Research paper

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

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A B S T R A C T

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

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Extensive investigations on the anti-amyloidogenic effects of EGCG in culture medium [24]. The structure and anti-amyloidogenic activity of EGCG is susceptible to oxidation in air under neutral or alkaline pH. For instance, 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. Oxidized EGCG have so far remained largely unclear. In the present investigation, 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 1.0 mg/mL prior to incubation at 37 °C. After 1 h of incubation, the solutions were transferred into Eppendorf tubes and centrifuged at 700g 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 1000g 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%.
2.8. Statistical analysis

Unless otherwise indicated, the experiments were performed in triplicate and the values are presented as means ± SD. Student’s t-test was utilized when two samples were compared.

3. Results

3.1. HPLC analyses of EGCG and its oxidized products

The chemical instability of EGCG has been extensively investigated in a neutral or alkaline medium. Dube et al. [26] reported that there was approximately 80% degradation of EGCG in a phosphate buffer (pH 7.4) after incubating for 2 h at 37 °C. The HPLC data of this study demonstrated that EGCG degraded and transformed into metabolites with lower hydrophobicity upon incubation in 50 mM Tris–HCl (pH 8.0) at 37 °C. Under the chromatographic conditions described in the experimental section, EGCG was eluted at a retention time of 12.7 min (Fig. 1A). The peak area of EGCG decreased with an increase of incubation time, along with a generation and increasing area of new peak at 10.2 min (Fig. 1B and C). After incubating for 12 h, less than 3% of intact EGCG remained in the solution. The half-life of EGCG is approximately 1.7 h, according to the data shown in Fig. 1B. The peak area of EGCG remains unchanged upon incubating this compound with 4 mM ascorbic acid for 12 h, suggesting that the degradation of EGCG can be inhibited by an antioxidant and that EGCG degrades through an auto-oxidation pathway. UV spectra show that both EGCG and its 12-h degradation species have a maximum absorbance at 275 nm (Fig. 1D), indicating that the oxidative products still possess a phenolic structure. The degradation of EGCG at pH 7.4 was also determined by HPLC. The half-life of EGCG was approximately 1.9 h. More than 8% of intact EGCG remained in the solution (pH 7.4) after incubating at 37 °C for 12 h. We used the oxidation products of EGCG prepared at pH 8.0 in the following experiments.

3.2. Inhibition of lysozyme fibrillation by EGCG and its oxidized products

Incubation of lysozyme in an acidic medium and elevated temperature resulted in the formation of amyloid fibrils [4,5,27,28]. The in vitro synthetic lysozyme amyloid fibrils shared similar biochemical properties with those extracted from pathological deposits in tissue. Increasing the temperature, ionic strength, or lysozyme concentration significantly shortened the lag time of fibril growth. In the present study, we incubated 1 mg/mL lysozyme in 150 mM NaCl at 65 °C with agitation. The growth of amyloid fibrils was monitored and characterized by ThT fluorescence, ANS fluorescence, and transmission electron microscopy.

The fluorescence profile of ThT bound to fibrillar lysozyme is characterized by a lag phase, followed by a sigmoid-like elongation phase, and a saturation phase, as shown in Fig. 2A. EGCG and the pre-incubated EGCG inhibited lysozyme fibrillation in a dose-dependent manner (Fig. 2B), resulting in a significant decrease in the final intensity of ThT fluorescence. The pre-incubated EGCG exhibited a noticeably stronger inhibition behavior to the fibril formation than that of the freshly prepared EGCG. Co-incubation of lysozyme with 50 µg/mL native EGCG resulted in 49.3% reduction in the final intensity of ThT fluorescence. Whereas in the presence of oxidized EGCG, 86.7% reduction of the final intensity of ThT fluorescence were recorded for the samples pre-incubated for 12 h (Fig. 2A and B).

ANS is a specific fluorescent dye for probing changes in surface hydrophobicity of protein molecules. Upon binding to a hydrophobic
region of protein, the intensity of ANS fluorescence is significantly enhanced with a blue-shift of the maximum emission wavelength [5]. Incubation of lysozyme resulted in a significant increase in the ANS fluorescence (Fig. 2C) and a shift in the maximum emission wavelength from 515 nm to 476 nm, reflecting an increase in the solvent-exposed hydrophobic interior of the protein during fibril growth. In the presence of native EGCG, significant depreciation in ANS fluorescence was recorded, suggesting that EGCG inhibited exposure of the hydrophobic interior of the protein, subsequently inhibiting fibril assembly. Oxidized EGCG species were also examined and gave stronger inhibition to the intensity of ANS fluorescence than freshly prepared EGCG, as shown in Fig. 2C.

Fig. 3 shows the TEM images of the lysozyme fibrils prepared in the absence and presence of native and oxidized EGCG. In the absence of EGCG, the mature lysozyme fibrils had a typical amyloid morphology characterized by long, dense, and partially bundled fibrils (Fig. 3A). In the presence of native EGCG, the length and density of fibrils decreased significantly (Fig. 3B). Only amorphous aggregates were observed in the sample containing an equimolar concentration of pre-incubated EGCG (Fig. 3C). These data indicate that the pre-incubated EGCG exhibited a stronger inhibitory role on the fibril formation than that of the native form, consistent with the ThT and ANS data.

3.3. The fibril-disruptive roles of EGCG and oxidized EGCG

One fibril-disruptive role of a natural polyphenol is to transform amyloid fibrils into amorphous aggregates. Transformation of amyloid fibrils into amorphous aggregates is accompanied by a decrease in amyloid structure and fibril cytotoxicity. In previous work, we found that the fibril-disruptive activity of tea catechins depended on both their hydrophobicity and antioxidant capacity [12]. In this study, the fibril-disruptive roles of EGCG and its oxidized products have been compared according to the methods described previously [12]. As shown in Fig. 4A, after 1 h of incubation, amorphous aggregates were observed in the tubes containing EGCG (tube 2) and oxidized EGCG (tube 3). The formed amorphous aggregates were separated by centrifugation and the deposition rates were evaluated quantitatively by determining the remaining lysozyme in the aqueous phase. The resultant data in Fig. 4B showed that oxidized EGCG induced 38.7% of the fibrils to transform into amorphous deposits, in comparison with 29.1% deposition of the fibrils induced by native EGCG, indicating that the oxidized EGCG played a stronger fibril-disruptive effect than the native form. Interestingly, 4 mM ascorbic acid abolished the disruptive effect of native EGCG on lysozyme fibrils (tube 5) but had no obvious effect on oxidized EGCG (tube 6). This fact suggests that oxidation is a
absence of EGCG. For details see materials and methods section. (A) Amorphous assemblies were prepared by incubating lysozyme (1 mg/mL) at 65 °C for 6 days in the presence of native or oxidized EGCG. Asterisks represent the conversion of lysozyme brils to amorphous aggregates. The conversion of brils to amorphous aggregates was expressed as the percentage of lysozyme deposited. Asterisk represents p < 0.05 (n=3).

3.4. Hemolysis induced by lysozyme assemblies prepared in the absence and presence of an inhibitor

Both oligomers and mature brils of lysozyme have been reported to exert toxicity on cells [4,5,28], acting via different mechanisms. A previous study [5] demonstrates that hemolysis can be induced by amyloid species of lysozyme in a fibril-age-dependent manner. In this study, the cell-damaging capacity of lysozyme assemblies prepared in the absence and presence of an inhibitor were evaluated using human erythrocyte as an in vitro model. As shown in Fig. 5, incubation of human erythrocytes in an isotonic environment with the fibril species of lysozyme resulted in hemolysis. The hemolytic effects increased with the age of fibrils, and the 4-day-old fibrils showed maximum hemolysis (27.9%). The subsequent decline in the hemolysis was partly caused by increased levels of aggregation and deposition of hemoglobin, which led to lower absorbance at 540 nm. Upon co-incubating with EGCG and its oxidized products, the hemolytic capacity of resultant lysozyme assemblies decreased significantly (p < 0.01). In accordance with the ThT and TEM data, oxidized EGCG displayed a greater capacity than its native form in inhibiting the formation of cell-damaging species of lysozyme. For instance, after incubation for 6 days, the oxidized EGCG-generated lysozyme assemblies induced 9.5% cell lysis, significantly less than the figure (15.0%) for native EGCG-generated lysozyme assemblies. This fact suggests that the oxidized EGCG-generated lysozyme assemblies are less cytotoxic than those created in the presence of native EGCG.

As shown in Fig. 5, the hemolytic effects increased with the growth of fibrils, in contrast to the previous view [29,30] that only oligomers and protofibrils exhibit cytotoxicity while mature fibrils show a weak or no effect. This fact may be attributed to the hydrophobic interactions between cell membranes and lysozyme fibrils. The hydrophobicity of the fibrillar species, probed by the fluorescent dye ANS, increased with the fibril aging (Fig. 2C) and with the ability of the fibrils to induce hemolysis. This suggests that one of the possible pathways through which lysozyme fibrils disrupt the cell membrane is interaction between the hydrophobic domains of the protein and lipid bilayers.

3.5. MS and MS/MS analyses of oxidized EGCG

MS and MS/MS analyses were performed to track the structural change of EGCG in oxidation. The methods and results are shown in the Supplementary material. Native EGCG yields an [M–H]– ion at m/z 457, as shown in Fig. S1. The [M–H]– ions at m/z 225 and 169 can be observed in the MS profile of oxidized EGCG (Fig. S2). The m/z 169 ion corresponds to the deprotonated ion of gallic acid [31]. The MS/MS spectrum of ions at m/z 225 yields ions at m/z 193, 165, and 149 (Fig. S3). These ions have been observed in the MS/MS spectrum of EGCG and EGC (epigallocatechin) [32]. According to the MS/MS data of EGCG and EGC reported in Ref. [32], we suggest that the ion at m/z 225 is originated from the B-ring of EGCG and probably has a vicinal trihydroxy structure. Possible degradation pathway of EGCG is suggested in Fig. S4.

HPLC–ESI–MS analysis of the oxidized EGCG at 12 h was also performed. The UV absorption at 280 nm and total ion chromatograms of oxidized EGCG obtained from the LC/MS analysis are shown in Fig. S5A and B, respectively. The peak at 9.9 min contains two species with similar hydrophobicity, as shown by the presence of the [M–H]– ions at m/z 225 and 169 in the mass spectra (Fig. S5C). According to the data of Fig. S2 and S5C, we suggest that the chemicals corresponding to ions at m/z 225 and 169 are the main oxidation products of EGCG.

4. Discussion

Extensive studies have been published about the biological and pharmacological activities of EGCG. Many of these activities, including anti-carcinogenic, antioxidant, anti-inflammatory, and anti-microbial are based on the antioxidant property of EGCG [33,34]. Recent reports confirm that this antioxidant property is also involved in the anti-amyloidogenic role of EGCG [12,21,23]. It is well known that the antioxidant capacity of EGCG is related to its instability and oxidation under neutral and alkaline mediums. However, the poor stability of EGCG under common experimental conditions is frequently neglected, particularly in the evaluation of its anti-amyloidogenic activity. In the
The data presented herein suggest that the oxidation of an intact polyphenol and polypeptide chains in the amyloid inhibition actions. Recent investigations demonstrated that covalent binding polyphenol should be considered adequately in its anti-amyloidogenic example, Bon in a pH-dependent manner. However, the details vary widely. For than the native form. The anti-amyloidogenic e...
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