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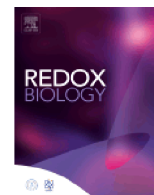

Research paper

Oxidized epigallocatechin gallate inhibited lysozyme fibrillation more strongly than the native form

Ting-Ting An[a,1], Shuang Feng[b,1], Cheng-Ming Zeng[a,*]

[a] Key Laboratory of Analytical Chemistry for Life Science of Shaanxi Province, School of Chemistry and Chemical Engineering, Shaanxi Normal University, Xi'an 710119, China

[b] School of Chemical Engineering, Guizhou Institute of Technology, Guiyang 550003, China

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ABSTRACT

Epigallocatechin gallate (EGCG), the most abundant flavanoid in green tea, is currently being evaluated in the clinic due to its benefits in the treatment of amyloid disorders. Its anti-amyloidogenic effect has been attributed to direct interaction of the intact molecule with misfolded polypeptides. In addition, antioxidant activity is also involved in the anti-amyloidogenic role. The detailed molecular mechanism is still unclear and requires further investigation. In the present study, the kinetics of EGCG oxidation and the anti-amyloidogenic effect of the resultant oxidation substances have been examined. The results indicate that EGCG degrades in a medium at pH 8.0 with a half-life less than 2 h. By utilizing lysozyme as an *in vitro* model, the oxidized EGCG demonstrates a more potent anti-amyloidogenic capacity than the intact molecule, as shown by ThT and ANS fluorescence, TEM determination, and hemolytic assay. The oxidized EGCG also has a stronger disruptive effect on preformed fibrils than the native form. Ascorbic acid eliminates the disruptive role of native EGCG on the fibrils, suggesting that oxidation is a prerequisite in fibril disruption. The results of this work demonstrate that oxidized EGCG plays a more important role than the intact molecule in anti-amyloidogenic activity. These insights into the action of EGCG may provide a novel route to understand the anti-amyloidogenic activity of natural polyphenols.

1. Introduction

Many diseases that occur mostly with age are caused by protein misfolding. These include Alzheimer's disease, Parkinson's disease, hemodialysis-related amyloid deposition, and a number of systemic amyloidoses [1–3]. The misfolding proteins, despite their unrelated amino acid sequences and tertiary structures, can assemble into amyloid fibrils with similar ultrastructures and identical biochemical properties, including long and unbranched fibrils with enriched β-sheet structure, increased surface hydrophobicity, fluorescence upon binding to thioflavin T (ThT), and the ability to disrupt cellular membranes.

Human lysozyme with point mutations has been reported to be associated with non-neuropathic systemic amyloidosis [3]. The amyloid fibrils formed by the wild-type of lysozyme *in vitro* shared similar ultrastructures and biochemical properties with those extracted from pathological deposits in tissue. Hen egg white lysozyme (referred to as lysozyme in this article) has been used as an alternative *in vitro* model for studying amyloidogenesis of a protein. Recent investigations showed that the synthetic lysozyme fibrils exhibited non-enzymatic cytotoxicity, including inducing aggregation and hemolysis of human erythrocytes, and reducing the viability of neuroblastoma cells through apoptotic and necrotic pathways [4,5].

Inhibition of amyloid formation and disruption of formed fibrillar assemblies are the therapeutic strategies proposed for the treatment of amyloid-related diseases. Recent investigations demonstrate that natural polyphenols are able to inhibit amyloid formation and disrupt preformed amyloid fibrils. Hydrogen bonding, hydrophobic interactions, and aromatic stacking are suggested to be the driving forces of the anti-amyloidogenic role of polyphenols [6–9]. In addition, antioxidant activity is also involved in the anti-amyloidogenic role [10–13]. It has been reported that the oxidized form of a polyphenol has a more potent disruptive effect on amyloid fibrils than the reduced form [8,14]. In previous works [15,16], we found that the inhibition of lysozyme amyloid fibrillation by polyphenols was associated with the formation of quinoproteins, and that quinone intermediates were actually the active form for phenolic compounds to interrupt amyloid structure.

A variety of epidemiologic investigations have demonstrated a beneficial effect of green tea or green tea extracts on neurodegenerative disorders. Epigallocatechin-3-gallate (EGCG, Scheme 1), which belongs to the flavanoid family, is the most abundant catechin in green tea and

* Corresponding author.
E-mail address: chengmingzeng@snnu.edu.cn (C.-M. Zeng).
[1] These authors contributed equally to this work.

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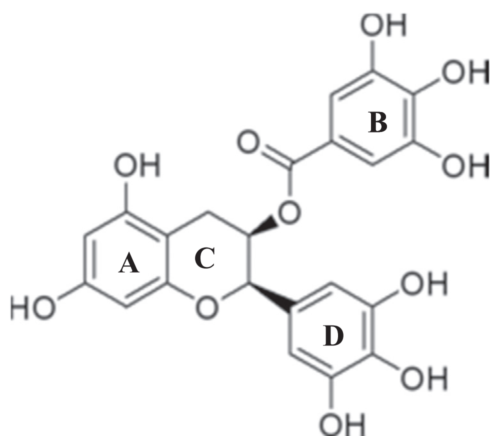

Scheme 1. Molecular structure of EGCG.

is a potent antioxidant that has been widely investigated. Of the anti-amyloidogenic natural polyphenols, only EGCG is currently being evaluated in the clinic due to its benefits in the treatment of amyloid disorders [17,18]. Recent *in vitro* investigations have indicated that EGCG exhibits an inhibitory effect on amyloid formation by β-amyloid peptide, α-synuclein, lysozyme, and other proteins [16,19–22]. It has also been reported that the fibril-disrupting efficiency of EGCG is positively correlated with its antioxidant capacity [12,23]. Despite extensive investigations on the anti-amyloidogenic effects of EGCG, the detailed molecular mechanism is still unclear and requires further investigation.

EGCG is composed of two vicinal trihydroxy structures in the B-ring and D-ring. These highly active trihydroxy moieties render EGCG susceptible to oxidation in air under neutral or alkaline pH. For instance, the half-life of EGCG was less than 30 min in McCoy's 5A culture medium [24]. The structure and anti-amyloidogenic activity of oxidized EGCG have so far remained largely unclear. In the present study, the anti-amyloidogenic effect of oxidized EGCG was evaluated *in vitro* using lysozyme as a model protein. The results suggest that oxidation of EGCG occurs at pH 8.0 and the oxidation products play a more potent inhibitory role on amyloid formation than its native form. In addition, oxidation of EGCG is found to be a prerequisite in the fibril-disruptive action.

2. Materials and methods

2.1. Chemicals

EGCG (MW 458.4 Da), hen egg white lysozyme (MW 14.3 kDa), thioflavin T (ThT), 1-anilino-naphthalene 8-sulfonate (ANS), and ascorbic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Unless otherwise indicated, all other reagents were of analytical grade. Fresh blood was drawn from healthy volunteers using sodium citrate as an anticoagulant.

2.2. Preparation of oxidized EGCG

EGCG was dissolved in 50 mM Tris–HCl (pH 8.0 or 7.4) at a concentration of 2 mg/mL, and was pipetted into Eppendorf tubes. Oxidation of EGCG was carried out at 37 °C for 0–12 h. The resultant solutions were stored at −40 °C until use. The oxidation products of EGCG prepared at pH 8.0 were used in next experiments.

2.3. UV spectra

EGCG and its oxidized products were diluted to 100 μg/mL prior to spectral scanning at 200–300 nm by using a U3900/3900 H spectrophotometer (Hitachi, Tokyo, Japan).

2.4. HPLC assay

Chromatographic separation was achieved on an Inertsil ODS column (4.6×250 mm, 5 μm; GL Sciences, Japan) using a Shimadzu LC−20 A system (Kyoto, Japan) at ambient temperature. Samples were filtered over 0.22 μm filters (Millipore) prior to injection. The mobile phase consisted of 0.1% formic acid in 40% methanol (HPLC grade) in water. The injection volume was 10 μL and the flow rate was maintained at 0.5 mL/min in an isocratic elution mode. EGCG and its oxidized species were detected at 280 nm.

2.5. Preparation and characterization of lysozyme fibrils

Lysozyme fibrils were prepared according to previous reports with minor modifications [15,16]. Briefly, lysozyme was dissolved in HCl solution (10 mM, pH 2.0) containing 150 mM NaCl with or without an inhibitor to a final concentration of 1.0 mg/mL. The mixture was incubated for 6 days at 65 °C in a water bath with agitation (60 rpm). Lysozyme fibril growth was monitored by ThT fluorescence, ANS fluorescence, and transmission electron microscopy (TEM). ThT fluorescence was measured in a mixture of 20 μg/mL lysozyme and 10 μM ThT with excitation at 440 nm and emission at 484 nm in a Perkin Elmer LS55 spectrofluorometer (Waltham, MA, USA). The emission spectra of ANS fluorescence in the presence of lysozyme fibrils were recorded between 400 and 600 nm using an excitation wavelength of 350 nm [5]. EGCG and oxidized EGCG had no obvious effects on ThT and ANS fluorescence under the experimental conditions of this study. For TEM measurements, an aliquot of lysozyme fibrils was diluted with water and dropped onto copper mesh grids. Samples were negatively stained with 5% (w/v) phosphotungstic acid and air-dried at room temperature. Observations were carried out using a JEOL JEM−2100 electron microscope (Tokyo, Japan) with an accelerating voltage of 80 kV.

2.6. Fibril-disruptive assay

The fibril-disruptive assay was carried out according to the methods described previously [12]. Lysozyme fibrils (10 mg/mL), prepared specifically for the fibril-disruptive assay, were diluted with 50 mM Tris–HCl (pH 8.0) and supplemented with aliquots of freshly prepared or pre-incubated EGCG (12 h) to a concentration of 1 mg/mL prior to incubation at 37 °C. After 1 h of incubation, the solutions were transferred into Eppendorf tubes and centrifuged at 700*g* for 5 min to separate the precipitates. Aliquots of the supernatant were obtained and the protein content was quantitatively measured using the Bradford assay [25]. Percentage of fibril deposition is calculated using (Ct-Cs)/Ct×100%, where Ct is the total concentration of lysozyme, and Cs is the concentration of lysozyme in the supernatant.

2.7. Hemolytic assay

Fresh blood was centrifuged at 1000 *g* for 10 min, and erythrocytes were separated from plasma and buffy coat and washed three times with isotonic phosphate-buffered saline (pH 7.4). For the hemolytic assay, the cell suspensions (1% hematocrit) were incubated at 37 °C for 3 h in the presence of lysozyme amyloid species (0.2 mg/mL) prepared with or without an inhibitor. An aliquot of the reaction mixture was removed and centrifuged at 1000*g* for 10 min. Absorbance of the supernatant was determined at 540 nm. The hemolytic rate was calculated in relation to the hemolysis of erythrocytes in 10 mM phosphate buffer (pH 7.4) which was taken as 100%.

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