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Tea Catechins Induce the Conversion of Preformed Lysozyme Amyloid Fibrils to Amorphous Aggregates

JING HE,[†] YAN-FEI XING,[‡] BO HUANG,[‡] YI-ZHENG ZHANG,[†] AND CHENG-MING ZENG*,[†,‡]

[†]College of Life Science, Sichuan University, Chengdu 610064, China and [‡]College of Chemistry and Material Science, Shaanxi Normal University, Xi'an 710062, China

Natural polyphenols are major constituents of plant foods and herbs. Numerous studies have demonstrated that natural polyphenols inhibited amyloid formation and destabilized the preformed amyloid fibrils. However, the molecular mechanism for the antiamyloidogenesis of polyphenols is still unclear and remains to be further explored. In the present study, the preformed lysozyme fibrils were used as an in vitro model to study the disruptive effects of tea catechins on amyloid fibrils. Results showed that tea catechins induced the conversion of lysozyme fibrils to amorphous aggregates and inhibited fibril-induced hemolysis. Hydroquinone also showed disruptive effect on the fibrils, whereas phenol and two typical antioxidants, ascorbic acid and α-tocopherol, did not affect the fibrillar structure, suggesting that polyphenolic structure is essential for fibril deposition. Correlation analyses indicate that the fibril-depositing effects were related to both the antioxidative potency and hydrophobicity of tea catechins. These findings provide new evidence for comprehensive understanding of the interaction between natural polyphenols and amyloid fibrils.

KEYWORDS: Tea catechins; lysozyme; amyloid fibril; amorphous aggregates; hemolysis; hydrophobicity; antioxidative activity

INTRODUCTION

Amyloid fibrils of proteins or peptide fragments have been found to be associated with more than 20 human diseases, such as Alzheimer's disease, type 2 diabetes, Parkinson's disease, hemodialysis-related amyloid deposition, and a number of systemic amyloidoses (*1*, *2*). These proteins, despite their unrelated amino acid sequences and tertiary structures, can unfold and assemble into fibrils with similar ultrastructures and identical tinctorial properties, including long and unbranched fibrils with enriched highly ordered β-sheet structure (*3*), increased surface hydrophobicity, fluorescence upon binding to thioflavin T, and gold-green birefringence when stained with Congo Red. Accumulating evidence has strongly suggested that amyloid fibrils are cytotoxic due to their binding and disrupting cellular membranes (*4*−*6*).

The therapeutic strategies proposed for the treatment of amyloid-related diseases are mainly based on inhibition of the amyloid formation and disruption of the fibrillar structures. Enzymatic inhibitors, hormones, antagonists, antibodies, peptide fragments, synthetic ligands, and natural antioxidants have appeared in the list of screened candidates. Natural polyphenolic compounds from foods and traditional herbal medicines, having broad pharmacological activities and exhibiting inhibition of amyloid formation, have been extensively investigated in the disruption of mature amyloid fibrils and reduction of the toxicity of fibrils to living cells (*7*−*15*).

Recent publications (*16*−*19*) have studied the antiamyloid effects of natural polyphenols on three consecutive processes: formation of nascent fibrils, elongation or extension of the fibrils, and destabilization of the formed mature assemblies. Eleven polyphenols were tested, and data analyses (*19*) indicated that the polyphenols can be categorized into three groups: slightly and highly active inhibitors and selective inhibitors with different effects on these three stages. The hydrophobic and/or aromatic character of the phenolic compounds contributes to the antiformation and antiextension effects, whereas their antioxidative potency relates to the promotion of destabilization of mature fibril. The destabilizing effects include disaggregation/fragmentation of the fibrils and conversion of the fibrils into amorphous deposition. A recent report demonstrated that, in the presence of trace transition metal ions, fibril disaggregation by polyphenols was associated with reactive oxygen species (ROS) formation (*20*). Furthermore, the oxidative form of polyphenol was found to have a more potent effect on amyloid formation than the reductive form (*21*). These facts suggested that the redox property of polyphenol is operative in the fibril-inhibiting and -destabilizing process.

Although many attempts have been made to elucidate the molecular mechanism of natural polyphenols against amyloidogenesis, the structure−activity relationship is still obscure and remains further explored. In the present work by using amyloid fibril of hen egg white lysozyme (termed lysozyme here) as an in vitro model, we have investiaged the disrupting effects of eight tea

*Address correspondence to this author at the College of Chemistry and Material Science, Shaanxi Normal University, Xi'an 710062, China (fax +86 29 85307774; telephone +86 29 85303871; e-mail chengmingz@hotmail.com).

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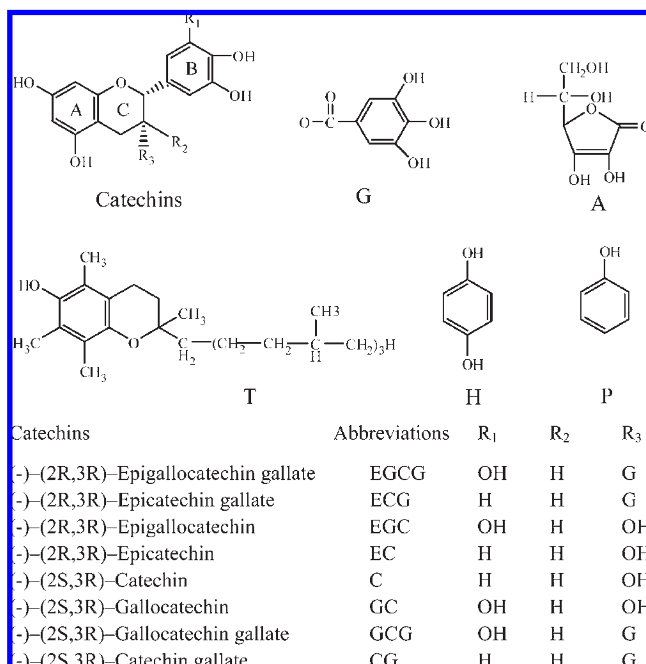

atechins	Abbreviations	R_1	R_2	R_3
-)–(2R,3R)–Epigallocatechin gallate	EGCG	OH	H	G
-)–(2R,3R)–Epicatechin gallate	ECG	H	H	G
-)–(2R,3R)–Epigallocatechin	EGC	OH	H	OH
-)–(2R,3R)–Epicatechin	EC	H	H	OH
-)–(2S,3R)–Catechin	C	H	H	OH
-)–(2S,3R)–Gallocatechin	GC	OH	H	OH
-)–(2S,3R)–Gallocatechin gallate	GCG	OH	H	G
-)–(2S,3R)–Catechin gallate	CG	H	H	G

Figure 1. Molecular structures of compounds tested in this study. Catechins with or without galloylate moiety (G); A, ascorbic acid; T, α-tocopherol; H, hydroquinone; P, phenol.

catechin compounds (**Figure 1**) on lysozyme fibrils. Our finding is that the polyphenolic structure is essential for the phenols depositing lysozyme fibrils and that both antioxidative activity and hydrophobicity of catechins can be involved in the fibril-destabilizing process.

MATERIALS AND METHODS

Protein and Chemicals. Hen egg white lysozyme (MW 14.3 kDa), thioflavin T (ThT), ascorbic acid, α-tocopherol, potassium persulfate, and all of the tea catechin compounds were purchased from Sigma-Aldrich (St. Louis, MO). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox C) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) were from Fluka (Buchs, Switzerland). Other reagents were of analytical or HPLC grade. Fresh blood was drawn from healthy volunteers using sodium citrate as an anticoagulant.

Preparation of Lysozyme Fibrils. Lysozyme fibrils were prepared according to our previous paper (*6*). Briefly, 200 mg of hen egg white lysozyme was dissolved in 10 mL of HCl solution (10 mM, pH 2.0) containing 0.1% NaN_3, to a final concentration of 2% (w/v). The mixture was incubated for 10 days at 65 °C in a water bath without agitation. The growth of lysozyme fibril was monitored by ThT fluorescence and transmission electron microscopy (TEM).

TEM. An aliquot (10 μL) of lysozyme fibril sample was diluted 20-fold with water in an Eppendorf tube. A freshly prepared solution (10 μL) was dropped onto carbon-coated copper-mesh grids. Samples were negatively stained with 10 μL of 2% (w/v) uranyl acetate for 1 min and air-dried at room temperature, after which the grids were washed with water and dried for 20 min. Observations were carried out using a Hitachi H-600 electron microscope with an accelerating voltage of 80 kV.

Quantitative Evaluation of Amorphous Aggregate Formation. The quantitative evaluation of the formation of amorphous aggregates from the preformed lysozyme amyloid fibrils was performed as follows: aliquots of preformed lysozyme fibrils and catechin solutions (5 mg/mL in 12.5% DMSO in water) were mixed with Tris-HCl buffer (50 mM, pH 8.0) in Eppendorf tubes. After incubation at room temperature, the tubes were centrifuged at 7000*g* for 5 min. Aliquot of the supernatant was taken, and the content of protein was measured according to the Bradford assay (*22*). Unless otherwise stated, all of the control samples of this work contained the same volume of DMSO but were devoid of catechins. We confirmed that 0.4% DMSO had no obvious effect on the fibrillar structure of

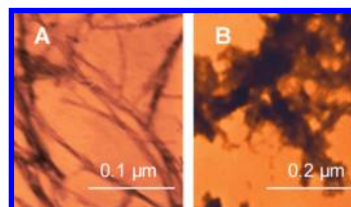

Figure 2. TEM images of lysozyme mature fibrils (**A**) and the amorphous aggregates induced by EGCG (**B**). Amorphous aggregates were derived from the fibrils (500 μg/mL) treated with 100 μg/mL EGCG at ambient temperature for 2 h.

lysozyme, membrane permeability of red blood cells (RBC), or ThT fluorescence.

ThT Fluorescence Measurements. Aliquots of lysozyme fibril solutions were diluted with Tris-HCl buffer (50 mM, pH 8.0), followed by the addition of 1 mM ThT solution. The resultant mixture contained 10 μM ThT and 50 μg/mL lysozyme. ThT fluorescence was measured using a Perkin-Elmer LS 55 fluorescence spectrometer with excitation at 440 nm and emission at 482 nm.

Hemolytic Assay. Fresh blood was centrifuged at 1000*g* for 10 min, and RBC were separated from plasma and buffy coat and washed three times with isotonic phosphate-buffered saline (pH 7.4). For hemolytic assay, RBC suspensions (1% hematocrit) were incubated at 37 °C for 40 min in the presence or absence of lysozyme fibrils and tea catechins. 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 hemolysis of RBC in 10 mM phosphate buffer, which was taken as 100%.

Determination of Partition Coefficients of Tea Catechins. Stoke solutions of catechin compounds were added to a 50% aqueous *n*-octanol solution in Eppendorf vials to obtain the final concentration of 0.1 mM. The vials were stirred for 1 min using a vortex to equilibrate the catechin between the aqueous and octanol phases. The emulsions were centrifuged at 3000*g* for 5 min. Following separation, both phases were collected for HPLC analysis in a reversed-phase mode. Partition coefficient ($K_{o/w}$) was calculated and expressed as the ratio of the HPLC peak area of catechin in octanol to the corresponding peak area in water.

Trolox Equivalent Antioxidant Capacity (TEAC) of Catechins. The TEAC values of tea catechins were determined according to the method described by Re et al. (*23*). Briefly, $ABTS^{\bullet+}$ radicals were generated by mixing ABTS (7 mM) and potassium persulfate (2.5 mM) and allowing them to react for 16 h in the dark. The solution of $ABTS^{\bullet+}$ was diluted, and catechins (dissolved in ethanol) were added. After incubation for 6 min, the extent of quenching of the $ABTS^{\bullet+}$ was measured spectrophotometrically at 734 nm and compared to standard amounts of Trolox C.

Statistical Analysis. Unless otherwise indicated, experiments were performed in triplicates, and values are presented as means ± SD. Student's *t* test was utilized when two samples were compared.

RESULTS AND DISCUSSION

Incubation of hen egg lysozyme for 10 days under the conditions described herein resulted in the formation of amyloid fibrils. The growth of protofibrils into mature fibrils from lysozyme monomer can be tracked and characterized by ThT assay and TEM. The final preformed fibrils had a typical long fibrillar structure with a diameter of 4−20 nm (**Figure 2A**).

The morphological conversion of preformed lysozyme fibrillar structures into amorphous aggregates by treatment with tea catechins has been observed under TEM. **Figure 2B** shows a representative image of the amorphous aggregates induced by EGCG, one of the most disruptive catechin compounds tested in this study. Other catechin compounds shared the same effect as EGCG on the morphological conversion of lysozyme fibrils.

Catechins with a gallic ester bond demonstrated high efficiency in converting lysozyme fibrils to amorphous aggregates. As

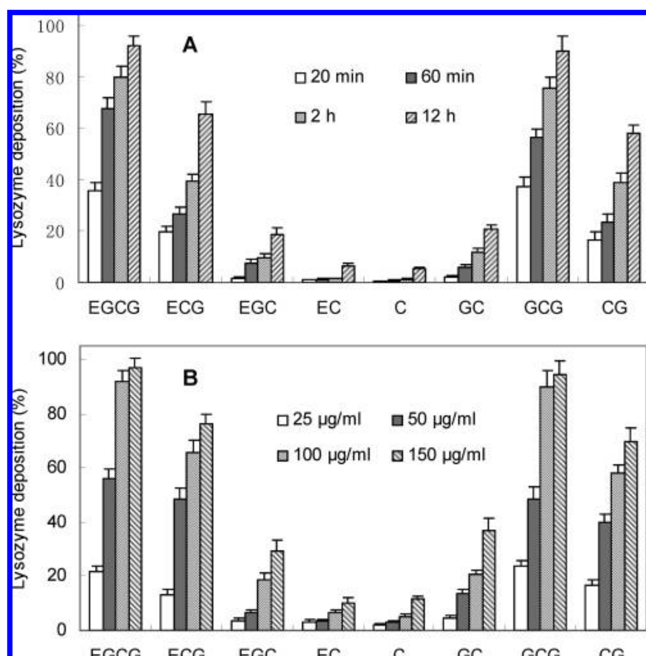

Figure 3. Transformation of lysozyme fibrils to amorphous aggregates by tea catechins: (**A**) time course of lysozyme fibrils (500 μg/mL) transformed to amorphous deposition in the presence of tea catechins (100 μg/mL); (**B**) dose dependence of the catechin-induced fibril deposition. Deposition of the fibrils is expressed as percentage of lysozyme in amorphous form. The fibrils (500 μg/mL) were treated with tea catechins at ambient temperature for 12 h. No deposit was observed in the mixture of native lysozyme (nonincubated, 500 μg/mL) and catechins (100 μg/mL) upon incubation at ambient temperature for 12 h.

shown in **Figure 3A**, 58−92% of the fibrils (500 μg/mL) were transformed to amorphous deposits by 100 μg/mL EGCG, ECG, and their corresponding epimers GCG and CG in 12 h. In these reaction systems, the molar ratios of lysozyme monomer to catechins were 1:6.2−6.5. Other catechins, having no gallic ester bond at the C-ring, showed significant low effects on the fibrils. The depositing rates of EC and C were 6.5 and 5.1%, whereas the values for EGC and GC were 18.9 and 20.6% ,respectively (the molar ratios of lysozyme monomer to catechins in the reaction systems were 1:9.3−9.9), suggesting that catechins with a trihydroxylated B-ring had higher fibril-depositing effects than their dihydroxylated analogues. Other substances were also checked in the fibril-depositing experiment. Phenol (100 μg/mL), the simplest phenolic compound, showed no effect on the fibrils. In contrast, hydroquinone (100 μg/mL) showed a medium effect and had a fibril-depositing rate of 15.9% in 12 h. Two conventional antioxidants, ascorbic acid and α-tocopherol, did not transform the fibrils at a concentration of 100 μg/mL during 12 h of incubation.

The fibril-depositing effects of catechins were dose-dependent, as shown in **Figure 3B**. A 50% deposition of lysozyme fibril was achieved by 45 μg/mL EGCG and 55 μg/mL ECG, respectively, on the basis of the 12 h incubation curves.

The transformation of the fibrils into amorphous aggregates was accompanied by a decrease of the fibrillar affinity for ThT, implicating a decrease of the β-sheet structure of the fibrils. As shown in **Figure 4**, ThT fluorescence of lysozyme fibrils decreased upon incubation with catechins ($p < 0.05$). Similar to the fibril-depositing effects, catechins with a gallic ester bond had higher efficiency to disrupt the β-sheet structure than other catechins.

Incubation of RBC in an isotonic environment with lysozyme fibrils resulted in hemolysis and cell aggregation. All tea catechins

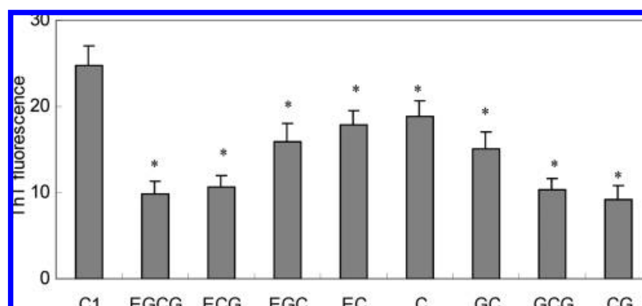

Figure 4. Effects of tea catechins on the affinity of lysozyme fibrils for ThT. The fibrils (500 μg/mL) were incubated in the presence or absence of catechin compounds (100 μg/mL) at room temperature for 2 h prior to ThT assay. Values are shown as means ± SD ($n = 3$). Asterisks represent $p < 0.05$ versus the ThT value of the fibrils without treatment by a catechin (C1).

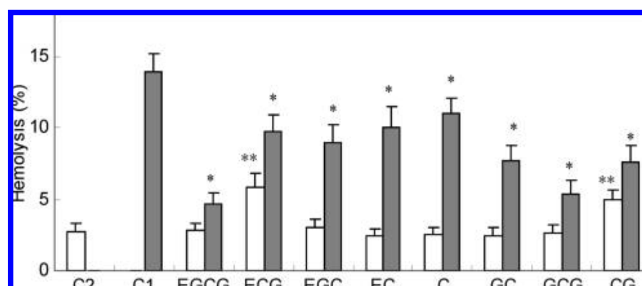

Figure 5. Inhibitory effects of tea catechins on fibril-induced hemolysis: (black bars) fibril-induced hemolysis in the absence (C1) and presence of a catechin; (white bars) hemolytic rates of RBC incubated with tea catechins (100 μg/mL) only. Hemolysis of RBC was induced by incubating the cell suspensions (1% hematocrit) with lysozyme fibrils (500 μg/mL) in the presence or absence of tea catechins (100 μg/mL). Values are shown as means ± SD ($n = 3$). *, $p < 0.05$ versus the hemolytic rate C1 by lysozyme fibrils in the absence of catechin; **, $p < 0.05$ versus the control hemolytic rate C2 in the absence of the fibrils and catechins.

tested herein showed inhibitory effects on the fibril-induced hemolysis ($p < 0.05$), as shown in **Figure 5**. Except for ECG and CG, catechins with a gallic ester bond demonstrated stronger inhibition than the nongalloylated catechins. For unknown reasons, ECG and CG induced hemolysis of RBC (<7%) when applied at the testing concentrations (**Figure 5**). This effect may result in low inhibitory rates of ECG and CG on the hemolysis induced by lysozyme fibrils. Other catechins showed no hemolytic effect in the experiments (**Figure 5**).

The catechin compounds tested herein showed different efficiencies in depositing the preformed lysozyme fibrils and inhibiting the fibril-induced hemolysis, with activity in the order of galloylated > nongalloylated and trihydroxylated B-ring (pyrogallol) > dihydroxylated B-ring (catechol). This order is basically consistent with the antioxidative scale TEAC of catechin compounds (**Table 1**). In addition, hydrophobicity of catechin is also related positively to the fibril-depositing effect. The galloylated catechins had higher *n*-octanol/water partition coefficient ($P_{o/w}$) values than the nongalloylated catechins (**Table 1**) because the gallic ester bond increased the lipophilic region of catechins. Correlation analyses (**Figure 6**) indicated that both antioxidative potency and hydrophobicity are related to the catechin-induced deposition of lysozyme fibrils, with correlation coefficients of $R^2 = 0.8076$ for TEAC (**Figure 6A**) and $R^2 = 0.6201$ for log P (**Figure 6B**). The higher correlation value for TEAC may suggest that the antioxidative property of catechin plays a determinant role in the catechin-induced deposition of lysozyme fibrils, in

Table 1. Properties of Tea Catechins[a]

compound	fibril deposition[b] (%)	$P_{o/w}$[c]	log *P*	TEAC
EGCG	92.1	15.9	1.20	4.89
ECG	65.6	21.3	1.33	5.13
EGC	18.9	1.0	−0.01	3.87
EC	6.5	4.3	0.63	2.63
C	5.1	3.9	0.59	2.36
GC	20.6	1.7	0.23	3.56
GCG	89.9	17.2	1.24	4.79
CG	57.9	23.6	1.37	4.57
hydroquinone	15.9	3.3	0.52	1.39
phenol	0	19.5	1.29	0.87
ascorbic acid	0	0.013	−1.87	1.02
α-tocopherol	0	14.6	1.16	1.05

[a] Only averages are shown. [b] Lysozyme fibrils (500 μg/mL) were incubated with a catechin (100 μg/mL) for 12 h at room temperature. [c] Partition coefficients in *n*-octanol/water system.

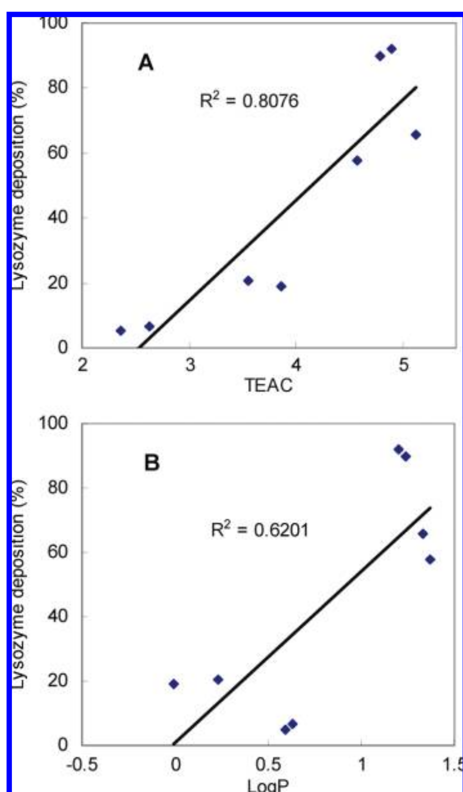

Figure 6. Correlations between the fibril-depositing activity and TEAC values (**A**) and *n*-octanol/water partition coefficients log *P* (**B**) of tea catechins.

agreement with the hypothesis that antioxidative potency of polyphenol relates to the promotion of destabilization of mature fibrils (*19*).

The fact that ascorbic acid, α-tocopherol, and phenol failed to deposit amyloid fibrils indicates that a structure with multiple hydroxyl groups on an aromatic ring is a prerequisite for specific interaction of an antioxidant with amyloid fibrillar structure. A redox process is possibly involved in the polyphenol-induced amyloid deposition, through a pathway of formation of a quinone structure that binds covalently (*7*) and/or noncovalently with amyloid fibrils.

Ascorbic acid and α-tocopherol are two important antioxidative vitamins in biological systems. Clinical applications have demonstrated that they delayed the progression of dementia (*24−26*). Moreover, several in vivo assays showed their protection of neurons from Aβ-induced cytotoxicity (*27, 28*). In this study, these two vitamins showed no effect on lysozyme amyloid fibrils, suggesting that ascorbic acid and α-tocopherol may function in alternative pathways in the process of antiamyloidogenesis, for instance, acting as scavengers of reactive oxygen species.

Polyphenols, including tea catechins, are capable of binding to proteins to form soluble or insoluble complexes. The binding strength is positively correlated with hydrophobicity of polyphenol (*29*). Heat treatment of the protein could increase the binding affinity and binding capacity of polyphenols (*30*). The transformation of native lysozyme to amyloid fibril involves unfolding and reorganizing the polypeptide chains, in which some hydrophobic domains and residues became exposed, resulting in an increase of surface hydrophobicity of the fibrillar species (*6*). Accordingly, the amyloid fibrillation of lysozyme enhanced the interaction between the protein and catechins, triggering the formation of amorphous aggregations. As a consequence, the fibril-depositing efficiency of a catechin is positively correlated to its hydrophobicity.

In addition to hydrophobic interactions, specific interactions between lysozyme and polyphenols have been evidenced. Proline (*30*) and tryptophan (*31*) were recognized as the specific binding sites of lysozyme for polyphenols. These residues in amyloid or denatured lysozyme are more accessible for catechins than the native form; therefore, the specific bindings are reinforced. It has been reported (*21*) that the flavonoid compound myricetin bonded specifically and reversibly to mature β-amyloid fibrils rather than to the monomeric peptide. Inhibition of the fibril growth and destabilization of mature β-amyloid fibrils by myricetin were also observed (*21*).

The transformation of lysozyme from its native form to amyloid fibrils increased the interaction of the protein with cellular membranes and therefore became cytotoxic (*6, 32*). The β-sheet content and hydrophobicity of the fibrillar assemblies increased with fibril growth and with the ability of the fibrils to induce hemolysis of RBC. Catechins disrupted the hydrogen bonds and van de Waals forces in the fibrillar assemblies, resulting in decreased β-sheet content and fibrillar cytotoxicity attenuation.

A recent review (*33*) suggested that there are several mechanisms for the protecting effects of polyphenols against the cytotoxicity induced by amyloid assemblies, including free radical scavenging, action as antioxidants against nitric oxide, and influence on various intracellular signaling pathways. Several in vivo studies (*34−36*) have demonstrated the antioxidative activity of polyphenols to be the primary role in cell rescue from Aβ toxicity. In a LDL oxidation ex vivo model, the protein-binding activity of phenol compounds was showed to be significantly correlated with the antioxidative potency (*37*). These results combined with our observation of this study support the hypothesis that the antioxidative property of polyphnol plays an important role in the disruption of fibrillar structures. However, the molecular mechanism of how a polyphenol disrupts amyloid structure via a redox pathway is still unclear and remains to be unveiled.

In summary, the preformed lysozyme fibrils were used as an in vitro model in this work to study the disruptive effects of tea catechins on amyloid fibrils. Eight tea catechins used herein showed a disruptive effect on the amyloid fibrillar structure, inducing lysozyme fibrils transformed to amorphous aggregates. A ThT fluorescence assay indicated that the β-sheet content of lysozyme fibrils decreased upon catechin binding. As a consequence, the hemolytic effect of the fibrils on RBC was attenuated. Both antioxidative potency and hydrophobicity of tea catechins were positively related to the fibril-disruptive activity. Ascorbic acid, α-tocopherol, and phenol did not demonstrate any effect on

lysozyme fibrils, indicating that a multihydroxylated phenolic structure is a prerequisite for the fibril-depositing activity of catechins. On the basis of these results, we suggest here that global properties of polyphenolic compounds, including their phenolic structures, antioxidative potency, and hydrophobicity, must be taken into account when they are applied for disrupting or destabilizing the fibrillar structures of proteins. This knowledge may be utilized for the design of novel antiamyloidogenic drugs with a polyphenolic structure.

ABBREVIATIONS USED

ThT, thioflavine-T; TEM, transmission electron microscopy; RBC, red blood cell; C, catechin; EC, epicatechin; EGCG, epigallocatechin gallate; EGC, epigallocatechin; ECG, epicatechin gallate; CG, catechin gallate; GC, gallocatechin; GCG, gallocatechin gallate. ABTS, 2,2′-azinobis(3-ethylbenzothiazoline- 6-sulfonate); Trolox C, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TEAC, Trolox equivalent antioxidant capacity.

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