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Quinopeptide formation associated with the disruptive effect of epigallocatechin-gallate on lysozyme fibrils

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ABSTRACT

Numerous studies demonstrate that natural polyphenols can inhibit amyloid formation and disrupt preformed amyloid fibrils. In the present study, the fibril-disruptive effects of epigallocatechin-3-gallate (EGCG) were examined using lysozyme as a model protein. The results indicated that EGCG dose dependently inhibited lysozyme fibrillation and modified the peptide chains with quinonoid moieties under acidic conditions, as measured by ThT fluorescence, transmission electron microscopy, and an NBT-staining assay. Moreover, EGCG transformed the preformed lysozyme fibrils to amorphous aggregates through quinopeptide formation. The thiol blocker, *N*-ethylmaleimide, inhibited the disruptive effect of EGCG on preformed fibrils, suggesting that thiol groups are the binding sites for EGCG. We propose that the formation of quinone intermediates *via* oxidation and subsequent binding to lysozyme chains are the main processes driving the inhibition of amyloid formation and disruption of preformed fibrils by EGCG. The information presented in this study may provide fresh insight into the link between the antioxidant capacity and anti-amyloid activity of polyphenols.

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1. Introduction

Amyloid fibrillation of polypeptides is associated with more than 20 human diseases, including Alzheimer's disease, type 2 diabetes mellitus, Parkinson's disease, hemodialysis-related amyloid deposition, transmissible spongiform encephalopathies, and a number of systemic amyloidoses [1–4]. Despite their unrelated amino acid sequences, these polypeptides are able to assemble into fibrils with identical amyloid properties including long and unbranched fibrillar morphology, enriched β-sheet structure, increased surface hydrophobicity, and the ability to disrupt cellular membranes.

Inhibition of amyloid formation and disruption of formed fibrillar assemblies are the therapeutic strategies proposed for the treatment of amyloid-related diseases. Recent investigations have demonstrated that polyphenolic compounds, particularly 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 [5–7]. In addition, antioxidant capacity is also thought to be involved in the fibril-disrupting activity of a polyphenol [8,9]. It has been reported that the oxidized form of a polyphenol has a more potent disruptive effect on amyloid fibrils than the reduced form [7,10,11]. In the previous work [12], we found that the inhibition of lysozyme amyloid fibrillation by monocyclic diphenols was associated with the formation of quinoproteins and that quinone intermediates were actually the active form for phenolic compounds to interrupt amyloid structure. Many natural polyphenols have a polycyclic molecular structure, for example, some members of the flavonoid and flavanoid families. It is of significance to determine whether these natural polyphenols share a similar pathway with monocyclic diphenols in their anti-amyloidogenic activity.

Epigallocatechin-3-gallate (EGCG, Fig. 1A), a member of the flavanoid family, is the most abundant catechin in green tea and is a potent antioxidant that has been widely investigated due to its benefits on human health. Recent investigations have indicated that EGCG exhibits an inhibitory effect on amyloid formation by the β-amyloid peptide, α-synuclein, and other proteins [13–16]. It has also been reported that the fibril-disrupting efficiency of a tea catechin is positively correlated with its antioxidant capacity [9,17,18]. However, there is no clear understanding of the link between redox property and the corresponding anti-amyloid activity of

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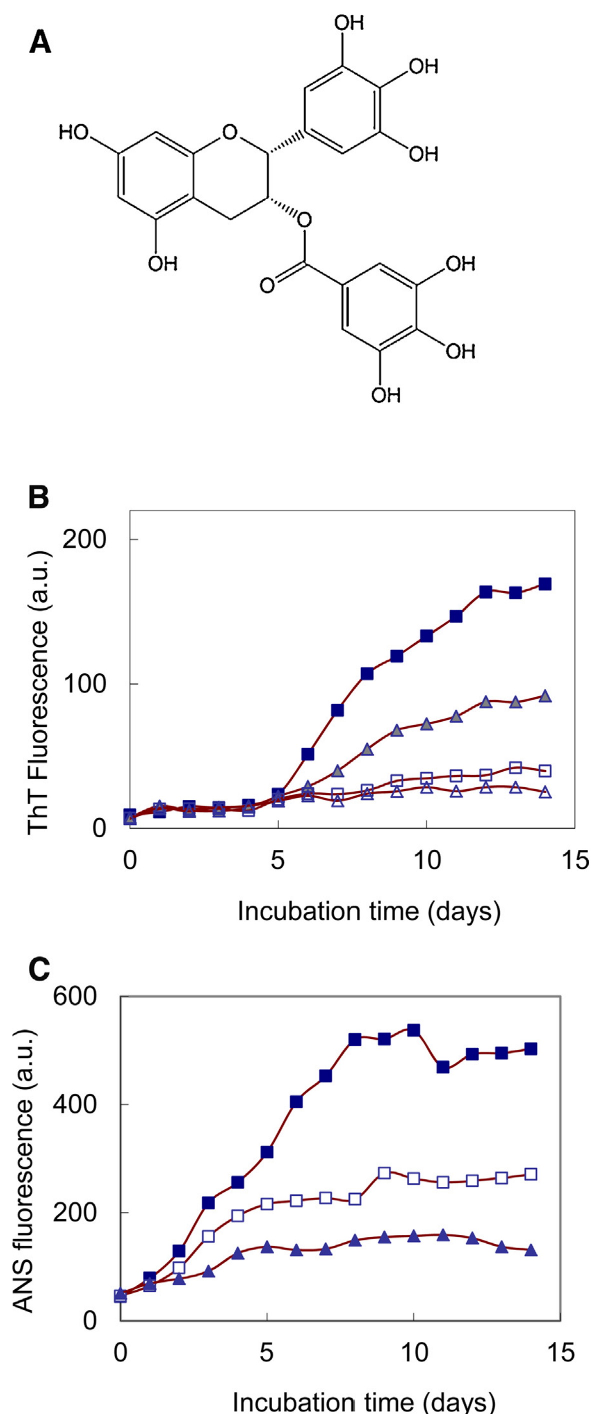

Fig. 1. (A) Molecular structure of EGCG. (B) ThT curves of lysozyme fibril growth in the absence (■) and presence of EGCG at the following molar ratios of EGCG to lysozyme (0.7 mM): 1:7 (▲), 1:2 (□), and 1:1 (△). (C) ANS fluorescence of lysozyme fibril growth in the absence (■) and presence of EGCG at molar ratios of EGCG to lysozyme (0.7 mM): 1:7 (□) and 1:1 (△).

polyphenols. In the present study, the anti-amyloidogenic effects of EGCG were evaluated *in vitro* using lysozyme as a model protein. The results suggest that EGCG inhibits lysozyme amyloid fibrillation and disrupts preformed fibrils in a dose-dependent manner. Quinopeptide formation was observed in both the inhibition of amyloid formation and disruption of preformed fibrils by EGCG. We further identified thiol groups originating from the breakage of disulfide bonds as the target of EGCG binding to lysozyme chains.

2. Materials and methods

2.1. Chemicals

(−)-Epigallocatechin-3-gallate (EGCG, MW 458.4), hen egg white lysozyme (MW 14.3 kDa), thioflavin T (ThT), *N*-ethylmaleimide (NEM), 1-anilino-naphthalene 8-sulfonate (ANS), and nitroblue tetrazolium (NBT) were purchased from Sigma–Aldrich (St Louis, MO, USA). Electrophoresis reagents were from Bio-Rad (Hercules, CA, USA). All other reagents were of analytical grade.

2.2. Preparation and characterization of lysozyme fibrils

Lysozyme fibrils were prepared according to our previous reports with minor modifications [9,19]. Briefly, hen egg white lysozyme was dissolved in HCl solution (10 mM, pH 2.0) with or without EGCG to a final concentration of 10 mg/mL (0.7 mM). EGCG (25 mM in water) was added according to the following molar ratios of EGCG to lysozyme (c EGCG/c lysozyme): 1:1, 1:2, and 1:7, respectively. The mixture was incubated for 12–14 days at 65 °C in a water bath without agitation. Lysozyme fibril growth was monitored by ThT fluorescence, ANS fluorescence, and transmission electron microscopy (TEM). ThT fluorescence was measured in a mixture of 33 μg/mL lysozyme and 10 μM ThT with excitation at 440 nm and emission at 484 nm in a Perkin Elmer LS55 spectrofluorimeter. 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 [19]. We confirmed that 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 20-fold with water and dropped onto copper-mesh grids. Samples were negatively stained with 2% (w/v) uranyl acetate and air-dried at room temperature. Observations were carried out using a JEOL JEM-2100 electron microscope with an accelerating voltage of 80 kV. For seeding experiments, mature lysozyme fibrils were sonicated for 15 min in a water bath (37 °C) and added to fresh lysozyme solutions at a ratio of 3% (seeds/fresh lysozyme; w/w).

2.3. Gel electrophoresis and NBT staining assay

SDS–PAGE was performed in tricine buffer (pH 8.2) using a 5% stacking gel and a 15% separating gel. Bands were visualized by Coomassie brilliant blue R-250 staining. For the blotting assay, the gel bands were transferred onto a polyvinylidene fluoride membrane (0.45 μm, Millipore) with a mini transfer cell (GE Healthcare). Quinopeptides were detected by staining the membrane with NBT (0.24 mM in 2 M potassium glycinate, pH 10). The blotting membrane was immersed in the glycinate/NBT solution for 45 min in the dark, resulting in a blue-purple stain of quinopeptide bands and no staining of other peptides.

2.4. Chromatographic analysis of EGCG stability in the inhibition of amyloid formation

Chromatographic separation was achieved on an Inerstil ODS column (4.6 × 250 mm, 5 μm; GL Sciences, Japan) using a Shimadzu LC-20A 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 in water. The injection volume was 20 μL, and the flow rate was maintained at 0.5 mL/min in an isocratic elution mode. EGCG and its degrade species were detected at 280 nm.

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