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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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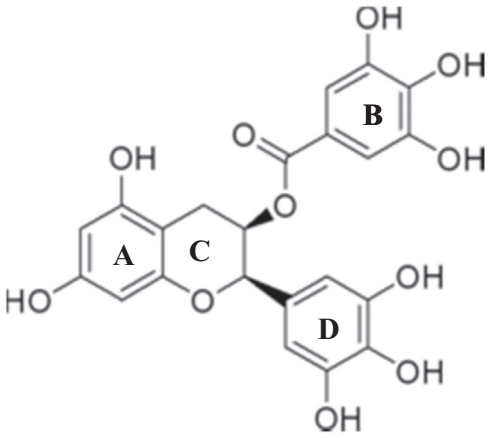

Scheme 1. Molecular structure of EGCG.

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%.

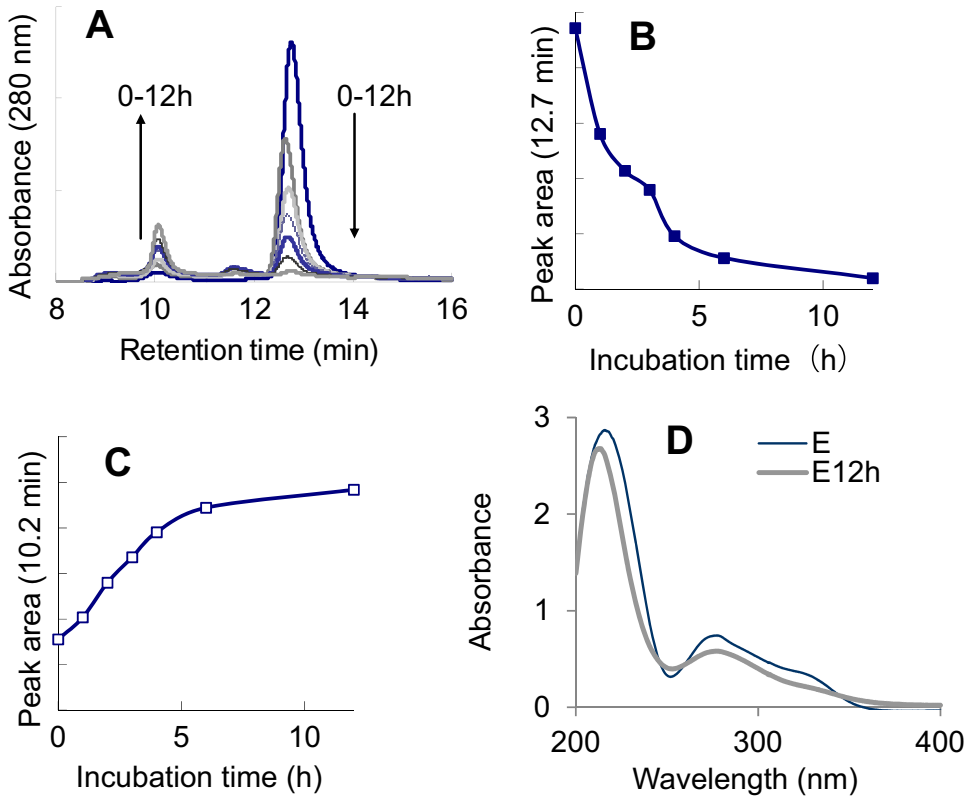

Fig. 1. Analysis of EGCG oxidation. (A) HPLC analysis of the kinetics of EGCG oxidation at 37 °C and pH 8.0. EGCG (12.7 min) and its oxidation substances (10.2 min) were detected at 280 nm. Time-dependent changes of peak area of EGCG and the oxidation substances are shown in (B) and (C), respectively. (D) UV-spectra of native EGCG (E) and the oxidation products (E12h).

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. 1A–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 of 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

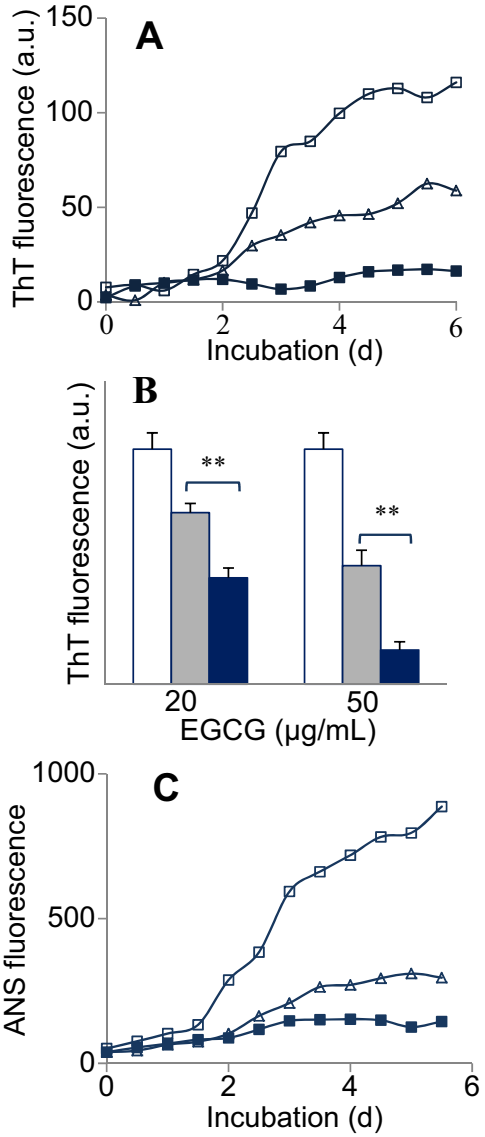

Fig. 2. Kinetics of lysozyme fibrillation in the absence and presence of 50 μg/mL native EGCG or oxidized EGCG at 12 h. Incubation temperature was 65 °C. (A) ThT assay. Open squares, lysozyme (Ly) only; open triangles, Ly+E; filled squares, Ly+E12h. (B) Dose-dependent inhibitory effects of EGCG and oxidized EGCG on lysozyme fibrillation.

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

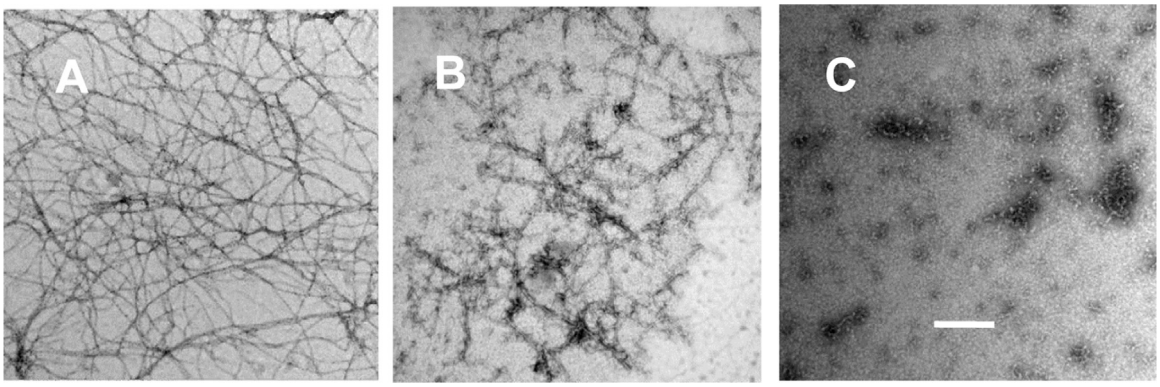

Fig. 3. TEM images of lysozyme assemblies. Samples were prepared by incubating lysozyme (1 mg/mL) at 65 °C for 6 days in the absence (A) and presence of 50 μg/mL EGCG (B) and oxidized EGCG at 12 h (C). Scale bar represents 200 nm.

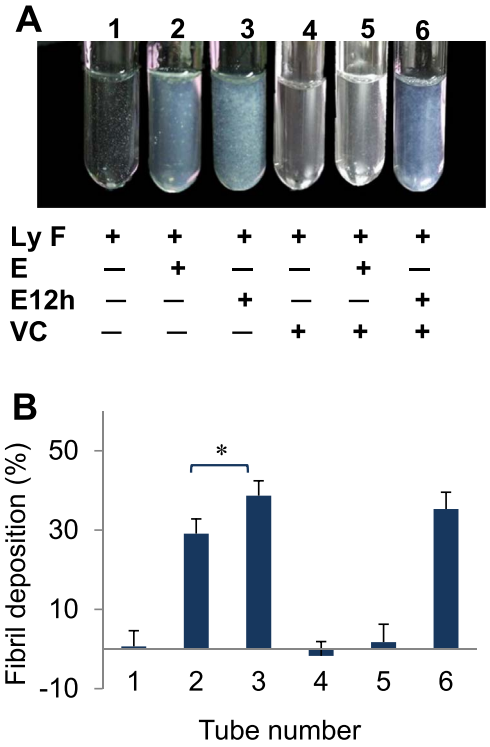

Fig. 4. Fibril-disrupting effects of EGCG and oxidized EGCG. Mature fibrils were prepared specifically by incubating lysozyme (10 mg/mL) at 65 °C for 6 days in the absence of EGCG. For details see materials and methods section. (A) Amorphous aggregates were induced by co-incubating preformed fibrils with EGCG at 37 °C for 1 h. The final concentration of mature lysozyme fibrils (Ly F) was 1 mg/mL; EGCG and oxidized EGCG, 50 μg/mL; VC (ascorbic acid), 4 mM. (B) Quantitative determination of the conversion of lysozyme fibrils to amorphous aggregates. The conversion of fibrils to amorphous aggregates was expressed as the percentage of lysozyme deposited. Asterisk represents $p < 0.05$ (n=3).

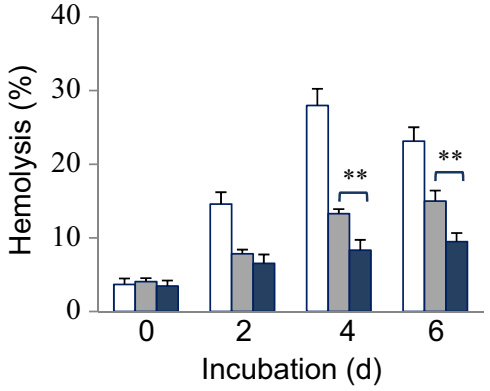

Fig. 5. Hemolytic assay. Hemolysis of erythrocytes was induced by incubating (37 °C) the cell suspensions (1%, v/v) with lysozyme assemblies (0.2 mg/mL) for 3 h. Lysozyme assemblies were prepared by incubating lysozyme (1 mg/mL) at 65 °C for 6 days in the absence and presence of 50 μg/mL native or oxidized EGCG. Asterisks represent $p < 0.01$ (n=3). The control solution showed no hemolytic effect (data not shown).

prerequisite for the fibril-disruptive action of EGCG.

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

Both oligomers and mature fibrils 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

present study, the instability of EGCG and the anti-amyloidogenic role of oxidized species are examined. The results demonstrate that, upon incubation in 50 mM Tris–HCl (pH 8.0) at 37 °C for 12 h, more than 97% of EGCG degrades through an auto-oxidation pathway. The resultant oxidized species has a more potent anti-amyloidogenic effect than the native form.

Numerous reports have examined the instability of EGCG by means of HPLC and mass spectrometry [24,35,36]. The reported results are similar, namely, this compound degrades through oxidation pathways in a pH-dependent manner. However, the details vary widely. For example, Bonfili et al. [35] report that, at pH 8 and 37 °C, EGCG has a half-life of 75 min; whereas in the report of Wei et al. [36], more than 75% of EGCG degrades after 1 h incubation at pH 8 and 37 °C. The chemical structures of oxidation products are also different from one another, probably originating from the experimental conditions and the methods of MS determination. Under the experimental conditions of this study, 50% EGCG degrades in less than 2 h. Two main oxidation products have been detected by mass spectrometry. Gallic acid, corresponding to the ion at m/z 169, has been reported to inhibit amyloid formation of proteins with lower activity than EGCG [37–39]. The $[M-H]^-$ ion at m/z 225 is rarely reported. Further research is required to characterize this chemical and determine its anti-amyloidogenic activity.

The anti-amyloidogenic effect of a polyphenol has been attributed to direct interaction of the intact molecule with misfolded polypeptides [6,7,9]. Molecular dynamic simulations have also usually been utilized to provide an atomistic explanation of the interactions between an intact polyphenol and polypeptide chains in the amyloid inhibition [40–42]. The data presented herein suggest that the oxidation of a polyphenol should be considered adequately in its anti-amyloidogenic actions. Recent investigations demonstrated that covalent binding plays an important role in the inhibition of protein fibrillation by EGCG and other polyphenols [15,16,21,43]. Through an auto-oxidation pathway, EGCG transforms to quinone or quinonoid substances which covalently bind to polypeptide chains, resulting in the inhibition of amyloid growth [16,21,43]. Upon incubation of lysozyme with oxidized EGCG, quinoapeptides have been detected (data not shown) by the NBT-staining assay [44], suggesting that the oxidation products are able to transform to quinone or quinonoid substances. How EGCG oxidation and quinopeptides formation drive the anti-amyloidogenic process merits further investigation.

In conclusion, EGCG is susceptible to oxidation in air under neutral or weak alkaline pH. The half-life of EGCG is less than 2 h under the experimental conditions of this work. By utilizing lysozyme as an *in vitro* model, the oxidized EGCG demonstrates a more potent anti-amyloidogenic role than the intact molecule. The oxidized EGCG also has a stronger disruptive effect on mature lysozyme fibrils than the native form. Ascorbic acid eliminates the disruptive effect of native EGCG on lysozyme fibrils, suggesting that oxidation is a prerequisite in the fibril-disruptive role of EGCG. As one of the most intensively investigated natural polyphenols, EGCG has attracted more and more attention due to its potential benefits on neurodegenerative diseases and other amyloid disorders. However, the molecular mechanism of the anti-amyloidogenic action of EGCG is still open to debate. Many reports have attributed the anti-amyloidogenic role of EGCG to the interactions between polypeptide and the intact molecule of this compound. The results of this work indicate that the oxidation of EGCG cannot be neglected in its anti-amyloidogenic effects. These insights into the action of EGCG may provide a novel route to understand the anti-amyloidogenic activity of natural polyphenols.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2016.12.016.

References

[1] C.M. Dobson, Protein folding and misfolding, Nature 426 (2003) 884–890.
[2] M. Stefani, Protein misfolding and aggregation: new examples in medicine and biology of the dark side of the protein world, Biochim. Biophys. Acta 2004 (1739) 5–25.
[3] M.B. Pepys, P.N. Hawkins, D.R. Booth, D.M. Vigushin, G.A. Tennent, A.K. Soutar, et al., Human lysozyme gene mutations cause hereditary systemic amyloidosis, Nature 363 (1993) 553–557.
[4] A.L. Gharibyan, V. Zamotin, K. Yanamandra, O.S. Moskaleva1, B.A. Margulis, I.A. Kostanyan, L.A. Morozova-Roche, Lysozyme amyloid oligomers and fibrils induce cellular death via different apoptotic/necrotic pathways, J. Mol. Biol. 365 (2007) 1337–1349.
[5] B. Huang, J. He, J. Ren, X.Y. Yan, C.M. Zeng, Cellular membrane disruption by amyloid fibrils involved intermolecular disulfide cross-linking, Biochemistry 48 (2009) 5794–5800.
[6] Y. Porat, A. Abramowitz, E. Gazit, Inhibition of amyloid fibril formation by polyphenols: structural similarity and aromatic interactions as a common inhibition mechanism, Chem. Biol. Drug Des. 67 (2006) 27–37.
[7] J.A. Lemkul, D.R. Bevan, Destabilizing Alzheimer's $A\beta_{42}$ protofibrils with morin: mechanistic insights from molecular dynamics simulations, Biochemistry 49 (2010) 3935–3946.
[8] M. Hirohata, K. Hasegawa, S. Tsutsumi-Yasuhara, Y. Ohhashi, T. Ookoshi, K. Ono, et al., The anti-amyloidogenic effect is exerted against Alzheimer's β-amyloid fibrils in vitro by preferential and reversible binding of flavonoids to the amyloid fibril structure, Biochemistry 46 (2007) 1888–1899.
[9] A.J. Doig, P. Derreumaux, Inhibition of protein aggregation and amyloid formation by small molecules, Curr. Opin. Struct. Biol. 30 (2015) 50–56.
[10] H. Shoval, L. Weiner, E. Gazit, M. Levy, I. Pinchuk, D. Lichtenberg, Polyphenol-induced dissociation of various amyloid fibrils results in a methionine-independent formation of ROS, Biochim. Biophys. Acta 1784 (2008) 1570–1577.
[11] M. Zhu, S. Rajamani, J. Kaylor, S. Han, F. Zhou, A.L. Fink, The flavonoid baicalein inhibits fibrillation of α-synuclein and disaggregates existing fibrils, J. Biol. Chem. 279 (2004) 26846–26857.
[12] J. He, Y.F. Xing, B. Huang, Y.Z. Zhang, C.M. Zeng, Tea catechins induced the conversion of preformed lysozyme amyloid fibrils to amorphous aggregates, J. Agr. Food Chem. 57 (2009) 11391–11396.
[13] W. Zhou, A. Gallagher, D.P. Hong, C. Long, A.L. Fink, V.N. Uversky, At low concentrations, 3,4-dihydroxyphenylacetic acid (DOPAC) binds non-covalently to alpha-synuclein and prevents its fibrillation, J. Mol. Biol. 388 (2009) 597–610.
[14] M. Zhu, S. Han, A.L. Fink, Oxidized quercetin inhibits α-synuclein fibrillization, Biochim. Biophys. Acta 2013 (1830) 2872–2881.
[15] S. Feng, X.H. Song, C.M. Zeng, Inhibition of amyloid fibrillation of lysozyme by phenolic compounds involves quinoprotein formation, FEBS Lett. 586 (2012) 3951–3955.
[16] N. Cao, Y.J. Zhang, S. Feng, C.M. Zeng, Quinopeptide formation associated with the disruptive effect of epigallocatechin gallate on lysozyme fibrils, Int. J. Biol. Macromol. 78 (2015) 389–395.
[17] J. Bieschke, Natural compounds may open new routes to treatment of amyloid diseases, Neurotherapeutics 10 (2013) 429–439.
[18] F. aus dem Siepen, R. Bauer, M. Aurich, S.J. Buss, H. Steen, K. Altland, et al., Green tea extract as a treatment for patients with wild-type transthyretin amyloidosis: an observational study, Drug Des. Dev. Ther. 9 (2015) 6319–6325.
[19] D.E. Ehrnhoefer, J. Bieschke, A. Boeddrich, M. Herbst, L. Masino, R. Lurz, et al., EGCG redirects amyloidogenic polypeptides into unstructured, off-pathway oligomers, Nat. Struct. Mol. Biol. 15 (2008) 558–566.
[20] N. Ferreira, I. Cardoso, M.R. Domingues, R. Vitorino, M. Bastos, G. Bai, et al., Binding of epigallocatechin-3-gallate to transthyretin modulates its amyloidogenicity, FEBS Lett. 583 (2009) 3569–3576.
[21] I.R. Chandrashekaran, C.G. Adda, C.A. MacRaild, R.F. Anders, R.S. Norton, EGCG disaggregates amyloid-like fibrils formed by *Plasmodium falciparum* merozoite surface protein 2, Arch. Biochem. Biophys. 513 (2011) 153–157.
[22] P. Cao, D.P. Raleigh, Analysis of the inhibition and remodeling of islet amyloid polypeptide amyloid fibers by flavanols, Biochemistry 51 (2012) 2670–2683.
[23] H. Shoval, D. Lichtenberg, E. Gazit, The molecular mechanisms of the anti-amyloid effects of phenols, Amyloid 14 (2007) 73–87.
[24] J. Hong, H. Lu, X. Meng, J.H. Ryu, Y. Hara, C.S. Yang, Stability, cellular uptake, biotransformation, and efflux of tea polyphenol epigallocatechin-3-gallate in HT-29 human colon adenocarcinoma cells, Cancer Res. 62 (2002) 7241–7246.
[25] M.A. da Silva, M.A. Arruda, Mechanization of the bradford reaction for the spectrophotometric determination of total proteins, Anal. Biochem. 351 (2006) 155–157.
[26] A. Dube, K. Ng, J.A. Nicolazzo, I. Larson, Effective use of reducing agents and nanoparticle encapsulation in stabilizing catechins in alkaline solution, Food Chem. 122 (2010) 662–667.
[27] E. Frare, P.P. deLaureto, J. Zurdo, C.M. Dobson, A. Fontana, A highly amyloidogenic region of hen lysozyme, J. Mol. Biol. 340 (2004) 1153–1165.
[28] S. Shariatizi, A.A. Meratan, A. Ghasemi, M. Nemat-Gorgani, Inhibition of amyloid fibrillation and cytotoxicity of lysozyme fibrillation products by polyphenols, Int. J. Biol. Macromol. 80 (2015) 95–106.
[29] R. Kayed, E. Head, J.L. Thompson, T.M. McIntire, S.C. Milton, C.W. Cotman, C.G. Glabe, Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis, Science 300 (2003) 486–489.
[30] B. Caughey, P.T. Lansbury, Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders, Annu.

Rev. Neurosci. 26 (2003) 267–298.
[31] Y. Mizooku, M. Yoshikawa, T. Tsuneyoshi, R. Arakawa, Analysis of oxidized epigallocatechin gallate by liquid chromatography/mass spectrometry, Rapid Commun. Mass Spectrom. 17 (2003) 1915–1918.
[32] G. Liu, J. Dong, H. Wang, L. Wan, Y. Duan, S. Chen, Study on the fragmentation pathways of EGC/GC and EGCG/GCG using ESI–IT–TOF, J. Chin. Mass Spectrom. Soc. 30 (2009) 287–294.
[33] M. Afzal, A.M. Safer, M. Menon, Green tea polyphenols and their potential role in health and disease, Inflammopharmacolgy 23 (2015) 151–161.
[34] S. Khurana, K. Venkataraman, A. Hollingsworth, M. Piche, T.C. Tai, Polyphenols: benefits to the cardiovascular system in health and in aging, Nutrients 5 (2013) 3779–3827.
[35] L. Bonfili, M. Cuccioloni, M. Mozzicafreddo, V. Cecarini, M. Angeletti, A.M. Eleuteri, Identification of an EGCG oxidation derivative with proteasome modulatory activity, Biochimie 93 (2011) 931–940.
[36] Y. Wei, P. Chen, T. Ling, Y. Wang, R. Dong, C. Zhang, et al., Certain epigallocatechin-3-gallate (EGCG) auto-oxidation products retain the cytotoxic activities of EGCG, Food Chem. 204 (2016) 218–226.
[37] Y. Liu, J.A. Carver, A.N. Calabrese, T.L. Pukala, Gallic acid interacts with α-synuclein to prevent the structural collapse necessary for its aggregation, Biochim. Biophys. Acta 2014 (1844) 1481–1485.
[38] Y. Liu, T.L. Pukala, I.F. Musgrave, D.M. Williams, F.C. Dehle, J.A. Carver, Gallic acid is the major component of grape seed extract that inhibits amyloid fibril formation, Bioorg. Med. Chem. Lett. 23 (2013) 6336–6340.
[39] S. Chan, S. Kantham, V.M. Rao, M.K. Palanivelu, H.L. Pham, P.N. Shaw, et al., Metal chelation, radical scavenging and inhibition of Aβ42 fibrillation by food constituents in relation to Alzheimer's disease, Food Chem. 199 (2016) 185–194.
[40] G. Ortore, E. Orlandini, A. Braca, L. Ciccone, A. Rossello, A. Martinelli, S. Nencetti, Targeting different transthyretin binding sites with unusual natural compounds, ChemMedChem 11 (2016) 1865–1874.
[41] Q.I. Churches, J. Caine, K. Cavanagh, V.C. Epa, L. Waddington, C.E. Tranberg, et al., Naturally occurring polyphenolic inhibitors of amyloid beta aggregation, Bioorg. Med. Chem. Lett. 24 (2014) 3108–3112.
[42] M. Carbonaro, A. Di Venere, A. Filabozzi, P. Maselli, V. Minicozzi, S. Morante, et al., Role of dietary antioxidant epicatechin in the development of β-lactoglobulin fibrils, Biochim. Biophys. Acta 2016 (1864) 766–772.
[43] F.L. Palhano, J. Lee, N.P. Grimster, J.W. Kelly, Toward the molecular mechanism(s) by which EGCG treatment remodels mature amyloid fibrils, J. Am. Chem. Soc. 135 (2013) 7503–7510.
[44] M.A. Paz, R. Fluckiger, A. Boak, H.M. Kagan, P.M. Gallop, Specific detection of quinoproteins by redox-cycling staining, J. Biol. Chem. 266 (1991) 689–692.