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Protective Activity of Green Tea against Free Radical- and Glucose-Mediated Protein Damage

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Protein oxidation and glycation are posttranslational modifications that are implicated in the pathological development of many age-related disease processes. This study investigated the effects of green tea extract, and a green tea tannin mixture and its components, on protein damage induced by 2,2′-azobis(2-amidinopropane) dihydrochloride (a free radical generator) and glucose in in vitro assay systems. We found that green tea extract can effectively protect against protein damage, and showed that its action is mainly due to tannin. In addition, it was shown that the chemical structures of tannin components are also involved in this activity, suggesting that the presence of the gallate group at the 3 position plays the most important role in the protective activity against protein oxidation and glycation, and that there is also a contribution by the hydroxyl group at the 5′ position in the B ring and the sterical structure. These findings demonstrate the mechanisms of the usefulness of green tea in protein oxidation- and glycation-associated diseases.

KEYWORDS: Antioxidative activity; 2,2′-azobis(2-amidinopropane) dihydrochloride; glycation; green tea; tannin

INTRODUCTION

Green tea, prepared from the leaves of *Camellia sinensis* L, is a drink that is popular worldwide. Recently, it has received considerable attention because it contains large amounts of tannin, classified as the flavan-3-ol class of flavonoids, which contributes to its excellent antioxidative activities.

In the body, molecules such as lipids, DNA, and proteins may be susceptible to free-radical attack, resulting in cell death or injury. Free radical-induced damage is involved in various pathological phenomena (*1*), and much interest has focused on the development of effective antioxidants against lipid and DNA oxidation. Indeed, it has been shown that green tea extract or green tea tannin suppress lipid peroxidation in biological tissues and subcellular fractions (*2*−*5*) and the formation of 8-oxodeoxyguanosine in DNA (*6*). However, there is comparatively little information about their effect on protein oxidative damage.

Protein oxidative damage is directly involved in the pathogenesis of many diseases. Free radicals can induce protein modifications including losses of protein function, such as the activity of enzymes, receptors, and membrane transporters (*7*, *8*), in turn resulting in biological dysfunctions. Proteins in the body are also modified by glucose through the glycation reaction. This reaction finally produces advanced glycation endproducts (AGEs) which are characterized by fluorescence, a brown color, and intra- or intermolecular cross-linking. Recently, the accumulation of AGEs has been observed in age- and diabetes-associated diseases, such as diabetic complications and Alzheimer's disease (*9*−*11*). Oxidative reactions considerably participate in the process of the AGEs formation (*12*, *13*), indicating that biological proteins are susceptibly modified in vivo to AGEs under oxidative stress.

In this study, we examine the ability of green tea extract, and a green tea tannin mixture and its components, to protect against protein damage using two in vitro model systems.

MATERIALS AND METHODS

Green Tea. Fifty grams of dry green tea leaves, which were produced in the Haibara district (Shizuoka, Japan), were added to 1 L of hot distilled water (70 °C) and shaken for 5 min. The resulting supernatant was freeze-dried to obtain green tea extract with a yield of about 20%, by weight, of the original preparation. The green tea tannin mixture was prepared from a hot-water extract of green tea, and was composed mainly of (−)-epigallocatechin 3-*O*-gallate (EGCg, 18.0%), (−)-gallocatechin 3-*O*-gallate (GCg, 11.6%), (−)-epicatechin 3-*O*-gallate (ECg, 4.6%), (−)-epigallocatechin (EGC, 15.0%), (+)-gallocatechin (GC, 14.8%), (−)-epicatechin (EC, 7.0%), and (+)-catechin (C, 3.5%). For purification of these components, recycling high-performance liquid chromatography (HPLC) was done using a JAI-LC-908 high-performance liquid chromatograph (Japan Analytical Industry Co., Tokyo, Japan) equipped with JAI RI and JAI UV

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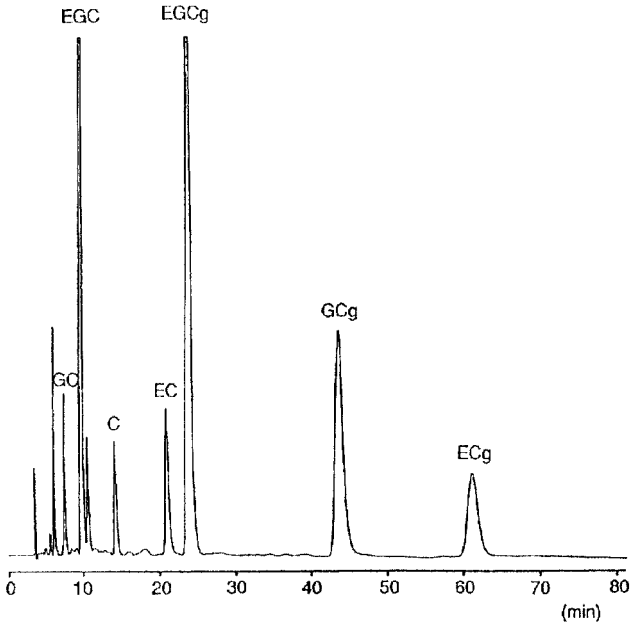

Figure 1. High-performance liquid chromatogram of green tea tannin mixture. GC, (+)-gallocatechin; EGC, (–)-epigallocatechin; C, (+)-catechin; EC, (–)-epicatechin; EGCg, (–)-epigallocatechin 3-*O*-gallate; GCg, (+)-gallocatechin 3-*O*-gallate; ECg, (–)-epicatechin 3-*O*-gallate.

detectors, operating at 280 nm, as described previously (*14*). A prepacked PVA HP-GPC column (JAIGEL GS-320, 50 × 2 cm i.d.) was used. Methanol was used as the eluting solvent at a flow rate of 3 mL/min. Each isolated component was identified by analysis using fast atom bombardment mass spectroscopy (FAB-MS) and HPLC. FAB-MS was recorded on a mass spectrometer (JMS-DX 303, JEOL, Tokyo, Japan) using glycerol as the matrix. A typical high-performance liquid chromatogram and the chemical structures of these constituents are illustrated in **Figures 1** and **2**. Caffeine was purchased from Sigma Chemical Co. (St. Louis, MO) and theanine was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2,2′-Azobis(2-amidinopropane) Dihydrochloride (AAPH)-Induced Protein Oxidation. According to the method of Courderot-Masuyer et al. (*15*), the reaction mixture containing 37.5 nM allophycocyanin, 3 mM AAPH, and aqueous solution of test sample in 75 mM phosphate buffer (pH 7.0) was incubated at 37 °C. The fluorescence obtained just before the addition of the radical generator AAPH was used as the 100% value for that sample. Loss of fluorescence was measured every 5 or 10 min at an emission wavelength of 651 nm and an excitation wavelength of 598 nm using a fluorescence spectrophotometer (model RF-5300PC, SHIMAZU, Kyoto, Japan).

Non-Enzymatic Glycation of Protein. According to the method of Vinson and Howard (*16*), bovine serum albumin (10 mg/mL) in 50 mM phosphate buffer (pH 7.4) with 0.02% sodium azide to prevent bacterial growth was added to glucose (25 mM) and fructose (25 mM) solution. This reaction mixture was mixed with an aqueous solution of test sample. Three concentrations were prepared for green tea extract, tannin, caffeine, and theanine. Six concentrations were prepared for tannin components. After incubating at 37 °C for 2 weeks, the fluorescent reaction products were assayed on a fluorescence spectrophotometer with an excitation wavelength of 350 nm and an emission wavelength of 450 nm. The data were expressed in terms of percentage inhibition, calculated from a control measurement of the fluorescence intensity of the reaction mixture with no test sample.

Statistics. Data are presented as mean ± SE of 5 determinations.

RESULTS

Figure 3 shows that the intrinsic allophycocyanin fluorescence was rapidly diminished by AAPH treatment to nearly 0% at 20 min, and green tea extract protected this quenching. Furthermore, this effect of green tea extract was shown to be dose-dependent. Of the components of green tea, the tannin mixture proved to be the most potent against AAPH-induced protein damage. Caffeine and theanine were found to lack such an effect, as shown in **Figure 4**. As shown in **Figure 5**, the addition of each tannin component at a concentration of 2.5 μM exerted a significant degree of protective activity. In particular, EGCg, GCg, and ECg retarded fluorescence diminishment much more than the other components, with more than 50% fluorescence intensity at 60 min. EC and C showed relatively low activity compared with that of EGCg, GCg, and ECg; whereas EGC and GC showed a considerable reduction of free radicals generated by AAPH, although their protective effects decreased with prolongation of the reaction time.

Figure 6 shows the effect of green tea extract and its components on AGEs formation after incubation for 2 weeks. It was found that green tea tannin showed significant inhibition of AGEs formation at a concentration of 1 μg/mL, and 25% inhibition at a concentration of 5 μg/mL. Neither caffeine nor theanine had inhibitory effects. Comparisons of tannin components at different concentrations showed that EGCg, GCg, and ECg had stronger activities than the other tannin components, with 12% inhibition even at a low concentration (0.25 μM). This inhibitory activity was dose-dependent. Among the gallate-free tannins, GC and C had higher activities than EGC and EC, as shown in **Table 1**.

DISCUSSION

Free radical exposure induced structural changes including amino acid modification, fragmentation, changes in absorption and fluorescence spectra (*8*), and functional changes in proteins (*7*). These changes act as indices of protein damage by free radicals. In this study, to examine the protective effect of green

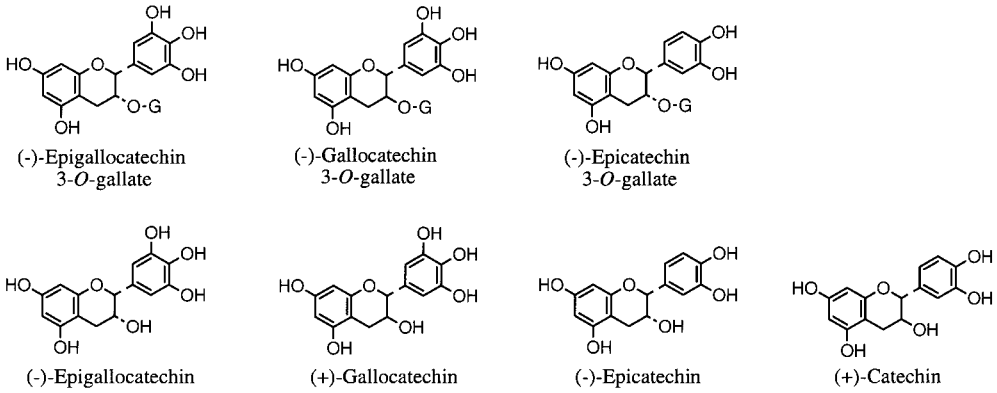

Figure 2. Chemical structures of green tea tannin.

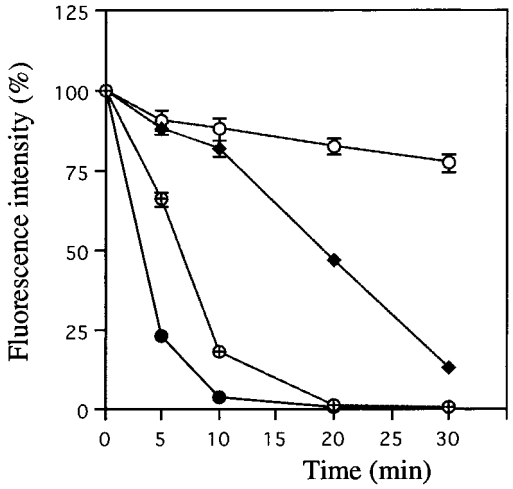

Figure 3. Time-response curve of green tea extract 0.2 μg/mL (⊕), 1 μg/mL (◆), 5 μg/mL (○), and non-additive control (●) on allophycocyanin quenching induced by AAPH.

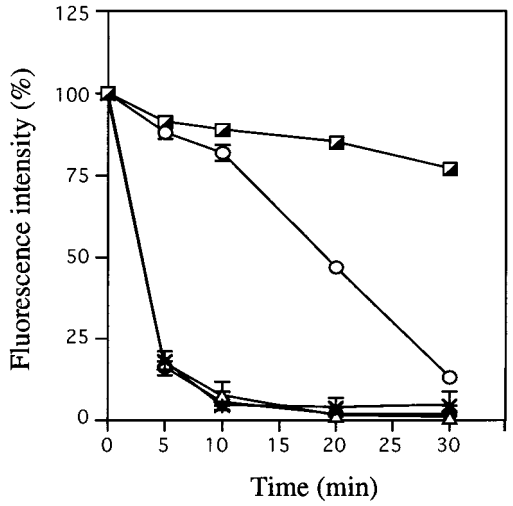

Figure 4. Time-response curve of green tea extract (○), tannin mixture (◪), caffeine (∗), and theanine (△) at 1 μg/mL and non-additive control (●) on allophycocyanin quenching induced by AAPH.

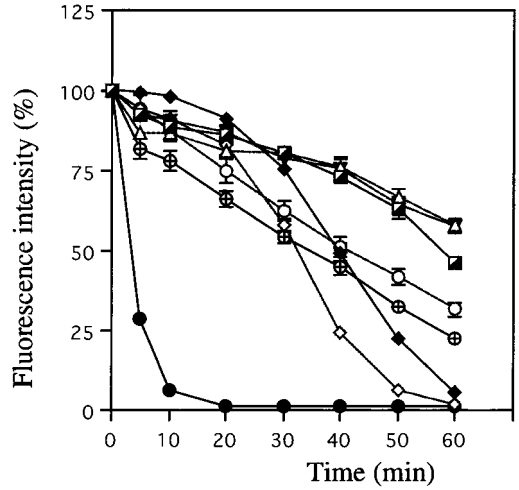

Figure 5. Time-response curve of EGCg (◪), GCg (△), ECg (▼), EGC (◆), GC (◇), EC (○), and C (⊕) at 2.5 μM and non-additive control (●) on allophycocyanin quenching induced by AAPH.

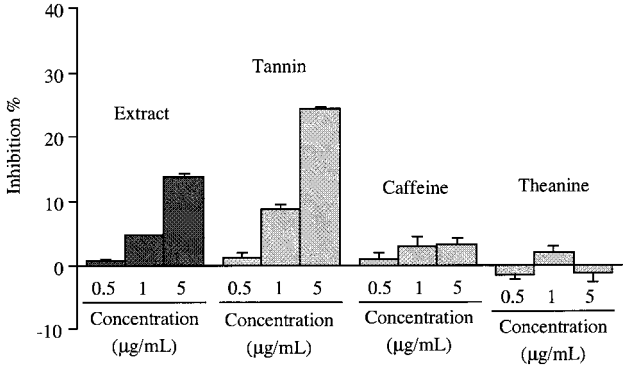

Figure 6. Effect of green tea extract and its components on AGEs formation.

tea against protein oxidation induced by AAPH, we measured the fluorescence intensity of allophycocyanin, a protein with natural fluorescence. AAPH has frequently been used as a free radical generator in studies associated with oxidative stress because it produces free radicals at a constant and measurable rate as a result of its temperature-dependent decomposition (*17*, *18*). Following treatment with AAPH, its intrinsic fluorescence was rapidly diminished, reflecting the oxidation of allophycocyanin. We found that green tea extract protected protein oxidative damage dose-dependently, and that EGCg, GCg, and ECg were the effective antioxidants among the various green tea tannin components, indicating that the gallate group at the 3 position plays a considerable antioxidative role. It is known that the free radicals generated from AAPH react with oxygen molecules rapidly to yield peroxyl radicals (*19*). Therefore, it can be assumed that the free radicals related to protein damage in this study were peroxyl radicals, and that scavenging peroxyl radicals play the important role of protective activity.

The reaction of amino groups of proteins with reducing sugars leads to the formation of Shiff bases and Amadori products. These early products further undergo various rearrangements to generate AGEs. It is now apparent that the protein glycation reaction occurs in biological tissues. Its contribution to some pathological conditions, including diabetic complications, aging, and Alzheimer's disease, has received considerable interest in recent years (*9*−*11*). AGEs have specific physicochemical properties including fluorescence, a brown color, and intra- or intermolecular cross-linking formation, and they have the biological characteristics of ligands recognized by cell membrane receptors expressed in vascular endotherial cells and macrophages. We measured AGEs formation using fluorescence, as proposed by Monnier and Cerami (*9*), to examine the inhibitory activity of green tea. In this study, green tea tannin showed stronger activity than green tea extract, whereas caffeine and theanine showed weak activity. There have been many recent indications that some radical species, including hydrogen peroxide, superoxide anion radicals, and singlet oxygen, participate in AGEs formation (*20*, *21*). In addition, it has been reported that antioxidant and radical scavengers inhibit these processes (*22*, *23*). Previously, we have reported that tannin-containing crude drugs can strongly inhibit the formation of AGEs among 21 galenicals (*24*). Green tea extract and tannin are known to be excellent antioxidants, with chelating activity, antioxidative activity, and radical scavenging activity (*2*−*5*, *25*−*30*). These activities are presumed to contribute to the inhibition mechanisms of the glycation reaction, although the mechanisms of AGEs formation are extremely complex and are affected by dehydration, cyclization, and oxidation. Our finding supports the theory that oxidative reactions have significant influence in AGEs formation and the possibility that antioxidants would be AGEs inhibitors, although this needs further study in vivo.

Table 1. Effect of Green Tea Tannin Components on AGEs Formation

material	concentration (μM)	inhibition (%)
(–)-epigallocatechin 3-*O*-gallate	0.25	12.4 ± 0.6
	0.5	15.3 ± 0.7
	1	20.0 ± 0.2
	2.5	27.5 ± 1.1
	5	32.7 ± 0.3
	10	46.5 ± 0.5
(–)-gallocatechin 3-*O*-gallate	0.25	12.5 ± 0.3
	0.5	16.7 ± 0.3
	1	21.3 ± 1.3
	2.5	33.9 ± 0.7
	5	42.2 ± 1.1
	10	48.2 ± 0.9
(–)-epicatechin 3-*O*-gallate	0.25	12.6 ± 0.5
	0.5	13.5 ± 1.1
	1	16.6 ± 1.6
	2.5	34.9 ± 0.5
	5	37.5 ± 0.6
	10	48.7 ± 0.2
(–)-epigallocatechin	0.25	2.2 ± 0.9
	0.5	5.5 ± 0.6
	1	9.0 ± 2.0
	2.5	9.3 ± 0.3
	5	14.8 ± 0.5
	10	19.6 ± 0.5
(+)-gallocatechin	0.25	0.2 ± 0.8
	0.5	7.6 ± 0.7
	1	12.4 ± 0.8
	2.5	28.1 ± 0.4
	5	40.2 ± 0.5
	10	39.9 ± 1.1
(–)-epicatechin	0.25	–4.9 ± 0.6
	0.5	3.4 ± 0.8
	1	2.7 ± 0.8
	2.5	4.4 ± 0.4
	5	18.6 ± 0.8
	10	23.0 ± 0.9
(+)-catechin	0.25	0.1 ± 0.1
	0.5	5.8 ± 1.4
	1	12.0 ± 0.8
	2.5	19.3 ± 0.4
	5	28.3 ± 0.5
	10	37.1 ± 1.5

Green tea contains relatively large amounts of tannin, almost all of the flavan-3-ol class of flavonoids, which are low-molecular-weight polyphenols with or without a gallate group, such as EGCg, ECg, EGC, and EC. This is a remarkable characteristic compared with other plants. Therefore, green tea is believed to be an excellent source of natural antioxidants. Our research has focused on the effect of green tea on renal damage, because renal failure is considered to be a condition of oxidative stress (*30*−*32*). We have demonstrated that green tea tannin decreases lipid peroxide and increases radical scavenging enzyme activities in the kidneys, which are closely related to renal failure. It is expected that the protective effect of green tea tannin on protein damage, shown in this study, may contribute to its beneficial effects against renal failure. The structure−activity relationship of the antioxidative activity of green tea polyphenols has also been studied (*33*−*35*). In this study, the seven kinds of tannin components isolated from green tea extract have been examined, as has the relationship between the chemical structures of tannin components and their activities. In both evaluation systems, we have observed that EGCg, GCg, and ECg effectively protect against protein damage, when compared with EGC, GC, EC, and C, indicating that the presence of galloyl groups plays an important role in protective activity. In addition, EGC and GC, which are isomers, had parallel diminishing curves of protein oxidative damage, with EC and C showing similar activities. Thus, the presence of the hydroxyl group at the 5′ position in the B ring affects peroxyl radical scavenging activity. In both cases, an α-hydroxyl group at C-3 is much more effective than a β-hydroxyl group at C-3. GC and C with a β-hydroxyl group at C-3 have a stronger activity against AGEs formation than do EGC and EC with an α-hydroxyl group at C-3. The differences between their sterical structures play a more important role than the presence of the hydroxyl group at the 5′ position in the B ring in the case of AGEs formation. The varying effects of individual compounds on oxidation and glycation in our study were closely related to their chemical structures.

In conclusion, our investigation has shown that green tea can effectively protect against AAPH- and glucose-mediated protein modifications in model systems in vitro, and that tannin plays an important role in this action. In addition, there is a clear correlation between chemical structure and activity, although these mechanisms have not yet been fully determined.

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