Stabilizing Effect of Ascorbic Acid on Green Tea Catechins

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Green tea catechins (GTCs) as a mixture of (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin gallate (EGCG) possess a variety of biological activities. We have previously studied the stability of GTCs either as a mixture or as individual epicatechin derivatives in various pH, demonstrating that GTCs as a mixture in alkaline solutions were extremely unstable and degraded almost completely in a few minutes, whereas in acidic solutions (pH < 4) they were very stable. For the pH ranging from 4 to 7, the stability of GTCs was inversely associated with the pH value of the incubation solutions. The present study examined the effect of ascorbic acid and citric acid on the stability of GTCs incubated in sodium phosphate buffer (pH = 7.42). Ascorbic acid added to the incubation mixture significantly increased the stability of GTCs whereas citric acid exhibited no effect. Four epicatechin derivatives examined demonstrated varying stability, with EGCG and EGC being equally instable and EC and ECG being relatively stable. The addition of ascorbic acid significantly increased the stability of all four derivatives, particularly EGC and EGCG. The present results, although not directly transferable to in vivo conditions, may suggest that the presence of ascorbic acid may stabilize the GTCs in the intestine where the pH is neutral or alkaline before absorption.

Keywords: *Catechin; epicatechin, epicatechin gallate; epigallocatechin gallate; epigallocatechin; longjing tea*

INTRODUCTION

Green tea catechins (GTCs) including mainly (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epigallocatechin gallate (EGCG) (Figure 1) are believed to have a wide range of pharmaceutical properties including being antihypertensive (Henry and Stephens-Larson, 1984), antioxidative (Chen and Chan, 1996; Chen et al., 1996; Ding et al., 1992; Miura et al., 1994; Zhang et al., 1997a), antiarteriosclerotic (Hertog et al., 1993), anticarcinogenic (Shi et al., 1994; Wang et al., 1994), and hypocholesterolemic (Imai and Nakachi, 1985; Kono et al., 1992). The content of GTCs varies among green tea, black tea, and oolong tea. Green tea, which is the nonfermented product, has the highest content of GTCs while black tea has the lowest content of tea catechins, which are extensively oxidized during fermentation. Oolong tea has an intermediate content of GTCs because it is a partially fermented product.

Our previous study has demonstrated that the stability of longjing GTCs is pH-dependent (Zhu et al., 1997). In alkaline solution (pH > 7), longjing GTCs were very instable and degraded almost completely in a few minutes, whereas in acidic solution (pH < 4) they were stable. The present study examined the effects of two commonly used organic acids (ascorbic acid and citric acid) on the stability of GTCs under various conditions.

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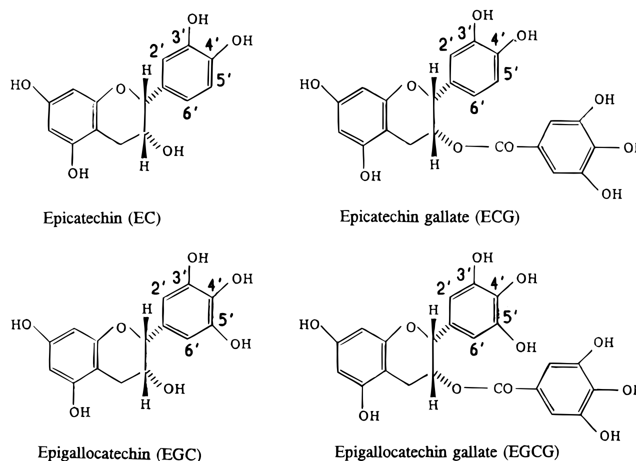

Figure 1. Chemical structures of (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), epigallocatechin (EGC), and (−)-epigallocatechin gallate (EGCG).

MATERIALS AND METHODS

HPLC Analysis of Longjing Tea GTCs. Longjing tea (Huangshan Forestry Farm, Xiaoshan, Zhejiang, China) was purchased from the local market. The GTCs were extracted, and the individual epicatechin derivatives were analyzed using HPLC as we previously described (Zhang et al., 1997b; Zhu et al., 1997). In brief, 10 g of longjing tea leaves was soaked three times with 140 mL of hot distilled water (80 °C). The infusion was cooled to room temperature, filtered, and then extracted with an equal volume of chloroform (HPLC grade; BDH Laboratory Supplies, Poole, England) to remove caffeine and pigments. The remaining aqueous layer was extracted twice with an equal volume of ethyl acetate (HPLC grade; BDH Laboratory Supplies). The ethyl acetate containing GTCs was then pooled and evaporated using a vacuum rotary evaporator.

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The resulting GTCs were dissolved in 10 mL of distilled water and freeze-dried overnight.

The individual epicatechin derivatives in longjing GTCs were separated using a Shimadzu LC-10AD HPLC (Tokyo, Japan) equipped with a ternary pump delivery system. In brief, 15 μL of longjing GTCs (0.5 mg/mL) was injected onto a 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 (HPLC grade; BDH Laboratory Supplies), and ethyl acetate (86:12:2, vol/vol/vol) was used at a flow rate of 1 mL/min. The individual epicatechin derivatives were separated and quantified using a UV detector at 280 nm (UVIS-205, Alltech, Deerfield, IL) and (+)-catechin (Sigma, St. Louis, MO) as an internal standard. Identification of each compound was made by comparison of retention time and co-chromatography 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 (Zhu et al., 1997).

Effects of Citric and Ascorbic Acids on the Stability of Longjing GTCs in Sodium Phosphate Buffer. The stability of longjing GTCs was first assessed in sodium phosphate buffer (60 mM, pH = 7.42) in the absence or presence of citric acid and ascorbic acid. A total of 5 mg of longjing GTCs was dissolved in 10 mL of sodium phosphate buffer containing 0.2 mg/mL citric acid or ascorbic acid followed by incubation at 37 °C. An aliquot (0.4 mL) of the incubation solution 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.

Effect of Ascorbic Acid on the Stability of GTCs in Krebs−Ringer Bicarbonate Buffer. Protective effect of ascorbic acid on the stability of longjing GTCs was also assessed in Krebs−Ringer bicarbonate buffer (pH = 7.50), 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. A total of 5 mg of longjing GTCs was similarly dissolved in 10 mL of Krebs−Ringer bicarbonate buffer containing 0.2 mg/mL ascorbic acid followed by incubation at 37 °C. An aliquot of the incubation solution (0.4 mL) was periodically sampled, and GTCs were then extracted with ethyl acetate followed by HPLC analysis using (+)-catechin as an internal standard.

Measurement of Ascorbic Acid. Ascorbic acid was determined spectrophotometrically (Sullivan and Carpenter, 1993) based on the fact that ascorbic acid can reduce blue dye, 2,6-dichlorophenolindophenol (DCPIP), to colorless. In brief, an aliquot (0.1 mL) of the GTC incubation solution containing ascorbic acid was periodically taken and then mixed with 1.9 mL of citrate buffer (0.1 M, pH = 4.8) and 0.5 mL of DCPIP solution (0.25 mg/mL). The absorbance at 520 nm was then recorded, and the value of ascorbic acid was expressed as the percent remaining.

RESULTS

Effects of Ascorbic Acid on the Stability of GTCs. Longjing GTCs as a mixture were instable in sodium phosphate buffer (pH = 7.42). As shown in Figure 2, almost 50% longjing GTCs as a mixture were degraded within 2 h. Citric acid did not show protective effect on longjing GTCs. In contrast, the addition of 0.2 mg/mL ascorbic acid significantly improved the stability of GTCs up to 20 h (Figure 2). Contrary to that of the control sample (pH = 7.42), the pH of the sample

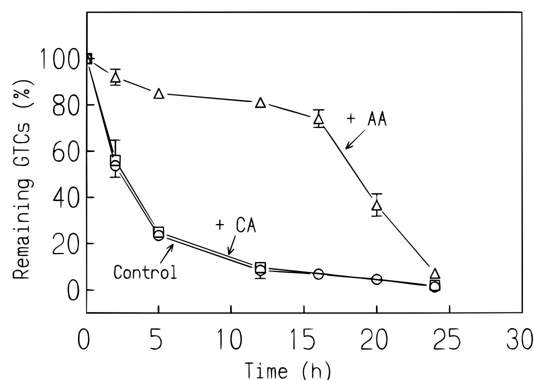

Figure 2. Effect of citric acid (CA) and ascorbic acid (AA; 0.2 mg/mL) on stability of longjing green tea catechins (GTCs, 0.5 mg/mL) in sodium phosphate buffer (pH = 7.42). Data are expressed as mean ± SD of $n = 4-5$ samples.

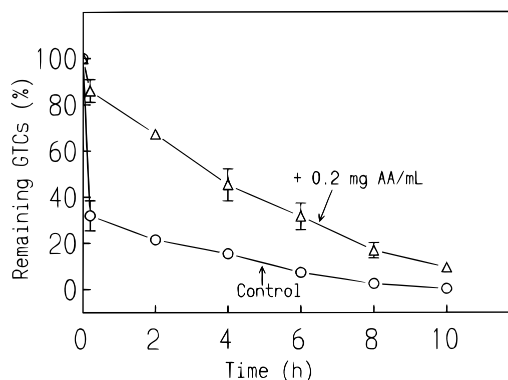

Figure 3. Effect of ascorbic acid (AA, 0.2 mg/mL) on stability of longjing green tea catechins (GTCs, 0.5 mg/mL) as a mixture in Krebs−Ringer bicarbonate buffer (pH = 7.50). Data are expressed as mean ± SD of $n = 4-5$ samples.

containing 0.2 mg/mL citric acid or ascorbic acid was reduced to 7.28 and 7.38, respectively.

The stability of longjing GTCs in the presence of ascorbic acid was also examined in Krebs−Ringer bicarbonate buffer (pH = 7.50). As shown in Figure 3, a similar protection effect of ascorbic acid on the stability of GTCs was also observed in Krebs−Ringer bicarbonate buffer.

To test whether it can regenerate longjing GTCs, ascorbic acid was added to the incubation mixture when almost 40% total GTCs were degraded in sodium phosphate buffer (Figure 4). The addition of ascorbic acid did not increase the total GTCs, but it reduced the rate of GTC degradation (Figure 4).

Dose-Dependent Effect of Ascorbic Acid on the Stability of Longjing GTCs. As shown in Figure 5, the addition of 0.005 mg/mL ascorbic acid did not show any protection to longjing GTCs while the addition of 0.05 mg/mL ascorbic acid slightly improved the stability of GTCs under the same conditions. When ascorbic acid was increased to 0.1−0.2 mg/mL, the stability of longjing GTCs was significantly improved as compared with the control sample. Meanwhile, the quantative change in ascorbic acid was compared with that of longjing GTCs (Figure 6). It was found that degradation of ascorbic acid was parallel with that of longjing GTCs.

Effect of Ascorbic Acid on the Stability of Individual Epicatechin Derivatives. The stability of four epicatechin derivatives was also examined in sodium phosphate buffer containing 0.2 mg/mL ascorbic acid (Figure 7). All four epicatechin derivatives demon-

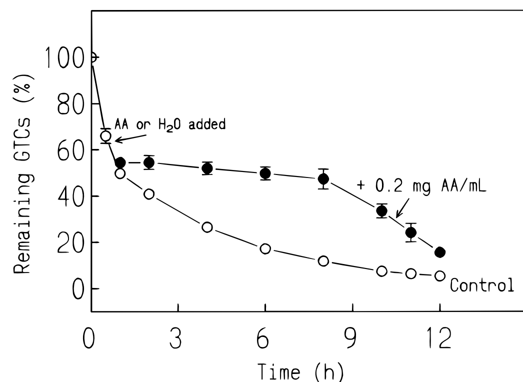

Figure 4. Stability of longjing green tea catechins (GTCs, 0.5 mg/mL) as a mixture in sodium phosphate buffers (pH = 7.42) as 0.2 mg/mL of ascorbic acid (AA) was added at the time almost 40% GTCs were degraded. Data are expressed as mean ± SD of $n = 4-5$ samples.

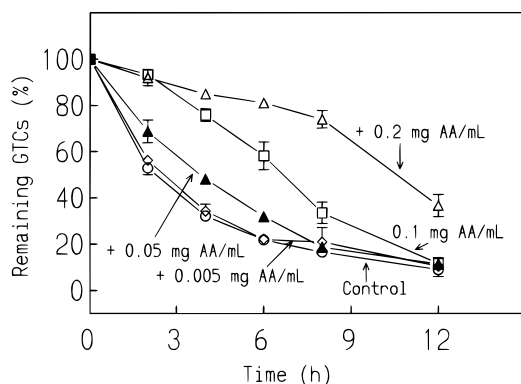

Figure 5. Dose-dependent effect of ascorbic acid (AA) on stability of longjing green tea catechins (GTCs, 0.5 mg/mL) in sodium phosphate buffer (pH = 7.4). Data are expressed as mean ± SD of $n = 4-5$ samples.

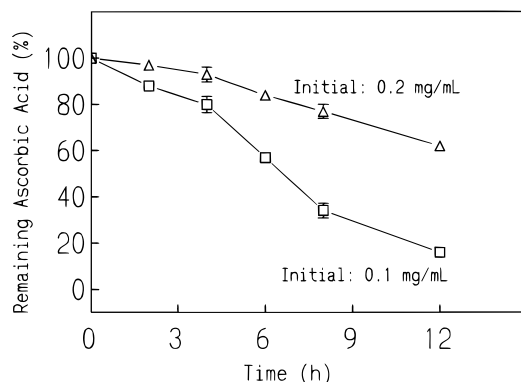

Figure 6. Quantitative change in ascorbic acid (initial concentration, 0.2 or 0.1 mg/mL) when incubated in sodium phosphate buffer (pH = 7.42) in the presence of 0.5 mg/mL of longjing green tea catechins. Data are expressed as mean ± SD of $n = 4$ samples.

strated varying stability in the alkaline solution. In the absence of ascorbic acid (control), EC was the most stable followed by ECG under the same conditions (Figure 7). In contrast, EGC was almost completely degraded when incubated for 2 h at pH 7.42 and so did EGCG when incubated for 5 h under the same conditions. Citric acid added to the incubation mixture did not show protection to EGC and EGCG, but it demonstrated a slight protection to EC and ECG. Under the same incubation conditions, the stability of four epicatechin derivatives was significantly increased when ascorbic acid was added instead (Figure 7).

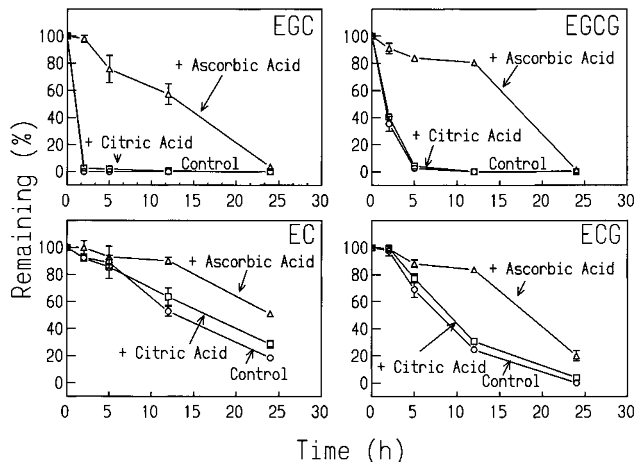

Figure 7. Effect of ascorbic acid (AA, 0.2 mg/mL) on stability of individual epicatechin isomers in longjing green tea catechins (GTCs, 0.5 mg/mL) in sodium phosphate buffer (pH = 7.42). Data are expressed as mean ± SD of $n = 4-5$ samples. EC, (−)-epicatechin; ECG, (−)-epicatechin gallate; EGC, epigallocatechin; EGCG = (−)-epigallocatechin gallate.

DISCUSSION

Green tea catechins have been shown to possess a variety of physiological functions. We have previously examined the stability of longjing GTCs either as a mixture or the individual epicatechin derivative in different solutions with varying pH. It was found that the stability of GTCs was pH-dependent, i.e., the lower the pH, the greater the stability (Zhu et al., 1997). This was in agreement with the reports by Suematsu et al. (1992) and Komatsu et al. (1991), who examined the stability of GTCs in canned tea drinks and found that the GTCs in the unfermented tea drinks were considerably more unstable than those in the fermented ones with varying pH values. It is speculated that the addition of organic acids may increase the stability of GTCs because they may decrease the pH of the incubation mixture. Two common organic acids were therefore chosen in the present study because they are often present in tea beverages. When 0.2 mg/mL citric acid was added to the sodium phosphate buffer solution containing 0.5 mg/mL longjing GTCs, the pH of the incubation solution was reduced from 7.42 to 7.28. HPLC analysis showed that citric acid did not demonstrate any protective effect to longjing GTCs (Figure 2). In contrast, when the same amount of ascorbic acid was added, the pH of the incubation mixture was only reduced to 7.38, but the stability of longjing GTCs was significantly improved (Figures 2 and 7). The protective effect of ascorbic acid on functional constituents was similarly observed in canned tea drinks during extraction, processing, and storage (Komatsu et al., 1991). The protective effect observed for ascorbic acid but not for citric acid indicates that the stabilizing activity of ascorbic acid on GTCs is not attributed to the drop in the pH value of the incubation mixture. It is well-known that four epicatechin derivatives are susceptible to the formation of their corresponding semiquinone free radicals in an alkaline solution when exposed to air (Guo et al., 1996; Nanjo et al., 1996; Yoshioka et al., 1991). It is also known that ascorbic acid functions partially as an antioxidant and can regenerate α-tocopherol by

converting the α-tocopherol free radical to the reduced form in vivo, thus sparing this lipophilic antioxidant (Packer et al., 1979). By similar deduction, when incubated together with GTCs, ascorbic acid perhaps serves as a reductant that can also protect these GTCs and recycle their free radical form. Another possibility is that ascorbic acid may reduce the concentration of the dissolved oxygen in the incubation solution, which in turn may contribute to lesser oxidation of GTCs (Figure 6). In addition to ascorbic acid, sodium sulfite, which is commonly used as a general food preservative, was also shown to improve the stability of tea catechins (Hara and Okushio, 1989).

The present results clearly demonstrated that ascorbic acid can stabilize longjing GTCs in alkaline solutions. First, the stability of GTCs was examined in both sodium phosphate buffer (pH = 7.42) and Krebs−Ringer bicarbonate buffer (pH = 7.50). The results showed clearly that ascorbic acid protected longjing GTCs from degradation regardless of the two buffers used. However, the GTCs seemed to degrade faster in Krebs−Ringer buffer than in sodium phosphate buffer (Figures 2 and 3). This may attribute to a higher pH value in the former buffer (Zhu et al., 1997) or a relatively higher level of iron in the former buffer because it is known that phosphate buffer has iron-chelating effect and therefore may reduce the iron-induced degradation of ascorbic acid. Second, the protective effect of ascorbic acid on GTCs exhibited a dose-dependent manner (Figure 5). Third, the rate of GTC degradation was immediately reduced as soon as 0.2 mg/mL ascorbic acid was added to the incubation mixture at time when almost 40% GTCs were already degraded (Figure 4).

Ascorbic acid significantly increased the stability of all four epicatechin derivatives particularly EGC and EGCG. As shown in Figure 7, EGC for 2 h and EGCG for 5 h of incubation in sodium phosphate buffer were almost completely destructed in the absence of ascorbic acid. For the same time, EGC and EGCG in the sample containing 0.2 mg/mL ascorbic acid showed only 25% and 15% destruction. Similarly, ascorbic acid also increased significantly the stability of EC and ECG (Figure 7). The present study confirmed that four epicatechin derivatives demonstrated varying stability with EGC and EGCG being equally unstable while EC and ECG were relatively stable (Zhu et al., 1997). The present result was in agreement with that of Kiatgrajai et al. (1982), who found that there was no significant degradation of EC but epimerization in alkaline solution in the absence of oxygen. In view of their chemical structures, EGCG and ECG have a similar backbone except an additional hydroxyl group at position 5′ in the former (Figure 1). By a similar observation, the structures of EGC and EC are same except an additional hydroxyl group at position 5′ in EGC (Figure 1). Perhaps, the three adjacent hydroxyl groups at positions 3′, 4′, and 5′ in EGCG and EGC were more vulnerable to form the semiquinone free radicals when a proton was donated. In fact, EGCG has been shown to be more susceptible to the formation of a semiquinone free radical than ECG in 1 mol of NaOH solution (Guo et al., 1996; Yoshioka et al., 1991).

Previous studies have suggested that GTCs are partially absorbed in both rats (Matsumoto et al., 1991; Okushio et al., 1996) and humans (Unno et al., 1996). We have studied the absorption of tea catechins and found that the maximum concentration of GTCs in plasma could reach 9−10 μg/mL after an oral administration of 100 mg of longjing GTCs as a mixture in rats (Zhang et al., 1997b). In contrast, the study by Matsumoto et al. (1991) showed that about 20% could be absorbed when rats were orally given 50 mg of EGCG. In another study (Zhu et al., 1997), it was found that GTCs exhibited a very poor stability in neutral or alkaline solution and speculated that it was probably one of mechanisms attributing to the poor absorption of GTCs. However, the experiment was conducted in the presence of oxygen and may not reflect the physiological conditions of the intestine where a lower concentration of oxygen is expected.

The present results, although not directly transferable to in vivo conditions, may have some implications in the process of GTCs absorption. This is because the intestinal pH is neutral or slightly alkaline, and the presence of ascorbic acid may partially prevent the degradation or epimerization (Kiatgrajai et al., 1982) of GTCs in the intestine before absorption. This speculation for better absorption of GTCs in the presence of ascorbic acid deserves further study. In addition, the present study confirms the stabilizing activity of ascorbic acid but not that of citric acid in tea beverages.

ABBREVIATIONS USED

GTCs, green tea catechins; EC, (−)-epicatechin; ECG, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin gallate; HPLC, high-performance liquid chromatography.

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