency of ascorbic acid against insulin aggregation may provide significant insight into the effect of vitamins on the protein aggregation and has potential implications in the field of amyloid biology of diseases as well in pharmaceutical industry.

2. Results and discussion

2.1. Effect of ascorbic acid on fibrillation propensity of insulin

The effect of ascorbic acid on insulin amyloid formation was examined via ThT fluorescence measurements. ThT interacts with amyloids in a specific manner and increase in ThT fluorescence has been widely used as an indicator of degree of amyloid formation [25–27]. ThT fluorescence of insulin in absence and presence of ascorbic acid at different time interval is represented in Fig. 1a. The inhibitory effect of ascorbic acid on fibrillation process followed concentration dependency (supplementary Fig. 1). In order to avoid any false positive results we also carried out ThT binding of ascorbic acid at different time intervals and the results demonstrate almost no enhancement in ThT fluorescence intensity (supplementary Fig. 2). When insulin was incubated alone, after lag phase, ThT fluorescence was observed in typical sigmoidal fashion that indicates the nucleation-dependent pathway [28]. These results are in agreement with previous study conducted by Wang et al. that demonstrated the nucleation dependent polymerisation of insulin [29]. The lag phase time of insulin was around 5 h after which ThT fluorescence intensity increases rapidly and finally reached plateau at 24 h. In contrast formation of insulin aggregates was suppressed in presence of ascorbic acid (400 μM), the lag time was increased to around 10 h and plateau time remains almost unchanged as compared to control. ThT fluorescence spectra of insulin and insulin in combination with ascorbic acid after 72 h of incubation at 60 °C are shown in Fig. 1b. It can be seen from the figure that ThT fluorescence intensity of insulin was reduced to 71% and 36% in presence of 200 and 400 μM of ascorbic acid respectively. These results suggests that as compared to control insulin, samples co incubated with ascorbic acid showed reduction in ThT fluorescence intensity, indicating the decreased formation of cross β-sheet structures. Delay in lag time and reduction in ThT fluorescence intensity in the presence of ascorbic acid suggests the anti-aggregation potential of ascorbic acid. Similar observations were reported by Choudhary et al. that showed osmolytes mediated inhibition of insulin fibrillation [30]. In order to extend the physiological relevance of this work inhibitory effect of ascorbic acid on amyloid formation was also studied at pH 7.4 and 37 °C (supplementary Fig. 3). These results suggest that ascorbic acid retard the amyloid formation even at physiological conditions, however the rate was slower in later.

2.2. Effect of ascorbic acid on the microenvironment of fibril formation

In nucleation stage the hydrophobic collapse results into the formation of protein aggregates and ThT binding confirms that ascorbic acid delayed the nucleation process. This might be either due to protein native state stabilisation or reduction in the surface

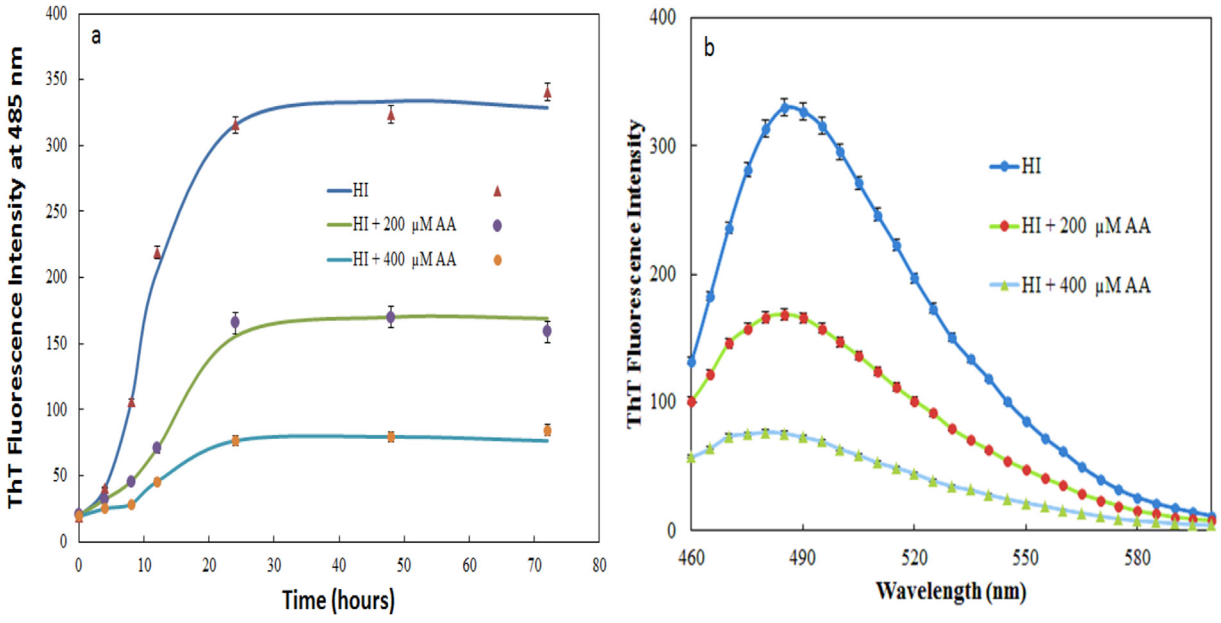

Fig. 1. (a) ThT fluorescence kinetics of insulin in absence and presence of ascorbic acid (200 and 400 μM). Results represent means ± s.d (n = 3) (b) ThT fluorescence spectra of insulin incubated at 60 °C over 72 h in absence and presence of ascorbic acid (200 and 400 μM). Experimental data represent the average ± s.d (n = 3).

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