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Gallic acid, one of the components in many plant tissues, is a potential inhibitor for insulin amyloid fibril formation

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ABSTRACT

Proteins under stressful conditions can lead to the formation of an ordered self-assembled structure, referred to as amyloid fibrils, to which many neurodegenerative diseases such as Type II diabetes, Alzheimer's, Parkinson's, Huntington's, etc., are attributed. Inhibition of amyloid fibril formation using natural products is one of the main therapeutic strategies to prevent the progression of these diseases. Polyphenols are the mostly consumed as antioxidants in a human nutrition. Herein, we have studied the effect of a simple polyphenol, gallic acid (GA), one of the main components in plant tissues, especially in tea leaves, on the insulin amyloid fibril formation. Different biophysical characterizations such as turbidity, atomic force microscopy (AFM), Thioflavin T (ThT) assays, circular dichroism, and Fourier transform-infrared spectroscopy have been used to analyze the inhibition of amyloid fibril formation. The occurrence of fibrils in an AFM image and ThT fluorescence enhancement confirms the formation of insulin amyloid fibrils when incubated under acidic pH 2 at 65 °C. In the presence of GA, absence of fibrils in AFM image and no change in the intensity of ThT fluorescence confirms the inhibition of insulin amyloid fibrils by GA. Spectroscopic results reveal that GA inhibits the conformational transition of α-helix → β-sheet, which is generally induced during the insulin fibril formation. It was found that the inhibitory effect of GA is concentration dependent and non-linear. Based on the observed results, we propose that GA interacts with native insulin, preventing nuclei formation, which is essential for fibril growth, thereby inhibiting the amyloid fibril formation. The present results thus demonstrate that GA can effectively inhibit insulin amyloid fibril formation *in vitro*.

1. Introduction

Protein aggregation can certainly be considered as one of the most interesting and challenging topics in current biological research. It has been established that well-defined ordered protein aggregates, referred to as amyloid fibrils, are the main cause for many neurodegenerative pathologies, including Type II diabetes, Alzheimer's, prion diseases, etc [1–6]. These amyloid fibrils are elongated assemblies mainly characterized by cross β-sheet structures aligned perpendicularly to the fibril axis [7,8]. In Alzheimer's and type II diabetes patients, deposits with ordered protein aggregates have been found in injured tissues [9]. In Alzheimer's and prion diseases, these deposits have been found in the brain [10]. In type II diabetes mellitus, the deposits are formed in the pancreas and contribute to β-cell dysfunction [11,12]. In addition to proteins associated with neurodegenerative diseases, other proteins such as insulin [13,14], lysozyme [15], β2 microglobulin [16], etc., can also form amyloid like fibrils under the well-defined conditions of pH, ionic strength, and elevated temperature. It has been postulated that the formation of fibril structures follows some common principles. Previous reports indicate that fibril formation is independent of primary structure. Contrarily, most of the amyloid and amyloid-like fibrils share a common cross β-sheet structure. Hence, an effective amyloid fibril inhibitor can target only the cross β-sheet secondary structure, not on the primary structure.

Insulin, a peptide hormone, is known to form amyloid like fibrils under certain conditions *in vitro* [17,18]. Insulin amyloid fibrils pose a variety of problems in biomedical and biotechnological applications. Amyloid deposits of insulin have been observed in patients with diabetes after repeated injections of insulin in normal aging [19] and after subcutaneous insulin infusion [20]. Insulin consists of two polypeptide chains, A (21

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residues) and B (30 residues), linked together by two inter-chain and one intra-chain disulfide bond. It exists as a mixture of hexameric, dimeric, and monomeric states in solution. The relative populations of different oligomeric species are correlated to the environmental conditions. For example, insulin is predominantly monomeric in 20% acetic acid [21,22], dimeric in 20 mM HCl [23], and hexameric at pH 7.5 in the presence of zinc [14]. Fibrillation of insulin was first identified in the 1940s [24,25,23]. Its formation depends on agitation, heating of the solution, and acid pH [24,25,23]. Insulin hexamers consisting of three identical dimers aggregated around two zinc ions [26]. The crystal structure of insulin consist predominantly of α-helices with a significant amount of antiparallel β-sheet, which is formed between the C-terminus of B-chain of the monomers in the dimer state [26,27]. Insulin forms amyloid like fibrils under a variety of conditions, with various overall morphologies depending on the arrangement of constituent protofilaments. *In vitro*, insulin is readily converted to inactive β-sheet rich amyloid fibrils upon incubation at high insulin concentrations, acidic pH, and elevated temperatures, and in the presence of organic solvents [28] along with agitation [29–31]. It has been proposed that insulin fibrillation occurs through the dissociation of oligomers into monomers, with the monomer undergoing structural changes to partially folded intermediates that have a strong propensity to fibrillate [27]. It has been suggested that the B-chain C terminus do not have a crucial role in fibril formation.

Polyphenols are widely consumed antioxidants in the human diet and are found in many foods, including vegetables, fruits and plants derived drinks such as tea, juices, coffee and wine. During the last two decades, research on naturally-occurring polyphenols towards the prevention of diseases has increased dramatically [32,33]. Notably, polyphenols prevent chronic diseases such as cardiovascular diseases [34] and cancers [35]. Furthermore, it is now well established that polyphenolic compounds can inhibit fibril formation associated with many amyloidogenic proteins and peptides [32,36,33]. Among the naturally-occurring polyphenols, Gallic acid (GA), is the simplest polyphenol and is found in many green plant tissues, especially in tea leaves, both in a free form and as a component of the polymers such as tannin, ellagitannin, Theaflavin-3-gallate, epigallocatechin-3-gallate (EGCG), etc. Although several complex polyphenols have been investigated for their ability to inhibit amyloid aggregation [32], only few studies have been done using GA. For example, it has been reported that GA can inhibit the aggregation of the reduced and carboxymethylated form of milk protein, namely kappa-casein [37]. In addition, GA inhibits amyloid β (Aβ) peptide fibril formation both *in vitro* and in vivo [38], reduces Aβ levels in the mouse brain [36], and also significantly improves the cognitive deterioration associated with AD. A recent study has shown that GA interacts with α-synuclein, associated with Parkinson's disease, to prevent the structural change required for its fibril formation [39].

The aim of the present study is to investigate the effect of the simplest polyphenol, GA, on the fibrillation of insulin *in vitro*. Different biophysical characterizations such as turbidity using UV–Visible spectroscopy, Thioflavin-T (ThT) binding using fluorescence spectroscopy, secondary structural changes using circular dichroism (CD), and Fourier-transformed infrared (FTIR) spectroscopy, and fibril morphology using atomic force microscopy (AFM) have been employed to unravel inhibitory activity. The results demonstrate that GA can inhibit insulin fibril formation *in vitro* under the experimental conditions studied here. We foresee that our research could pave way for future work on the computational study to understand the mode of interaction between GA and insulin.

2. Materials and methods

2.1. Materials

Insulin (I-5500) and ThT (T3516) were purchased from Sigma Aldrich and used as received. Gallic acid was purchased from SRL, Pvt. Ltd (Mumbai, India). Acetic acid and other chemicals were of analytical grade.

2.2. Insulin fibril formation

The stock solutions of insulin (10 mg/ml) and GA (5 mg/ml) were prepared in 20% acetic acid solution (pH 2) containing 100 mM NaCl by weighing. Appropriate volume of these stock solutions and 20% acetic acid (pH 2) containing 100 mM NaCl solutions were mixed to get 1:0, 1:5, 1:10, 1:20 and 1:50 M ratios of insulin:GA complexes. The final concentration of insulin was 2 mg/ml (0.34 mM) and the final concentrations of GA were 0–17 mM. Insulin fibril formation was monitored by incubating the insulin:GA complexes at 65 °C, without agitation and stirring, for over a period of 12 h.

2.3. Turbidity measurements

The fibrillation kinetics of amyloid proteins can be determined by using the absorbance assay at 600 nm (A_{600}). A_{600} can be used as a direct measurement of the mass of the aggregated insulin. Each experimental solution was freshly prepared in glass vials. All samples were pre-incubated in 20% (v/v) acetic acid containing 100 mM NaCl (pH 2) and heated at 65 °C up to 12 h. At different time intervals, the vials are first shaken gently to evenly distribute the sample followed by transferring the sample to UV quartz cell for optical density (OD) measurement. OD at 600 nm was measured using Perkin Elmer Lambda EZ 201 UV/Vis spectrometer. The percentage of insulin fibril inhibition was calculated by considering an average value of OD obtained during the saturation time (10.5–12 h, see Fig. 1B) of insulin fibril formation, in the absence of GA, as 100%.

2.4. Steady-state fluorescence measurements

A stock solution of ThT was prepared in 10 mm phosphate buffer (pH 7) containing 150 mM NaCl. This stock solution (1 ml) was diluted with 50 ml of above phosphate buffer. This solution was used as a working solution. The solution of insulin:GA complexes, after incubation at 65 °C for 12 h, were shaken well before measurements because the intensity of ThT fluorescence depends on the amount of fibrils added. At the time of measurements, a constant volume (20 μl) of insulin:GA complexes was added to 2 ml of working ThT solution. The fluorescence spectra were measured from 460 to 660 nm at a scan rate of 100 nm/min. A quartz cell with a path length of 1 cm was used. The excitation wavelength was 440 nm. All fluorescence spectra were recorded using Perkin Elmer LS 45 luminescence spectrometer at 25 °C.

2.5. Far-UV CD measurements

All CD measurements were carried out on a Jasco J-715 spectropolarimeter at 25 °C. Owing to high absorbance of insulin (2 mg/ml) in 1 mm path length cell, the insulin:GA mixtures, after incubation at 65 °C for 12 h, were diluted ten times with deionized water. The CD spectra of insulin:GA mixtures with >1:10 M ratio was not measured due to high absorbance of GA. Each CD spectrum was an average of three individual scans. The baseline of the CD spectrum was corrected by subtracting the CD spectrum of solvent.