Stability of Green Tea Catechins

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Green tea catechins (GTCs), which include (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC) and (−)-epigallocatechin gallate (EGCG), possess a variety of biological activities. We have previously studied the effect of dietary GTCs as a mixture on membrane oxidation of red blood cells and found that GTCs were partially absorbed and detected in the blood of rats given an oral ingestion of 100 mg of GTCs. To explain the partial absorption of GTCs and their varying free-radical scavenging capacity at different pH, the present paper was to study further the pH stability of these GTC isomers because there is a sharp increase in pH from the acidic stomach to the slightly alkaline intestine. Longjing GTCs as a mixture in alkaline solutions (pH > 8) were extremely unstable and degraded almost completely in a few minutes, whereas in acidic solutions (pH < 4) they were very stable. For the pH between 4 and 8, the stability of GTCs was pH-dependent, i.e., the lower the pH, the greater the stability. Four epicatechin isomers examined demonstrated varying stability in alkaline solutions with EGCG and EGC being equally instable, and EC and ECG being relatively stable. The present results suggest that part of the mechanism by which GTCs were partially absorbed may be attributed to instability of EGCG and EGC in the intestine where the pH is neutral or alkaline.

Keywords: *Epicatechin; epicatechin gallate; epigallocatechin gallate; epigallocatechin; longjing tea*

INTRODUCTION

Tea is believed to have a wide range of pharmaceutical properties including being antihypertensive (Henry and Stephens-Larson, 1984), antioxidative (Ding et al., 1992; Miura et al., 1994), antiarteriosclerotic (Hertog et al., 1993), anticarcinogenic (Shi et al., 1994; Wang et al., 1994), and hypocholesterolemic (Imai and Nakachi, 1985; Kono et al., 1992). These diverse biological activities are thought to be attributed to a group of polyphenol compounds, namely green tea catechins (GTCs), present in tea leaves. GTCs are a mixture of epicatechin isomers including mainly (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG) and (−)-epigallocatechin gallate (EGCG) (Figure 1). The content of GTCs varies among green tea, black tea, and oolong tea. Green tea refers to a nonfermented product in which GTCs are mostly preserved while black tea is a fermented product in which GTCs are extensively oxidized during manufacturing process. Oolong tea is a partially fermented product in which GTCs are partially degraded.

Green tea is an excellent source of polyphenol antioxidants. We have previously shown that jasmine green tea contains 7.4 g of GTCs/100 g of dry tea leaves and EGCG is the major isomer followed by ECG, EGC, and EC (Chen and Chan, 1996; Chen et al., 1996). We have also examined the antioxidative activity of GTCs as a mixture, or separately, and found that GTCs and individual epicatechin isomers exhibited a stronger inhibition on lipid oxidation in canola oil than in butylated hydroxytoluene (Chen and Chan, 1996). Furthermore, we demonstrated that these epicatechin isomers purified from green tea were effective agents to protect human low-density lipoprotein and red blood cell membrane from oxidative modification (Zhang et al., 1997a,b).

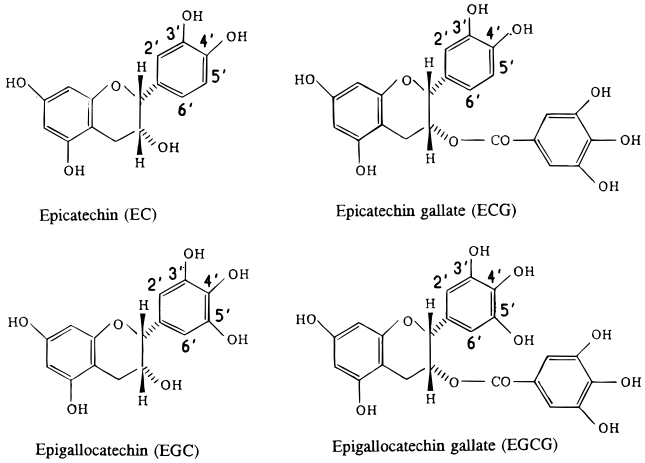

Figure 1. Chemical structures of (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate.

Several studies have suggested that tea catechins are only partially absorbed in both rats (Matsumoto et al., 1991; Okushio et al., 1996) and humans (Unno et al., 1996). Our data showed that the maximum concentration of GTCs in plasma could reach 9−10 μg/mL after an oral administration of 100 mg of GTCs as a mixture in rats (Zhang et al., 1997b). The study by Matsumoto et al. (1991) showed that about 20% could be absorbed when rats were orally given 50 mg of EGCG. However, the mechanism by which GTCs are poorly absorbed remains unknown, but partial degradation of GTCs in neutral or alkaline intestine could be offered as a possible explanation (Guo et al., 1996; Matsumoto et al., 1991). In addition, the free-radical scavenging capacity

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of GTCs has been shown to vary with different pH (Nanjo et al., 1996). This led us to conduct several experiments examining stability of GTCs either as a mixture or individual epicatechin isomers in various pH.

MATERIALS AND METHODS

HPLC Analysis of Longjing Tea GTC Extracts. The method described by Agarwal et al. (1992) was used to extract total GTCs from longjing green tea. The individual isomers in longjing tea extracts were separated using a Shimadzu LC-10AD HPLC (Tokyo, Japan) equipped with a ternary pump delivery system. In brief, 15 μL of longjing tea extracts (0.5 mg/mL) was injected onto column (Hypersil ODS, 250 × 4.6 mm, 5 μm, Alltech, Deerfield, IL) via a Rheodyne valve (20 μL capacity, Cotati, CA). An eluting mixture of H_2O containing 0.05% H_2SO_4, acetonitrile, and ethyl acetate (86:12:2, v/v/v) was used at a flow rate of 1 mL/min. The separated GTC isomers were monitored using a UV detector at 280 nm (UVIS-205, Alltech) and quantified using (+)-catechin (Sigma, St. Louis, MO) as an internal standard. Identification of each isomer was confirmed by comparison of retention time and cochromatography with authentic standards of EC, EGC, ECG, and EGCG (Kurita Industrial Co., Ltd, Tokyo, Japan). The extraction method used in the present study yielded 10.2 g of crude GTCs/100 g of longjing tea leaves in which EGCG, ECG, EGC, and EC accounted for 68.0%, 19.0%, 1.4%, and 3.2%, respectively.

Isolation and Purification of Individual GTC Isomers. Individual GTC isomers were isolated as we previously described (Zhang et al., 1997a,b). In brief, 50 mg of crude longjing tea GTC extracts in H_2O were loaded onto a semi-preparative column (Spherisorb ODS-2, 250 × 10 mm, 10 μm, Isco, Inc., Lincoln, NE) via a Rheodyne valve with a 250-μL sample loop. A 29% methanol solution in H_2O was used at a flow rate of 0.7 mL/min. The eluting peaks of epicatechin isomers were monitored at 280 nm using a UV detector (UVIS-205, Alltech) and collected manually. The fraction containing individual epicatechin isomer was checked immediately using analytical column as described above, and the purity of each isomer was found to be >99% as determined by HPLC. The methanol was removed using a rotary evaporator. The resulting pure epicatechin isomers were then freeze-dried overnight and stored in dark at −20 °C until used.

Stability of Longjing GTCs in Krebs−Ringer Bicarbonate Buffer. The stability of longjing GTCs was firstly assessed in Krebs−Ringer bicarbonate buffer (pH = 7.4), which is commonly used in metabolic studies. The Krebs−Ringer bicarbonate buffer was prepared by mixing the following ingredients: 2.5 mM $CaCl_2 \cdot 2H_2O$, 2.4 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM KH_2PO_4, 4.8 mM KCl, 119.0 mM NaCl, 32.5 mM $NaHCO_3$, and 5.6 mM glucose. Five milligrams of longjing GTCs was dissolved in 10 mL of Krebs−Ringer bicarbonate buffer followed by incubation at 37 °C. An aliquot of the incubation solution (0.4 mL) was periodically sampled, and 0.1 mL of the internal standard solution containing 0.5 mg/mL (+)-catechin was added. The sample was then extracted twice with 1 mL of ethyl acetate. The combined ethyl acetate extracts were then concentrated to 0.1 mL and subjected to HPLC analysis as described above.

pH Stability of Longjing GTCs. The pH stability of longjing GTCs was assessed by incubation of 5 mg of GTCs as a mixture in 10 mL of 60 mM sodium phosphate buffer at different pH varying from 5.0 to 7.4. For the pH between 8 and 12, 5 mg of GTCs as a mixture was incubated in 10 mL of NaOH solution, whereas for the pH between 1 and 5, the same amount of GTCs was incubated in 10 mL of HCl solution. An aliquot (0.4 mL) of the incubation mixture was periodically sampled, and 0.1 mL of (+)-catechin solution (0.5 mg/mL) was added followed by extraction twice with 1 mL of ethyl acetate. After evaporation, the extracts were redissolved into 0.1 mL of ethyl acetate and subjected to HPLC analysis.

Stability of Individual Epicatechin Isomers. The pH stability of individual epicatechin isomers was also assessed under the same conditions. In brief, 6 mg of EC, ECG, EGC, and EGCG as a mixture (1.5 mg each) was incubated in 10 mL of 60 mM sodium buffer at pH 7.4. An aliquot (0.4 mL) of the incubation mixture was periodically sampled, and 0.1 mL of (+)-catechin solution (0.5 mg/mL) was added as an internal standard. The mixture was similarly extracted twice with 1 mL of ethyl acetate and then subjected to HPLC analysis as described above.

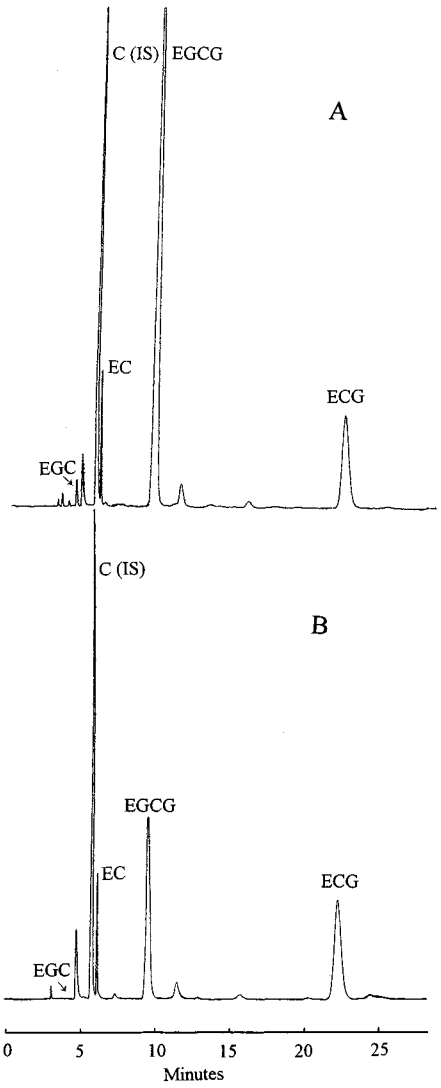

Figure 2. HPLC profile of longjing tea catechins. A, before addition to Krebs−Ringers bicarbonate buffer; B, 5 min after addition to Krebs−Ringers bicarbonate buffer. Peak identification: EGC, (−)-epigallocatechin; C, (+)-catechin (internal standard); EC, (−)-epicatechin; EGCG, (−)-epigallocatechin gallate; and ECG, (−)-epicatechin gallate.

Stability of GTCs in Boiling Water. To examine the stability of GTCs in hot water, 5 mg of GTCs/mL was maintained in 10 mL of boiling water for 7 h. The appropriate volume of water was periodically added to compensate for loss due to evaporation. An aliquot of 50 μL was periodically sampled, and 50 μL of catechin solution (1 mg/mL) was added as an internal standard. The sample was then subjected to HPLC analysis as previously described.

RESULTS

Stability of GTCs as a Mixture in Krebs−Ringer Bicarbonate Buffer. GTCs extracted from longjing tea were unstable in Krebs−Ringer bicarbonate buffer. Typical HPLC chromatograms of GTCs before or after incubation in Krebs−Ringer bicarbonate buffer (pH = 7.4) for 5 min are shown in Figure 2. When GTCs were dissolved in Krebs−Ringer bicarbonate buffer, the

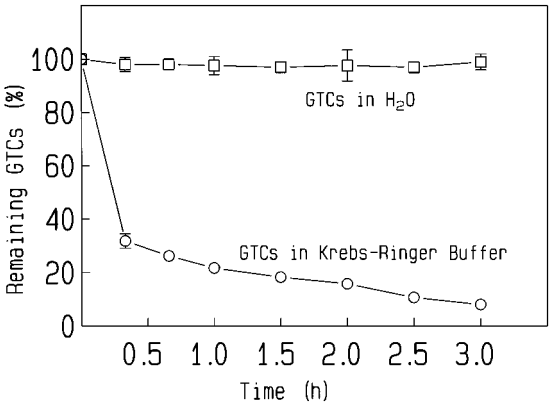

Figure 3. Stability of longjing green tea catechins (GTCs) as a mixture in Krebs−Ringer bicarbonate buffer (pH = 7.4) and H_2O (pH = 4.9). Data are expressed as mean ± SD of $n = 5$ samples.

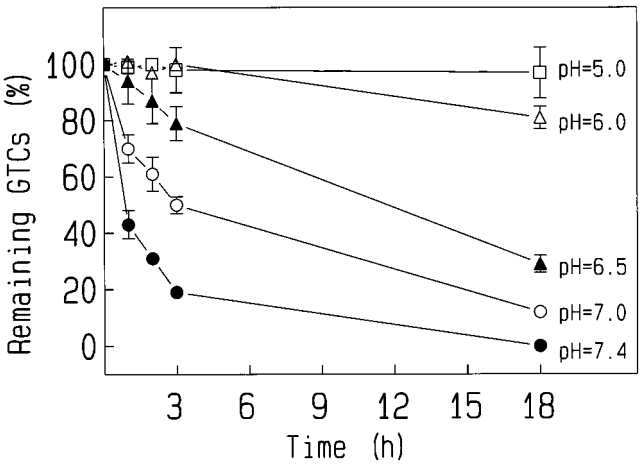

Figure 4. Stability of longjing green tea catechins (GTCs) as a mixture in sodium phosphate buffers with varying pH. Data are expressed as mean ± SD of $n = 5$ samples.

mixture was initially light-brown and rapidly became dark-brown. HPLC analysis, using (+)-catechin as an internal standard, showed that more than 75% of total GTCs was degraded within a first half hour (Figure 3). In contrast, the same amount of GTCs dissolved in HPLC grade H_2O remained unchanged for a period of 3 h under the same incubation conditions. Contrary to that of Krebs−Ringer bicarbonate buffer (pH = 7.4), the pH of GTCs in H_2O was found to be 4.9 (Figure 3).

pH Stability of Longjing GTCs as a Mixture. It was found that GTCs in the alkaline solution (pH > 8) were extremely instable and degraded almost completely in a few minutes, whereas in the acidic solution (pH < 4) they were very stable at least for 18 h (data not shown). The effect of pH on stability of GTCs was best illustrated when the pH value of sodium phosphate buffer was set between 5.0 and 7.4. As shown in Figure 4, GTCs incubated at pH = 5 remained almost unchanged in 18 h, while they were slightly degraded at pH = 6. In contrast, GTCs were much less stable when the pH values were between 6.5 and 7.4. It was obvious that the stability of GTCs was pH-dependent under the present experimental conditions.

pH Stability of Individual Epicatechin Isomers. Four epicatechin isomers examined demonstrated varying stability in the alkaline solution (Figure 5). To simplify the presentation, only data for pH 7.4 are shown. EGCG and EGC were equally instable. In contrast, EC was most stable followed by ECG under

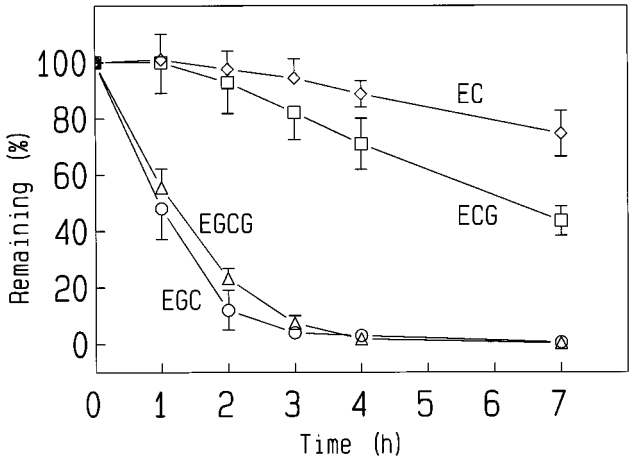

Figure 5. Stability of individual epicatechin isomers in sodium phosphate buffer (pH = 7.4). Data are expressed as mean ± SD of $n = 5$ samples.

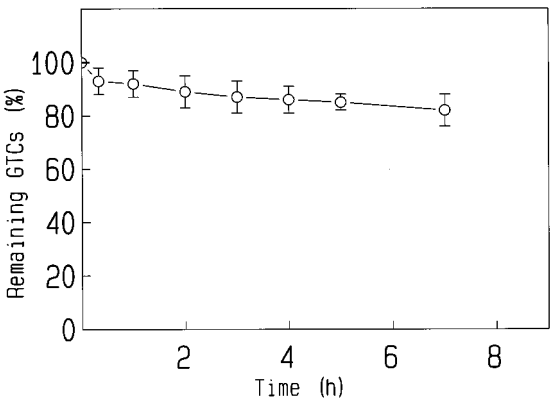

Figure 6. Stability of longjing green tea catechins (GTCs) in boiling water. Data are expressed as mean ± SD of $n = 5$ samples.

the same conditions. As shown in Figure 5, EGCG and EGC were almost completely degraded when incubated for 3 h at pH 7.4. Under the same incubation conditions, EC remained unchanged while ECG was decreased by 20% (Figure 5).

Stability of Longjing GTCs in Boiling Water. Thermal stability of GTCs was also examined in boiling H_2O (pH = 4.9) for 7 h. As shown in Figure 6, GTCs exhibited a remarkable stability in boiling H_2O. Boiling for 7 h resulted in about 15% reduction in total GTCs. However, no difference in thermal loss among individual epicatechin isomers was observed under the present conditions.

DISCUSSION

GTCs are a group of polyphenol compounds belonging to the flavonoid family. These epicatechin isomers have been shown to possess a variety of physiological functions. However, limited information is available on their metabolism. To study how GTCs were metabolized in liver homogenate (data not shown), GTCs as a mixture were firstly dissolved in Krebs−Ringer bicarbonate buffer. It was accidentally noticed that the color of GTCs in the solution changed from light-brown to dark-brown in a few minutes. Subsequent HPLC analysis clearly demonstrated that total longjing GTCs were rapidly degraded in Krebs−Ringer bicarbonate buffer compared with the same amount of GTCs dissolved in H_2O (Figures 2 and 3). To examine which ingredient would induce rapid degradation of GTCs, an

equal amount of longjing GTCs was then incubated in each of the solutions containing only one single ingredient of Krebs−Ringer bicarbonate buffer. After incubation for 3 h, GTCs remained unchanged or slightly changed in one of the following six solutions with acidic pH: 2.5 mM $CaCl_2 \cdot 2H_2O$ (pH = 4.5), 2.4 mM $MgSO_4 \cdot 7H_2O$ (pH = 4.6), 1.2 mM KH_2PO_4 (pH = 4.5), 4.8 mM KCl (pH = 4.7), 119.0 mM NaCl (pH = 4.6), and 5.6 mM glucose (pH = 4.6). However, substantial degradation of GTCs was only observed in the solution of 32.5 mM $NaHCO_3$ (pH = 8.3). From these observations, it appeared that the pH might significantly affect the stability of GTCs.

Information on absorption of GTCs is also very limited (Unno et al., 1996; Okushio et al., 1996). To study their absorption, the effect of pH on stability of GTCs must be addressed because there is a rapid change in pH from stomach to intestine. In fact, the present results clearly demonstrated that longjing GTCs were instable in a solution with pH > 6.5 and that EGCG and EGC were preferentially destroyed (Figures 4 and 5). In contrast, EC and ECG were relatively more stable in both acidic and alkaline pH. Together with the fact that EGCG accounts for 68% total longjing tea GTC extracts, if EGCG is not detected in blood after an oral dose of 100 mg of longjing GTCs (Zhang et al., 1997b), part of the mechanism may involve selective degradation of EGCG in the intestine and blood where the pH was neutral or slightly alkaline.

The reason why EGCG and EGC were less stable than EC and ECG in an alkaline solution remained unclear at the present time. EGCG and ECG have a similar backbone except for an additional hydroxyl group at position 5′ in the former. By a similar observation, the structures of EGC and EC are the same except for an additional hydroxyl group at position 5′ in EGC (Figure 1). Perhaps, the three adjacent hydroxyl groups at position 3′, 4′, and 5′ in EGCG and EGC were more vulnerable to destruction than the two adjacent hydroxyl groups at position 3′ and 4′ in ECG and EC. In fact, EGCG has been shown to be more susceptible to formation of a semiquinone free radical than ECG in 1 mol of NaOH solution (Guo et al., 1996; Yoshioka et al., 1991). This may also explain why the free-radical scavenging capacity of GTCs increases with increasing pH because at neutral or alkaline pH, tea catechins increase their proton-donating potential and become easier to form the corresponding semiquinone free radicals (Yoshioka et al., 1991). We are currently investigating the breakdown pathway of EGCG and EGC and their decomposition products. It will be also worthy to study further the metabolism and degradation of individual isomers *in vivo* if they are absorbed and circulated in blood.

It appeared unlikely that the gallate catechins, EGCG and ECG, were converted to their corresponding catechin, EGC and EC, as no increase in the latter two isomers was observed when longjing GTCs were incubated as a mixture in both acidic and alkaline solutions (Figure 1). Furthermore, the conversion of EGCG to EGC was undetected when pure EGCG was incubated alone in both acidic and alkaline pH (data not shown).

The effect of hot water used in common tea making on GTCs composition has not been assessed before. The present study was the first report to demonstrate that GTCs were stable in boiling water at least for 7 h (Figure 6). We have also studied the thermal stability of GTCs in heated canola oil and found that thermal loss of GTCs was significantly less compared to that of butylated hydroxytoluene (Chen and Chan, 1996). It should be noticed that the pH value of H_2O in the presence of 0.5 mg/mL GTCs was recorded to be 4.9.

In conclusion, EGCG and EGC were extremely vulnerable to degradation in the alkaline solutions while EC and ECG were relatively stable. If GTCs were not well absorbed, part of the mechanism may involve selective degradation of EGCG and EGC in the intestine where pH becomes neutral or alkaline.

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