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Stability of Tea Polyphenol (−)-Epigallocatechin-3-gallate and Formation of Dimers and Epimers under Common Experimental Conditions

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(−)-Epigallocatechin-3-gallate (EGCG), the most abundant and biologically active compound in tea, has been extensively studied for its activities related to disease prevention in animal models and in vitro. However, its stability under different experimental conditions has not been well-characterized. In the present study, the stability of EGCG in animal drinking fluid and under cell culture conditions and the factors that affect its stability under these conditions were investigated. Our results demonstrated that auto-oxidation and epimerization are the two major reactions causing the instability of EGCG. The structures of the major oxidation products, EGCG dimers, were identified. The rates of these reactions were affected by the temperature, pH, the partial pressure of oxygen, the level of antioxidants, the concentration of EGCG, and other components of tea. In future studies with EGCG, its stability should be considered in order to avoid possible artifacts.

KEYWORDS: Stability; EGCG; dimers; GCG; auto-oxidation; epimerization; cell culture; tea

INTRODUCTION

Tea, made from the leaves of *Camellia sinensis*, is one of the most widely consumed beverages in the world. The three major types of tea, green, oolong, and black, differ in their manufacturing process and chemical compositions. Many studies in humans, animal models, and cell lines suggest potential health benefits from the consumption of tea, including the prevention of cancer and heart disease (*1*−*3*). Many of the health benefits have been attributed to the polyphenolic components in tea. (−)-Epigallocatechin-3-gallate (EGCG) is the most abundant and biologically active constituent in tea (**Figure 1**). Green and oolong teas typically contain 30−130 mg of EGCG per cup (237 mL), whereas black tea may contain up to 70 mg of EGCG per cup (*4*).

Green tea, black tea, and EGCG, when administered to animals in drinking fluid, have shown cancer preventive activity against ultraviolet light-induced, chemically induced, and genetic models of carcinogenesis (*1*−*3*). For example, we demonstrated that orally administered green tea inhibited chemically induced lung tumorigenesis in mice, esophageal carcinogenesis in rats, and oral carcinogenesis in hamsters (*5*−*7*). Chung et al. (*8*) showed that 2% green tea inhibited 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumor multiplicity by ∼45%. The corresponding amount of EGCG inhibited lung tumor multiplicity by 28%. Intestinal tumorigenesis in the *Apc*^{Min} mice was shown by Orner et al. (*9*) to be inhibited by orally

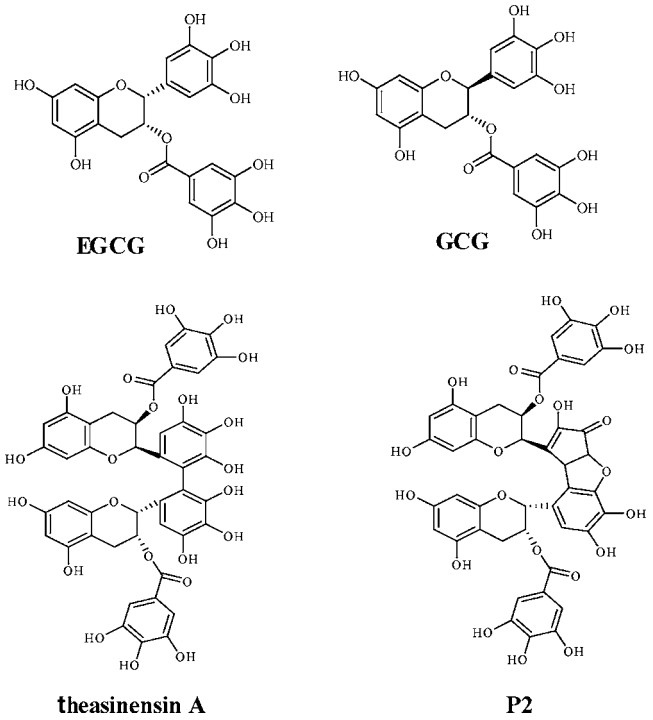

Figure 1. Structure of EGCG and its epimerization and oxidation products.

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administered green tea, and recently, such an activity was also demonstrated in our laboratory with EGCG administered in drinking fluid (*10*). The stability of EGCG and other tea polyphenols in the drinking fluid under some of these experimental conditions was not well-characterized.

The mechanisms of the chemopreventive activity have been studied extensively; however, they are not clearly understood. Most of the mechanisms have been proposed based on the studies in vitro. In cell lines, tea polyphenols, especially EGCG, have been shown to cause growth inhibition and apoptosis in many human tumor cell lines (*11*−*14*). Some of these activities are probably due to the inhibition of specific protein kinases leading to cell cycle arrest or blocking of the activation of transcription factors, such as activator protein-1 and nuclear factor-κB (*15*−*20*). The mechanisms of the action of EGCG in cell culture systems, however, are complicated by the fact that it is not stable under most cell culture conditions. We have shown previously that EGCG was unstable in several cell culture media, such as McCoy's 5A, HBSS, and Ham's H-12 (*21*). In McCoy's 5A culture media, the half-life of EGCG was less than 30 min, and the half-life increased to 130 min in the presence of HT-29 human colon adenocarcinoma cells (*21*).

To fully understand the biological effects of EGCG and other tea polyphenols, a careful study on their stability under common experimental conditions is of importance. This information, however, is lacking, even though the stability of tea catechins in tea drinks under either direct brewing or industrial canning processes as well as in bread production and storage after adding tea catechins to bread has been studied (*22*−*27*). In the present study, the stability of EGCG in drinking fluids used in most animal experiments and under cell culture conditions as well as the factors that affect its stability were investigated.

MATERIALS AND METHODS

Materials. Decaffeinated green tea extracts (GTE) (dehydrated water extract of green tea; 1 g of powder derived from 6 g of dry leaves) were obtained from Unilever Best Foods Inc. (Englewood Cliffs, NJ). EGCG (100% pure) was provided by Mitsui Norin Co. Ltd. (Shizuoka, Japan). Theasinensin A was synthesized previously in our laboratory (*28*). The structure was confirmed by ^{1}H, ^{13}C, and two-dimensional (2D) NMR. Superoxide dismutase (SOD), ascorbic acid, CD_3OD, RP C-18 silica gel, and Sephadex LH-20 gel were purchased from Sigma (St. Louis, MO). Thin-layer chromatography (TLC) was performed on 250 μm thickness, 2−25 μm particle size TLC plates (Sigma-Aldrich), with compounds visualized by spraying with 5% (v/v) H_2SO_4 in ethanol solution. High-performance liquid chromatography (HPLC) grade solvents and other agents were obtained from VWR Scientific (South Plainfield, NJ). HPLC grade water (Nanopure water (18 MΩ) was prepared using a Millipore Milli-Q purification system (Bedford, MA). RPMI 1640 and Ham's F12 medium were purchased from Mediatech Inc. (Herndon, VA).

HPLC Analysis. EGCG levels were analyzed using an HPLC system, consisting of a Waters 717 refrigerated autosampler, a Hitachi L-6200A intelligent pump, and an ESA 5600 coulochem electrode array system (CEAS). The potentials of the CEAS were set at −100, 200, 400, and 600 mV. Separation was achieved using previously described methods (*29*). In brief, a 150 mm × 4.6 mm i.d. Supelcosil C18 reversed-phase column (Supelco Co., Bellefonte, PA) was used. The column and CEAS detector were housed in a temperature-regulated compartment maintained at 25 °C. The autosampler was maintained at 6 °C. For binary gradient elution, mobile phases A (1.75% acetonitrile and 0.12% tetrahydrofuran in 30 mM NaH_2PO_4, pH 3.35) and B (58.5% acetonitrile and 12.5% tetrahydrofuran in 15 mM NaH_2PO_4, pH 3.45) were used. The flow rate was maintained at 1 mL/min, and the mobile phase began with a 5 min isocratic phase of 96% A and 4% B. It was followed by progressive, linear increases in B to 15% at 15 min, 22% at 25 min, 80% at 32 min, and 100% at 42 min. The mobile phase was maintained at 100% B for 10 min and then was reequilibrated to 4% at 53 min for another run.

Liquid Chromatography/Mass Spectrometry (LC/MS) and NMR Analyses. LC/MS analysis was carried out with a Finnigan Spectra System, which consisted of a Finnigan model P4000 pump, a model AS3000 refrigerated autosampler, and a Finnigan LCQ Deca mass detector (ThermoFinigan, San Jose, CA) incorporated with an electrospray ionization (ESI) interface. A 75 mm × 2.1 mm i.d., 3 μm Discovery HS C_{18} column (Supelco) was used for separation with a flow rate of 0.2 mL/min. The column elution started with a 6 min isocratic phase of 100% solvent A (5% aqueous methanol with 0.2% acetic acid), followed by progressive, linear increases in B (95% aqueous methanol with 0.2% acetic acid) to 20% at 15 min, 50% at 25 min, 55% at 30 min, and 100% at 36 min. The mobile phase was maintained at 100% B for 8 min and then was reequilibrated to 100% A at 45 min. The LC elute was introduced into the ESI interface. The negative ion polarity mode was set for ESI ion source with the voltage on the ESI interface maintained at approximately −4 kV. Nitrogen gas was used as the sheath gas at a flow rate of 80 arb, and the auxiliary gas was set at 2 arb, respectively. The heated capillary temperature and voltage were maintained at 260 °C and −24 V, respectively. The tube lens offset voltage was −55 V. The structural information of the standard (−)-gallocatechin-3-gallate (GCG) and theasinensin A were obtained by tandem mass spectrometry (MS/MS) through collision-induced dissociation with a relative collision energy setting of 25%. ^{1}H (600 MHz), ^{13}C (150 MHz), and all 2D NMR spectra were acquired on a Varian Unity INOVA 600 NMR spectrometer (Palo Alto, CA) equipped with a *z*-gradient inverse-detection triple resonance probe.

Isolation of GCG as a Product of EGCG in Phosphate Buffer. EGCG (0.2 g) was incubated in 100 mL of phosphate buffer (50 mM Na_2HPO_4, pH 7.4) at 37 °C for 2 days. After extraction with ethyl acetate and evaporating the solvent in vacuo, the residue was applied to a Sephadex LH-20 column and eluted with 35% aqueous acetone to obtain GCG (30 mg) and EGCG (80 mg). GCG: ^{1}H NMR (CD_3OD, 600 MHz): δ 5.05 (1H, brd, J = 4.8 Hz, H-2), 5.37 (1H, m, H-3), 2.76 (1H, dd, J = 4.8, 16.4 Hz, H-4a), 2.71 (1H, dd, J = 5.4, 16.4 Hz, H-4b), 5.96 (2H, s, H-6 and H-8), 6.40 (2H, s, H-2′ and H-6′), 6.97 (2H, s, H-2″ and H-6″). ^{13}C NMR (CD_3OD, 150 MHz): δ 79.2 (d, C-2), 71.0 (d, C-3), 23.7 (t, C-4), 157.6 (s, C-5), 95.6 (d, C-6), 158.0 (s, C-7), 96.4 (d, C-8), 156.3 (s, C-9), 99.5 (s, C-10), 131.0 (s, C-1′), 106.3 (d, C-2′ and C-6′), 146.9 (s, C-3′ and C-5′), 133.9 (s, C-4′), 121.4 (s, C-1″), 110.2 (d, C-2″ and C-6″), 146.3 (s, C-3″ and C-5″), 139.8 (s, C-4″), 167.7 (s, C-7″).

Stability of EGCG or GTE under Different Conditions. Different concentrations of EGCG (or GTE) were prepared and incubated under different conditions {in Nanopure water at room temperature or 37 °C or in 50 mM phosphate buffer (pH 7.4) in the absence or presence of nitrogen (the buffer solution was flushed with nitrogen for 24 h in a 37 °C water bath before adding EGCG) or EDTA or in cell culture medium [Ham's and RPMI-1640 (50:50), pH 7.4] in the absence or presence of SOD (5 units/mL) or as powder for long-term storage}. Then at different time points, 20 or 100 μL samples were taken and transferred to vials containing 380 μL or 9.9 mL of a solution containing 0.2% ascorbic acid and 0.05% EDTA to stabilize EGCG. These samples were immediately analyzed or stored at −80 °C before analyzing by HPLC. The quantification of EGCG and theasinensin A was based on the standard curve of each compound.

RESULTS AND DISCUSSION

Stability of EGCG and Other Tea Polyphenols in Drinking Fluid. To study the stability of EGCG and other tea polyphenols, we used three different concentrations of EGCG (0.025, 0.1, and 0.32%) and GTE (0.05, 0.2, and 0.6%), which covered the range of concentrations used in most animal studies. EGCG was rather stable in the 0.32% (6.99 mM) solution; no significant change in EGCG concentration was observed in the 7 day experiment (**Figure 2**). The 0.1 (2.06 mM) and 0.025% (0.51 mM) solutions were less stable, showing a gradual decrease of EGCG concentration in the first 2 or 3 days and a

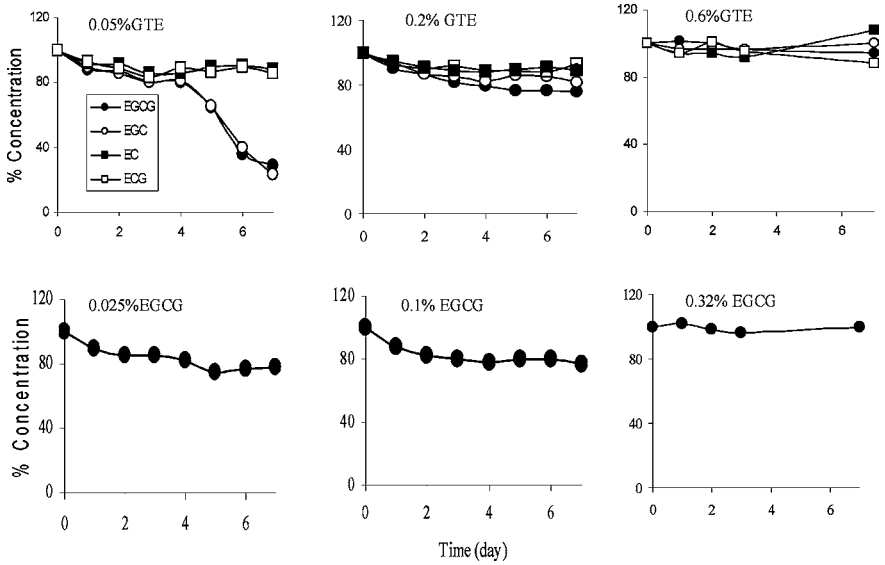

Figure 2. Stability of EGCG and GTE at room temperature. Three different concentrations of EGCG (0.025, 0.1, and 0.32%) and GTE (0.05, 0.2, and 0.6%) were prepared in Nanopure water and kept in animal feeding bottles at room temperature.

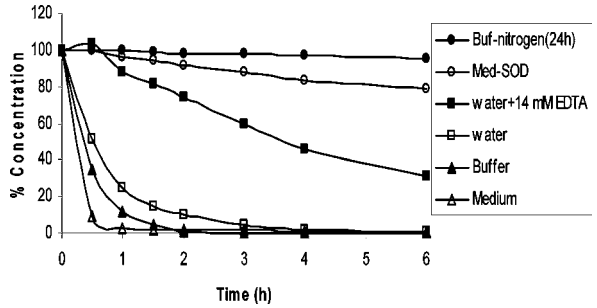

Figure 3. Factors affecting the stability of 20 μM EGCG. Stability of 20 μM EGCG after addition to 50 mM Na_2HPO_4 (pH 7.4) buffer flushed with nitrogen for 24 h at 37 °C (●). Stability of 20 μM EGCG in HAM's F12:RPMI 1640 (1:1) medium (pH 7.4) at 37 °C in the presence of SOD (5 U/mL) (○). Stability of 20 μM EGCG in water at 23 °C in the presence of 14 mM Na_2EDTA (■). Stability of 20 μM EGCG in water at 23 °C in the absence of 14 mM Na_2EDTA (□). Stability of 20 μM EGCG in 50 mM Na_2HPO_4 (pH 7.4) buffer without saturated nitrogen at 37 °C (▲). Stability of 20 μM EGCG in HAM's F12:RPMI 1640 (1:1) medium (pH 7.4) at 37 °C in the absence of SOD (△).

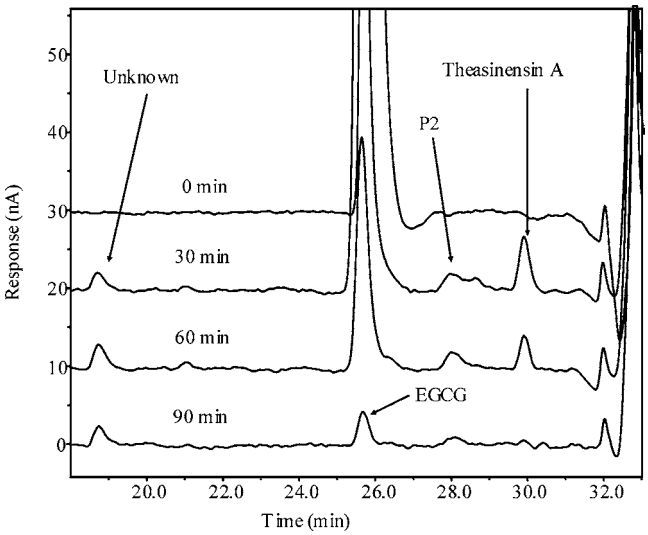

Figure 4. Time-dependent conversion of EGCG to dimers. HPLC spectra of EGCG (20 μM) samples after they were incubated in Ham's F12 and RPMI-1640 (50:50) culture medium (pH 7.4) at 37 °C in the absence of nitrogen at different time points (0, 30, 60, and 90 min).

maximal decrease of about 20% during the 7 day studies. In 0.6% GTE solution [containing 1.57 mM EGCG, 1.33 mM (−)-epigallocatechin (EGC), 0.77 mM (−)-epicatechin-3-gallate (ECG), and 0.56 mM (−)-epicatechin (EC)], EGCG was rather stable with no change or a few percent decrease in concentration in 7 days, and other catechins were also rather stable. In 0.2 and 0.05% GTE solutions, up to a 20% decrease of EGCG concentration was observed after 3 or 4 days. In general, EGC had a similar stability to EGCG at all three concentrations of GTE, whereas ECG and EC were more stable (**Figure 2**). It is interesting to note that in 0.05% GTE, the concentration of EGCG and EGC dropped rapidly during days 4−7, and the reason for this phenomenon is unknown.

Stability of EGCG in Cell Culture Medium. EGCG was unstable in cell culture medium. When added to cell culture medium [Ham's F12 and RPMI-1640 (50:50), which was the medium that we used to culture KYSE 150 and 510 human esophageal squamous cell carcinoma cells], the EGCG concentration decreased rapidly, with a half-life of about 30 min (**Figure 3**). After 6 h of incubation, EGCG was not detectable in the medium. During the incubation, three major new peaks appeared in the HPLC spectra (**Figure 4**). Two of them were confirmed by LC/MS/MS to be the dimers of EGCG. **Figure 5** shows the respective LC/MS/MS chromatograms and fragment ion mass spectra of the standard theasinensin A (EGCG dimer) and the corresponding dimer peak formed in cell culture medium from 20 μM EGCG at 30 min. Theasinensin A and the major dimer peak found in the cell culture medium exhibited the same chromatographic retention times and molecular masses and also had the same fragment ion mass spectra (**Figure 5**), confirming the formation of theasinensin A. **Figure 6** shows the LC/MS/MS chromatograms and fragment ion mass spectra of the other major dimer peak formed at 30 min. This dimer peak showed a molecular ion mass of 883 (ESI negative, $[M - H]^-$), which is the same as that of P2, one of the major auto-oxidation products of EGCG in mild alkaline fluids (*30*). The major mass fragment ions of 883 were the ions that lost fragments corresponding to one gallic acid group (*m*/*z* 713, $[M - H - 170]^-$) and two gallic acid groups (*m*/*z* 543, $[M - H - 170 \times 2]^-$) (**Figure 6**), respectively. On the basis of the

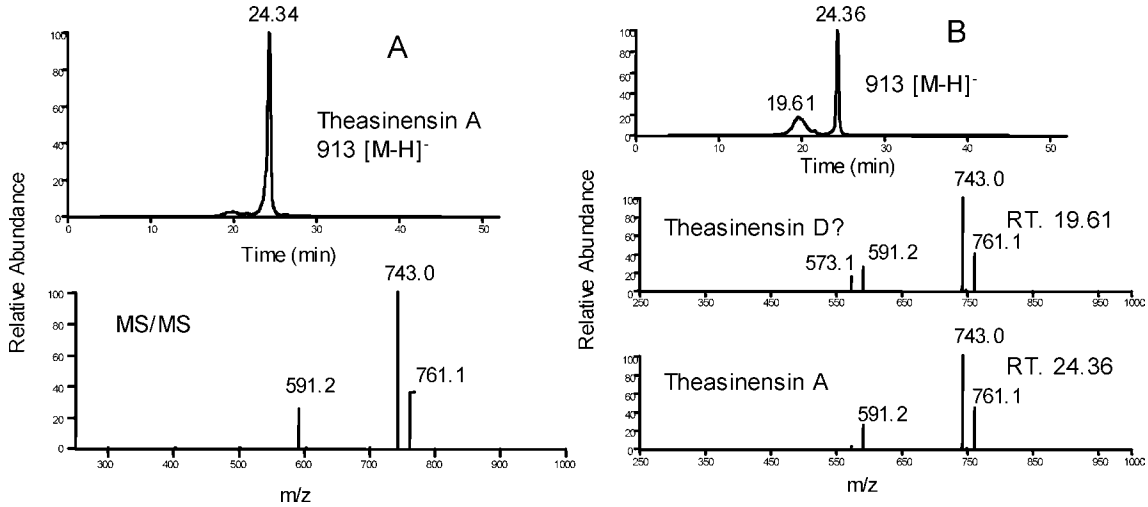

Figure 5. Identification of theasinensin A by LC/MS/MS. ESI negative LC/MS/MS spectra of standard theasinensin A and related peaks in Ham's F12 and RPMI-1640 (50:50) culture medium (20 μM EGCG at 30 min). (**A**) LC and MS/MS spectra of standard theasinensin A (MW 914). (**B**) LC and MS/MS spectra of the peak found in buffer solution after EGCG was added at 30 min.

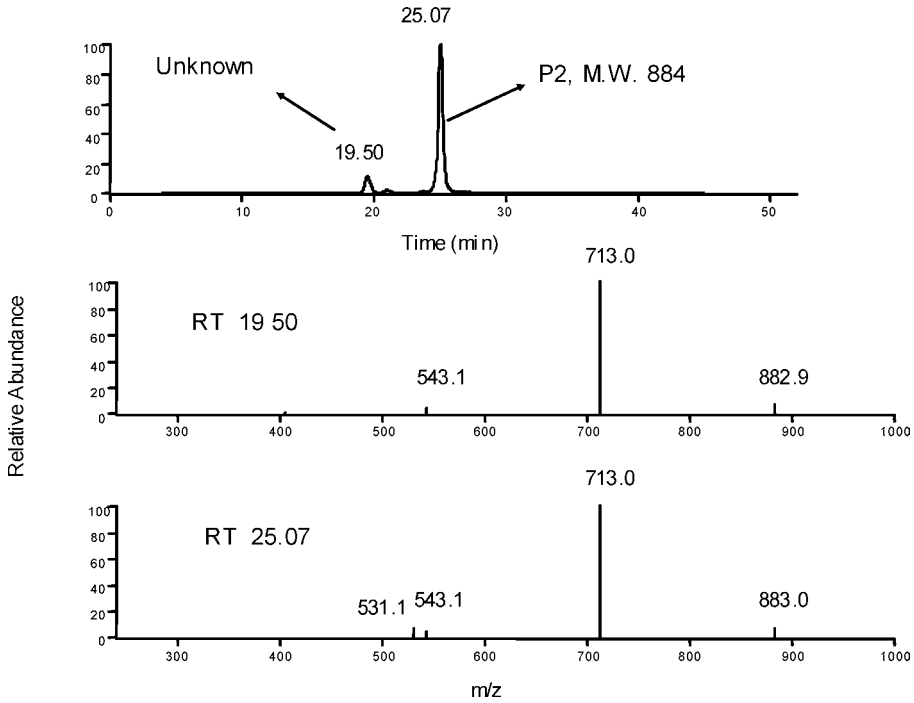

Figure 6. Identification of P2 by LC/MS/MS. ESI negative LC/MS/MS spectra of the major EGCG dimer peak corresponding to MW 884 in Ham's F12 and RPMI-1640 (50:50) culture medium (20 μM EGCG at 30 min).

molecular ion mass and the major mass fragments of this peak, we tentatively identified this peak as P2. The presence of minor peaks (one in each of **Figures 5** and **6**), which had the same molecular ion and mass fragments as that of theasinensin A or P2, but with different retention times, suggests that they are epimers of theasinensin A or P2. All of the dimers were unstable in the cell culture medium. Their peak height reached a maximum at 30−60 min and then decreased to an undetectable level at 6 h.

Factors Affecting the Stability of EGCG. As shown in **Figure 3**, the addition of SOD dramatically increased the stability of EGCG in cell culture medium. The effect of SOD suggests that the instability of EGCG is due to superoxide-mediated auto-oxidation of EGCG. The dimer peaks (**Figure 4**) were not detectable in the presence of SOD; instead, a new peak appeared and increased with time. This peak was identified as GCG (an epimer of EGCG) by using selected ion monitoring LC/MS/MS (**Figure 7**). Corresponding to the ESI negative molecular ion 457 $[M - H]^-$, two peaks showed up in the LC spectrum of EGCG samples in the presence of SOD at 24 h. The major peak was identified as EGCG by comparison with the standard EGCG. The other peak showed almost the same retention time and molecular masses and also had the same fragment ion mass spectra as those of standard GCG. With the inhibition of auto-oxidation by SOD, epimerization of EGCG apparently became the predominant reaction.

We found that the pH of Ham's F12 and RPMI-1640 (50:50) culture medium changed from 7.40 to 8.34 during the incubation with EGCG for 6 h. To maintain a constant pH, we used a phosphate buffer (50 mM Na_2HPO_4, pH 7.4). EGCG was slightly more stable in this phosphate buffer than in cell culture medium (**Figure 3**). The major oxidation products were the same as those formed in cell culture medium. To study the effect of O_2 on the stability of EGCG, the phosphate buffer (50

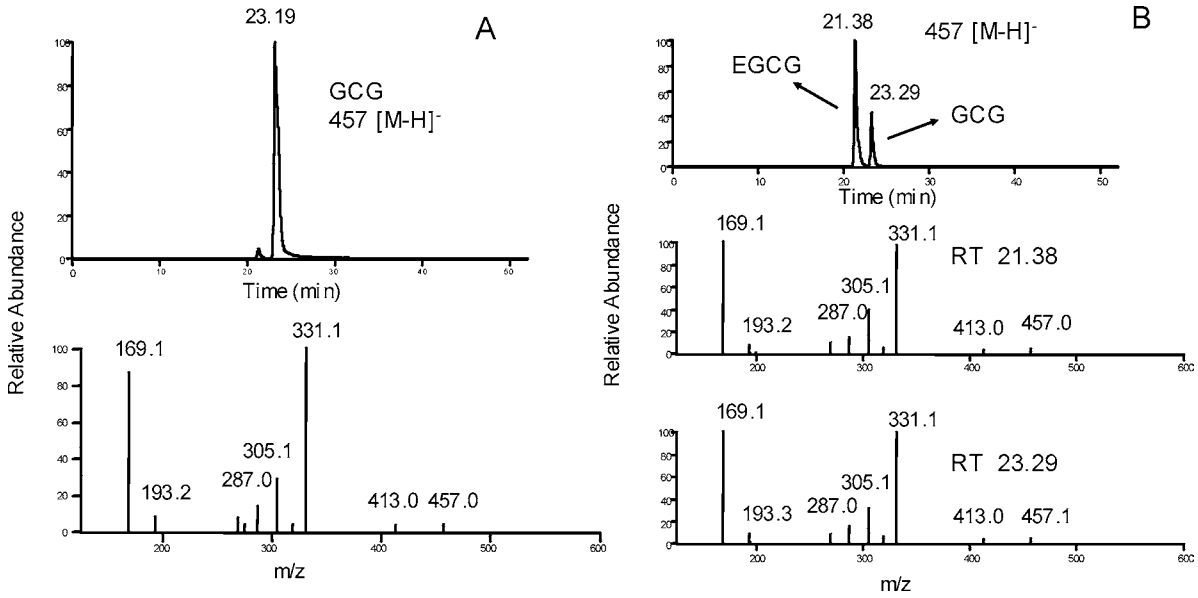

Figure 7. Identification of GCG by LC/MS/MS. ESI negative LC/MS/MS spectra of standard GCG and related peak in cell culture medium (20 μM EGCG, with SOD at 24 h). (**A**) LC and MS/MS spectra of standard GCG (MW 458). (**B**) LC and MS/MS spectra of the peak found in cell culture medium (20 μM EGCG with SOD at 24 h).

mM Na_2HPO4, pH 7.4) was flushed extensively with N_2 gas. Under low O_2 partial pressure, EGCG was greatly stabilized with 95% remaining after 6 h. No dimers were detected, and GCG was formed (**Figure 3**). An aqueous solution of EGCG (20 μM) showing a pH of 5.4 was not very stable at room temperature (23 °C). The stability was significantly increased by the presence of EDTA (14 mM), suggesting the involvement of metal in catalyzing the auto-oxidation of EGCG (**Figure 3**).

Concentration-Dependent Stability of Low Concentration of EGCG and Theasinensin A. The stability of EGCG in water at 37 °C was concentration-dependent, and the $t_{1/2}$ increased from 30 min in a 20 μM solution to 150 min in a 96 μM solution (**Figure 8**). The EGCG in GTE was even more stable than the pure 96 μM EGCG solution, even though the GTE solution contained only 20 μM EGCG (48 μM in terms of total catechins). The formation of theasinensin A was also concentration-dependent. The higher amounts of theasinensin A formed from higher concentrations of EGCG also apparently had longer half-lives (**Figure 8**).

EGCG was unstable in cell culture medium or in pH 7.4 buffer at low concentrations (20−100 μM), and EGCG dimers were the major products under these conditions (**Figures 3** and **4**). Incubating a higher concentration EGCG (0.2%, 4.12 mM) in phosphate buffer (pH 7.4), however, produced GCG but not the dimers. Corresponding to the decrease of EGCG, the amount of GCG increased, reaching a maximum at day 2 (at 37 °C) or day 5 (at 23 °C). At day 7 (at 37 °C) or day 10 (at 23 °C), both EGCG and GCG were not detectable. During the incubation, the color of the solutions became brown, with increasing absorbance in the 400−800 nm range during days 4−7 (at 37 °C) or 4−10 (at 23 °C). Apparently, larger molecular weight compounds were formed. These colored compounds appeared to be similar to the pinkish-brown material formed upon storage of EGCG. The white-colored pure EGCG usually turns to pinkish-brown upon long-term storage. The colored materials had an absorbance between 400 and 800 nm, but HPLC and ^{1}H NMR analysis did not reveal any new compounds besides the predominant EGCG and a trace amount GCG.

The purpose of this study was to determine the stability of EGCG under common experimental conditions, the products

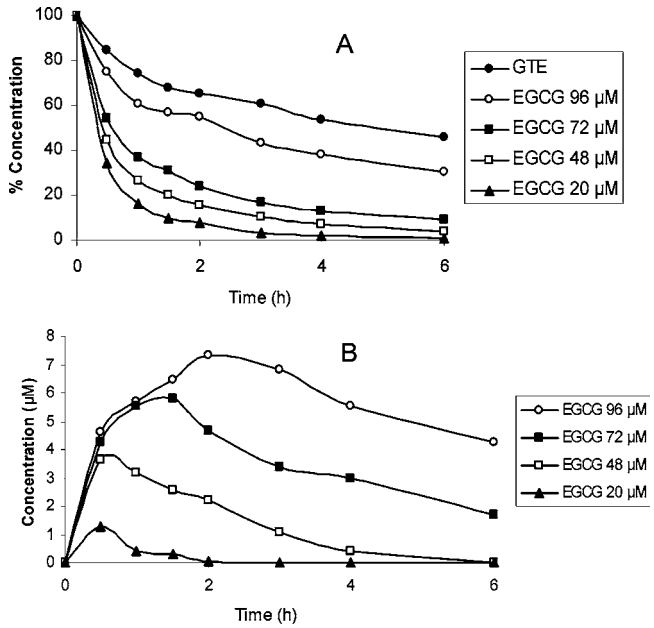

Figure 8. Concentration-dependent stability of a lower concentration of EGCG and theasinensin A. Stability of EGCG (**A**) and formation of theasinensin A (**B**) at different concentrations of EGCG (20, 48, 72, and 96 μM) and in GTEs (GTE contains 20 μM EGCG and around 48 μM total tea catechins) in water at 37 °C.

formed, and the factors that affect the stability. Our results (**Figure 2**) indicated that in animal studies, the catechins were rather stable at higher concentrations (0.32% EGCG or 0.6% GTE). At lower concentrations (0.1 and 0.025% EGCG or 0.2 and 0.05% GTE), up to a 20% decrease in EGCG concentration could occur in 2−3 days. In general, the stability of EGC was about the same as EGCG, whereas ECG and EC were more stable, possibly due to the oxidation of the trihydroxy structure on the B-ring of EGC and EGCG. We also observed that EGCG and other tea polyphenols were less stable if the solution was made in tap water, possibly due to the presence of metal ions.

Our previous study in cell culture systems indicated that EGCG underwent similar oxidation in the presence or absence

of cells, and cells could only slightly increase the stability of EGCG (*21*). To simplify the experimental system, we conducted the present study in the absence of cells. Under these conditions (pH 7.4, 37 °C), EGCG (20 μM) underwent auto-oxidation, with a half-life of less than 30 min; EGCG dimers and other products were formed. Several factors, including pH, temperature, oxygen levels, antioxidant levels, metal ions, concentration of EGCG, and other ingredients in tea, could affect the stability of EGCG (**Figures 3** and **8**). The presence of SOD or nitrogen stabilized EGCG and increased its half-life to longer than 6 h. On the basis of these observations, metal-catalyzed auto-oxidation of EGCG is possible.

We observed that once the auto-oxidation of EGCG was prevented by SOD or nitrogen, epimerization of EGCG to GCG became appreciable. GCG was also the major product formed from EGCG at high concentrations [0.2% (4.12 mM) solution or higher concentration], whereas EGCG dimers were formed mainly with low concentrations of EGCG (such as 20−100 μM). In aged EGCG powder, GCG was also found. All of these results suggest that there are two major reactions involved in the instability of EGCG; one is auto-oxidation, and the other is epimerization. The rates of these two reactions are affected by the level of oxygen, the concentration of EGCG, and the level of antioxidants.

As a pro-oxidant, EGCG can be oxidized to form phenolic radicals, superoxide radical, and hydrogen peroxide. These species may trigger a variety of biochemical reactions and biological responses. For example, hydrogen peroxide may contribute to cell apoptosis (*11*, *12*), and the radical species may contribute to the inactivation of epidermal growth factor receptor (EGFR) and telomerase as reported in the literature (*31*−*34*). The presently observed auto-oxidation and epimerization of EGCG, however, may not occur in animal tissue due to the higher antioxidative capacity (SOD, glutathione peroxidase, glutathione, and ascorbic acid) and lower oxygen partial pressure in the cells. The oxygen partial pressure in a cell culture system (160 mmHg) is much higher than that in the blood or tissues (<40 mmHg) (*35*). To avoid artifacts from auto-oxidation of EGCG, SOD may be added to the cell culture system. Recently, we observed that the inhibition of EGFR phosphorylation caused by preincubation of the cell with EGCG could be prevented by the presence of SOD, suggesting that the inhibition of the EGFR signaling pathway is caused by the auto-oxidation of EGCG (*36*). Other antioxidants, especially phenolic phytochemicals, may undergo similar auto-oxidation. For instance, curcumin has been reported to be unstable in phosphate buffer (pH 7.2) and cell culture medium (pH 7.2) at 37 °C, with about 90% decomposed within 30 min (*37*). In future studies of antioxidants, their stability should be considered in order to gain a better understanding of the mechanism of their actions.

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