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Probing the role of ortho-dihydroxy groups on lysozyme fibrillation

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ABSTRACT

Changes in the microenvironment of Trp in lysozyme are one of the key factors in the fibrillation process. Gallic acid through the oxidation of o-dhydroxy moiety into quinone was shown to inhibit lysozyme fibrillation by stabilizing the Trp microregions [Konar et al., Int. J. Biol. Macromol. 103 (2017) 1224–1231]. In this article we compare the inhibitory effects of several gallic acid-based phenolic compounds. The results show that pyrogallol, and 3,4-dihydroxy benzoic acid, each containing the o-dihydroxy moiety exhibited a significant inhibitory effect on lysozyme fibrillation which is further supported by docking studies. Interestingly, the inhibitory effect of pyrogallol is almost similar to that observed for gallic acid. The lower inhibitory effect of 3,5-dihydroxy benzoic acid and 4-hydroxy benzoic acid corroborates this finding as neither of the compounds can be transformed into quinone intermediates. The ineffectiveness of benzoic acid towards fibrillation questions the role of the —COOH group in the inhibition. The IC_{20} values determined show the similar trends. Results of the Thioflavin T binding assay and parameters from the docking studies reveal a strong correlation based on which a relation has been obtained that could be used to identify potential polyphenol based inhibitors by considering docking studies alone.

1. Introduction

Natural phenolic compounds have remained a topic of special attention because of their anti-oxidant properties that reduce the risks associated with several diseases. Aromatic systems containing one or more hydroxyl groups either in the free form or in the form of different functional derivatives of esters, methyl ethers, glycosides, etc. are generally referred to as 'phenolics'. These phenolic compounds are widely distributed in fruit, vegetables, seeds and leaves. These comprise the secondary metabolites of plants and exhibit a number of beneficial health effects like anti-inflammatory, anti-cancer, anti-microbial, anti-allergic, anti-artherogenic apart from their anti-oxidant activity [1–3].

The anti-oxidant activity of the phenolic compounds is attributed to their ability to donate hydrogen radicals that can react with the reactive oxygen species (ROS)/reactive nitrogen species (RNS) generated during different metabolic reactions that help to protect our body from oxidative damage. The anti-oxidative property is purported to depend upon the number as well as the position of the hydroxyl groups present in the molecule. The introduction of a second hydroxyl group at the *ortho* or *para* position of mono-substituted phenols increases the antioxidant efficiency by a significant extent [4]. Hydroxylation at three consecutive positions particularly in the 3-, 4-, and 5- positions of a phenolic acid compound confers the highest antioxidant activity [4]. Antioxidants can significantly delay or prevent biological macromolecules such as proteins, nucleic acids, lipids, and sugars from ROS mediated oxidative damage at very low concentrations [5]. ROS mediated oxidative stress arises in the body when the balance between the antioxidants and ROS are interrupted as a result of excessive generation of ROS with respect to the detoxification by the antioxidant defence mechanism [6]. Oxidative damage is believed to be an important cause of protein fibrillation that causes several neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and prion disease [7–9].

The primary interest is, therefore, to find out suitable therapeutics that can counteract amyloid growth and toxicity. Inhibition of the formation of amyloid fibrils or disruption of the formed fibrillar assemblies are the most widely used strategies for understanding the therapeutic processes. Significant efforts have been employed to screen anti-amyloidogenic candidates by developing different types of enzymatic inhibitors, antagonists, antibodies, peptide fragments and synthetic ligands [10]. The consumption of highly anti-oxidative phytochemicals and bioactive chemicals derived from plants offer beneficial effects in protecting against

Abbreviations: HEWL, hen egg white lysozyme; ThT, Thioflavin T; ANS, 8-anilino-1-naphthalene sulfonic acid; GA, gallic acid; PG, pyrogallol; 3,4-DHBA, 3,4-dihydroxy benzoic acid; 3,5-DHBA, 3,5-dihydroxy benzoic acid; 4-HBA, 4-hydroxy benzoic acid; BA, benzoic acid; TEM, transmission electron microscopy; FESEM, field emission scanning electron microscopy; CD, circular dichroism.

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neurodegenerative diseases involving oxidative stress [11]. Epidemiological studies indicate that drinking three to four glasses of wine per day is capable of an approximate 80% decrease in risk of dementia and Alzheimer's disease [12]. Phenolic compounds from fruits and vegetables are thus invaluable potential agents for neuroprotection that operate by modulating several cellular processes such as signaling, proliferation, apoptosis, redox balance [13]. Resveratrol, curcumin, rosmarinic acid and catechin have demonstrated protective effects against the cytotoxicity of the β-amyloid 1–41 fragment [14,15]. Past reports from this laboratory have also shown that green tea polyphenols inhibit the fibrillation of various proteins in vitro, including human serum albumin [16] and hen egg white lysozyme (HEWL) [17]. Despite extensive investigations on the inhibitory effects of these plant based phenolic compounds on amyloidosis, their exact molecular mechanisms are still largely unknown.

Gallic acid (GA) is a tri-hydroxy phenolic acid compound which possesses a number of health benefits such as, antioxidant [18], anti-inflammatory [19], anti-viral [20], and anti-cancer [21] properties. We have previously shown that under fibrillation conditions, the oxidation of the vicinal dihydroxy moieties of GA into quinone and H_2O_2 resulted in a significant reduction of the extent of hydrophobic association (an important factor in the fibrillation process) leading to an inhibition of HEWL fibrillation. The inhibition occurs mainly through two pathways involving either (i) the prevention of the exposure of hydrophobic residues of the protein towards the solvent through covalent binding of the quinone moiety to the Trp residues of HEWL and/or (ii) the stabilization of the partially folded intermediates due to oxidative modification of the Met residues of HEWL that results in an increased polarity on the protein [22].

To elucidate the specific role of the vicinal dihydroxy groups of natural polyphenolic compounds toward protein fibrillation, we have chosen a set of five different compounds, benzoic acid (BA), 4-hydroxy benzoic acid (4-HBA), 3,4-dihydroxy benzoic acid (3,4-DHBA), 3,5-dihydroxy benzoic acid (3,5-DHBA), and pyrogallol (PG) which are structurally related to GA. The ability of these compounds to inhibit the fibrillation of HEWL with that of GA under the same experimental conditions has been investigated. The chemical structures of all the compounds studied are shown in Fig. 1. From a systematic investigation using several biophysical techniques such as UV–vis spectroscopy, fluorescence spectroscopy, circular dichroism spectroscopy, and microscopic techniques such as, field emission scanning electron microscopy (FESEM), transmission electron microscopy (TEM), fluorescence microscopy, and molecular docking studies, we are able to demonstrate that among all compounds, the o-dihydroxy moiety containing phenolic compounds are the most efficient amyloid inhibitors.

2. Materials and methods

Hen egg white lysozyme (HEWL), Thioflavin T (ThT), Congo red, 8-anilino-1-naphthalene sulfonic acid (ANS) were purchased from Sigma Chemical Co. (St. Louis, USA) and used as received. Gallic Acid, pyrogallol, 3,4-dihydroxy benzoic acid, 3,5-dihydroxy benzoic acid, 4-hydroxy benzoic acid, benzoic acid and other chemicals were received from SRL, India.

2.1. Preparation of fibrillar solutions

Samples were prepared according to the procedure reported earlier [22]. Briefly, stock solutions of all phenolic compounds and benzoic acid were prepared in 30% (v/v) ethanol. The fibrillar solutions were prepared by dissolving 2.15 mg HEWL in 1 mL phosphate buffer (50 mM, pH 7.4) containing 50 μM NaCl and 30% of ethanol with and without 200 μM of phenolic compounds and benzoic acid. The resulting mixtures were first vortexed and then incubated at 65 °C for 6 h followed by room temperature incubation for one day to get mature fibrils. Before conducting spectroscopic and microscopic experiments, samples were diluted with 20 mM phosphate buffer (pH 7.4).

2.2. Congo red binding assay

Details of sample preparation methods and techniques used in the Congo red binding assay have been discussed in the Supplementary Material.

2.3. Thioflavin T (ThT) fluorescence and ANS binding studies

The fluorescence measurements were carried out using a Fluorolog 3 spectrofluorimeter. To perform Thioflavin-T (ThT) fluorescence assay, fibrillar solutions of 5 μM protein concentration were mixed with 10 μM ThT and the spectra were recorded from 460 to 600 nm on excitation at 440 nm. During the experiment the slit width and the integration time were kept at 5 nm and 0.3 s respectively. In the ANS binding assay, the change in fluorescence intensity of ANS was measured in presence of 2 μM fibrillar solutions incubated with 10 μM ANS in dark for 1 h. Excitation wavelength for these samples was set at 370 nm and the samples were scanned in the wavelength range of 400–600 nm keeping a 5 nm slit width and an integration time of 0.4 s. Methods and techniques of Trp fluorescence studies have been discussed in the Supplementary Material in detail.

2.4. Circular dichroism (CD)

Far-UV CD spectra were obtained using a JASCO-815-Spectropolarimeter with a scanning rate 50 nm/min and scanning range from 190 to 200 nm at 25 °C. The sample concentrations were kept at 10 μM. Quartz cuvette of 0.1 cm path length was used for this study. Protein secondary structure content was determined using an online server DICHROWEB [23].

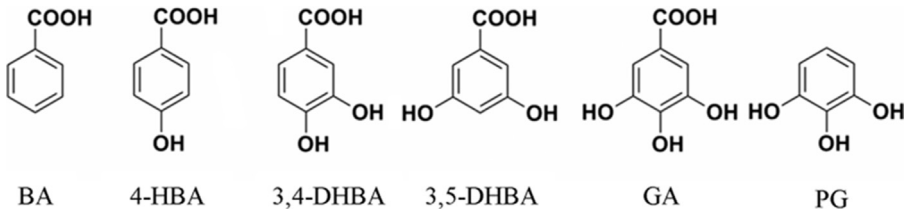

Fig 1. Chemical structures of phenolic acid compounds and benzoic acid: benzoic acid (BA), 4-hydroxy benzoic acid (4-HBA), 3,4-dihydroxy benzoic acid (3,4-DHBA), 3,5-dihydroxy benzoic acid (3,5-DHBA), Gallic acid (GA), and pyrogallol (PG).