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Stability of Black Tea Polyphenol, Theaflavin, and Identification of Theanaphthoquinone as Its Major Radical Reaction Product

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In the current study, we have focused on isolation and detection of major radical oxidation products from theaflavin in order to better understand antioxidation mechanisms of this compound. Theanaphthoquinone was identified as a major oxidation product of theaflavin from two different oxidant model systems: DPPH and peroxidase/hydrogen peroxide. This result indicated that the benzotropolone moiety in theaflavin may play an important role in its antioxidant properties. The stability of theaflavin was studied in varying pH solutions: simulated gastric juice and buffer solutions of pH 5.5, pH 7.4, and pH 8.5. The results indicated that theaflavin is unstable in alkaline conditions, while it was stable in acidic conditions. Theanaphthoquinone was identified as an autoxidation product of theaflavin during its stability study in alkaline conditions.

KEYWORDS: Theaflavin; theanaphthoquinone; oxidation; radical oxidation; pH stability

INTRODUCTION

As a popular beverage, tea (*Camellia sinensis*) has attracted public attention because of accumulating scientific evidence linking tea consumption with health benefits (*1*). Fresh tea leaves contain four major tea catechins with unique biological activities; however, black tea is manufactured through fermentation of the tea leaves. During the fermentation process, important chemical changes occur due to the action of polyphenol oxidase (PPO). PPO is responsible for oxidizing the dihydroxylated B ring (catechol) and trihydroxylated B ring (pyrogallol) of tea catechins to their *o*-quinones. Subsequent chemical reactions generate the various characteristic black tea polyphenolic pigments that are not found in unprocessed tea leaves. Four major theaflavins have been identified from black tea, namely, theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate (*2*).

Tea consumption may provide health benefits, and these biological activities are believed to arise from the antioxidant activity of polyphenolic compounds in tea, specifically their ability to effectively scavenge reactive oxygen species (*1*). A number of studies have demonstrated the antioxidant capacity of black tea and black tea polyphenols using in vitro and in vivo methods. Many in vitro studies have shown that black tea has a comparable antioxidant capacity to green tea. Researchers using the oxygen-radical absorbance capacity assay have shown that black tea has a similar peroxyl radical scavenging capacity to green tea (*3*). Trolox equivalent antioxidant capacity values of green tea and black tea are comparable (*4*). Black tea extracts have been shown to exhibit protection of human red blood cells against oxidative damage from lipid peroxidation (*5*). Sarkar and Bhaduri (*6*) reported that black tea extracts are more effective than green tea catechins in scavenging superoxide anions.

Several studies have reported a proposed mechanism for the reaction of tea catechins with radicals and have proposed mechanisms for the antioxidant properties of these compounds (*7*, *8*). When tea catechins were reacted with peroxyl radical generated by thermolysis of the radical initiator 2,2′-azobis-(2,4-dimethylvaleronitrile), the resulting seven-membered B ring anhydride and symmetrical dimer were identified as the oxidation products of EGCG and (−)-epigallocatechin (EGC) (*8*). These results indicated that the principal sites for radical reaction are on the B ring rather than the galloyl moiety. Although different oxidation model systems, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), gave different oxidation products, Sang et al. have demonstrated that the catechin B ring is the preferred site for oxidation (*9*). Theaflavins have been regarded as biologically important active components in black tea. Little information is available concerning a mechanism for the antioxidative action of black tea polyphenols. Because the antioxidant mechanisms of theaflavins are still unclear, we sought to identify the oxidation products from theaflavin in order to better understand these mechanisms. Identification of the oxidation products of theaflavins can provide useful information to understand the mechanism giving rise to the antioxidant

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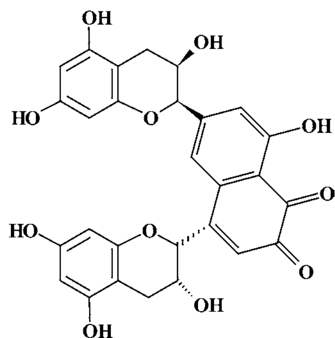

Figure 1. Chemical structure of compound **1**.

properties of these molecules. Also, limited information is available about the stability of theaflavins in various pH solutions. In this study, we focused on examination of the stability of theaflavin in different pH conditions.

MATERIALS AND METHODS

Materials and General Procedures. Epicatechin (EC), DPPH, and horseradish peroxidase were purchased from Sigma (St. Louis, MO). All high-performance liquid chromatography (HPLC) grade solvents were from Fisher Scientific (Fair Lawn, NJ). Tea catechin, EGC, was isolated from commercial green tea polyphenol extract using an LH-20 column chromatography. Thin-layer chromatography (TLC) was performed on Sigma-Aldrich silica gel TLC plates (250 μm thickness, 2−25 μm particle size), and the spots were detected by UV illumination and spraying with 5% (v/v) H_2SO_4 in an ethanol solution. ^{1}H NMR spectra were obtained on a Varian 600 instrument (Varian Inc., Palo Alto, CA). The compound was analyzed in acetone-d_6 with tetramethylsilane as an internal standard. Atmospheric pressure chemical ionization (APCI)-MS were recorded on a Micromass Platform II system (Micromass Co., Beverly, MA) equipped with a Digital DECPC XL 560 computer for data analysis.

Theaflavin Preparation. Theaflavin was prepared by a modified enzymatic oxidation method from Tanaka et al. (*10*). In brief, crude PPO was prepared from commercial banana fruit. Fresh banana flesh (400 g) was homogenized with 800 mL of cold 100 mM potassium phosphate buffer (pH 7.0, 4 °C). The homogeneous solution was centrifuged at 4 °C for 20 min (10000*g*), and clear supernatant was collected. The same volume of cold acetone (−40 °C) was slowly poured into the collected solution with stirring. Then, precipitated proteins were collected by centrifugation (10000*g*, 20 min, 4 °C). The resulting pellets were washed with the same buffer three times and dissolved in the same buffer. The protein solution was lyophilized; the resulting powder was used as a crude PPO.

EC (1 g) and EGC (1 g) were dissolved in 200 mL of phosphate−citrate buffer (50 mM, pH 5.0) along with 2 g of crude PPO enzyme. The enzymatic oxidation was carried out at room temperature for 6 h with stirring. The reaction solution was then subjected to partition three times with the same volume of ethyl acetate. The organic layer was concentrated under reduced pressure. The resulting residues were subjected to Sephadex LH-20 and RP-18 column chromatography successively to afford a reddish theaflavin (yield, 15.2%).

Theaflavin Oxidation Model Using DPPH Oxidant System. Theaflavin (720 mg) was dissolved in 30 mL of acetone, and the radical oxidation was initiated by adding DPPH (1000 mg) into this solution. The reaction was carried out for 20 h with stirring in the dark at room temperature. The reaction solution was dried under reduced pressure. The resulting residue was subjected directly to Sephadex LH-20 column chromatography eluting with 95% ethanol. Six fractions were collected (each 200 mL), and fraction 3 was subjected to LH-20 (95% ethanol) and RP-18 (50% aqueous methanol) successively for further purification and yielded a deep reddish amorphous solid, theanaphthoquinone (24 mg). Further purification of fraction 5 afforded unreacted theaflavin (155 mg).

Theaflavin Oxidation Model Using Peroxidase/Hydrogen Peroxide Oxidant System. Theaflavin (500 mg) was dissolved in 30 mL of 20% aqueous acetone along with 1 mg of peroxidase. One milliliter of 3.11% hydrogen peroxide solution was added to the reaction solution every 40 min. The reaction was carried out at room temperature for 3.5 h with stirring. Then, the reaction solution was dried under reduced pressure. The resulting residues were subjected to Sephadex LH-20 column chromatography eluting with 95% ethanol. Among the collected seven fractions (each 180 mL), fraction 3 was subjected to LH-20 (95%

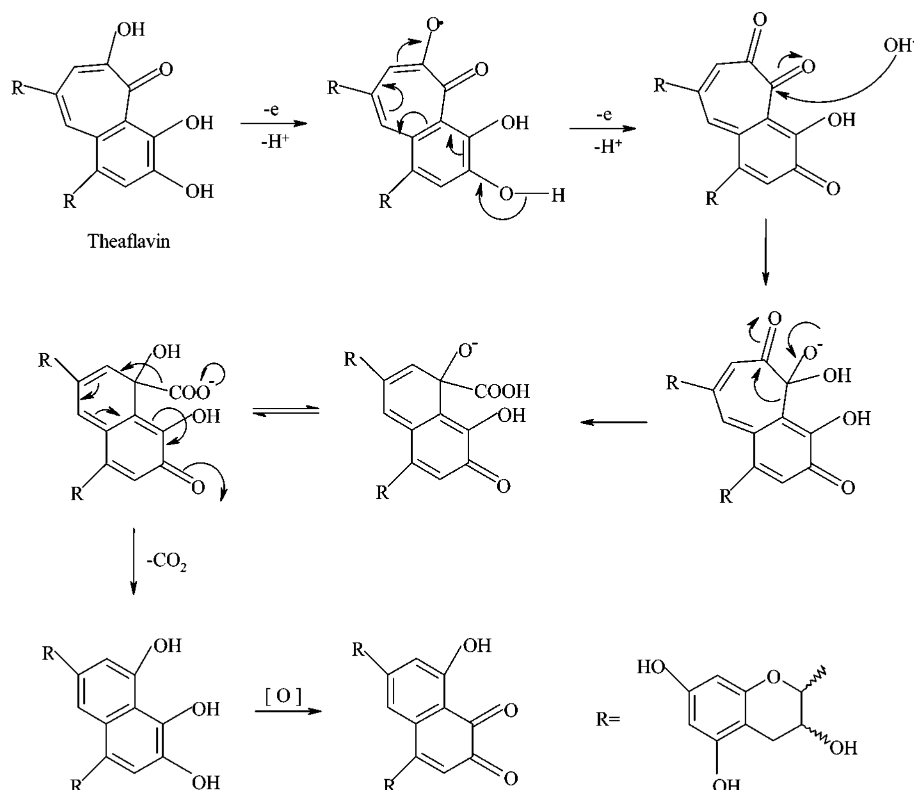

Figure 2. Proposed mechanism of theanaphthoquinone formation in radical reaction.

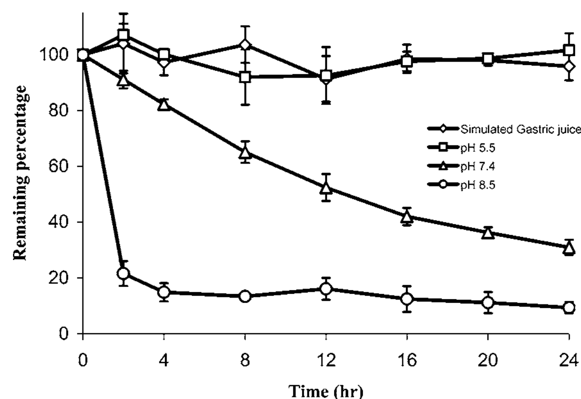

Figure 3. Theaflavin degradation in different pH solutions. Data were expressed as means ± SD ($n = 3$).

ethanol) and RP-18 (50% aqueous methanol) column chromatography to afford theanaphthoquinone (35 mg). Fractions 5 and 6 were combined and subjected to RP-18 column chromatography eluting with a gradient of 40−50% of aqueous methanol to yield unreacted theaflavin (123 mg).

Theanaphthoquinone. Deep red amorphous powder. APCI-MS $[M + H]^+$ at m/z 535. ^1H NMR (600 MHz in acetone-d_6): δ_H 7.40 (1H, s), 7.28 (1H, s), 6.74 (1H, s), 6.01 (1H, d, $J = 2.4$ Hz), 6.01 (1H, d, $J = 2.4$ Hz), 5.96 (1H, d, $J = 2.4$ Hz), 5.96 (1H, d, $J = 2.4$ Hz), 5.35 (1H, br s), 5.11 (1H, br s), 4.40 (1H, d, $J = 3.6$), 4.37 (1H, br s), 2.68−2.93 (4H, m).

Theaflavin Stability in Various pH Solutions. The stability of theaflavin (4 mg in 16 mL) was examined in different pH solutions, such as simulated gastric juice (0.2% sodium chloride, 0.24% hydrochloric acid), pH 5.5 sodium acetate buffer (60 mM), pH 7.4 phosphate buffer (60 mM), and pH 8.5 phosphate buffer (60 mM). Aliquots (1 mL) of the sample were collected and analyzed at various time intervals using HPLC to examine the degradation rate of theaflavin at ambient temperature. The HPLC system was fitted with a Zorbax ODS HPLC column (5 μm, 4.6 mm × 250 mm, RP-18) and equipped with an autosampler (Waters, 717) and UV detector (Varian, 2050) at a wavelength of 375 nm. Theaflavin analysis was performed with a linear increasing gradient from 10 to 60% acetonitrile in water with constant 0.1% acetic acid in 15 min. The flow rate was 1.0 mL/min. The degradation rate of theaflavin was measured based on decreasing theaflavin peak area.

To identify oxidation products of theaflavin in alkaline conditions, an aliquot of sample in pH 8.5 was partitioned with ethyl acetate, and the organic part was dried under reduced pressure. The sample was dissolved in water and analyzed with LC/electrospray ionization (ESI). The analysis was performed on the Finnigan TSG 7000 mass spectrometer equipped with an HP 1100 HPLC system (Hewlett-Packard, Palo Alto, CA). The mass spectrometer was operated in the negative ESI mode. Full scans were acquired from m/z 100 to 650/s. The information for MS condition is as follows: voltage, 3.5 kV; capillary temperature, 300 °C; sweep gas, 70 psi; and auxillary gas, 5 units. HPLC was performed with a Prodigy ODS(3) HPLC column (5 μm, 100 Å, 2.0 mm × 250 mm, Phenomenex, Torrance, CA). The mobile phase delivered at 0.2 mL/min was a linear gradient from 5 to 95% acetonitrile in water with constant 0.1% formic acid in 40 min.

RESULTS AND DISCUSSION

Identification of Theaflavin Oxidation Product from DPPH and Peroxidase/Hydrogen Peroxide Oxidant Model Systems. A major oxidation product of theaflavin was isolated using column chromatography methods from two different oxidant model systems, DPPH and peroxidase/hydrogen peroxide, and its structure was identified by interpretation of ^1H NMR and MS spectra. It has been reported that the dihydroxy B ring and the trihydroxy B ring are the major sites for antioxidant action of tea catechins (*7*). As theaflavin is dimeric compound formed from the catechin B ring, it was necessary to identify the principal site of oxidation that gives rise to the antioxidant properties of these compounds. Therefore, we carried out a theaflavin oxidation experiment using two different oxidation model systems. The DPPH is a stable radical, which is widely used for determination of the antioxidant activity of test compounds. The DPPH radical forms a reduced DPPH paired with a hydrogen from the test compounds. Heme-containing peroxidases reduce hydrogen peroxide to water, while oxidizing various substrates. The horseradish peroxidase/hydrogen peroxide system has been used to generate substrate-derived radicals that can undergo further reactions.

Theaflavin was subjected to oxidation using the above two model systems, and successive purification steps using LH-20 and RP-18 column chromatography afforded a compound (**1**) from both oxidation systems. After comparison with the ^1H NMR spectrum of theaflavin and those of isolated compound **1** along with published spectral data (*11*), it was found that the chemical shift of the characteristic three proton signals in the benzotropolone moiety of compound **1** was different from those of theaflavin. Specifically, the chemical shifts of three aromatic protons in the benzotropolone moiety of theaflavin were δ_H 8.04 (1H, s), 8.01 (1H, s), and 7.58 (1H, s); however, those of compound **1** were δ_H 7.40 (1H, s), 7.28 (1H, s), and 6.74 (1H, s). When we compared the ^1H NMR spectrum of compound **1** with those of theanaphthoquinone, which was reported by Tanaka et al. (*12*), we concluded that the spectral characters of this compound were consistent with those of theanaphthoquinone (**Figure 1**). Moreover, positive ion APCI-MS ($[M + H]^+$ at m/z 535) data of compound **1** strongly supported this conclusion. This compound has been reported as an oxidation product of theaflavin by Tanaka et al. (*10*). They reported that this compound is both an enzymatically and a nonenzymatically generated from theaflavin. As shown in **Figure 2**, we propose a radical oxidation mechanism of theaflavin and theanaphthoquinone formation through one-electron oxidation. From these results, it is probable that the benzotropolone moiety of theaflavin plays an important role in affording antioxidant protection for the preferred oxidation site. Jovanovic et al. (*13*) reported that although theaflavin radicals have a higher reduction potential than the tea catechin EGCG, theaflavins have significantly higher reaction rates with superoxide radicals than EGCG. These authors proposed that the benzotropolone moiety might be responsible for electron donation because of the existence of resonance forms. As part of our continuing chemical study of the antioxidant mechanisms of theaflavins in our laboratory, A ring fission products of theaflavin 3,3′-digallate have been reported using the hydrogen peroxide oxidant system (*14*). This result indicated that the preferred oxidation site of theaflavin 3,3′-digallate is placed in the A ring of flavan-3-ol rather than the benzotropolone moiety. Although the experiment was carried out with a different oxidant system, this observation suggests that gallated theaflavins may have a different antioxidation mechanism as compared to nongallated theaflavins. Interestingly, Jovanovic et al. (*13*) reported that gallated theaflavins have a lower superoxide scavenging activity than nongallated theaflavin. They proposed that the gallate moiety may prevent reaction between the radicals and the benzotropolone moiety in theaflavins. It might be that further chemical studies are needed to understand the antioxidation mechanism of monogallated theaflavin.

Stability of Theaflavin in Different pH and Its Oxidation Product. In the present study, solutions with various pH values, including simulated gastric juice and buffer solutions of pH 5.5,

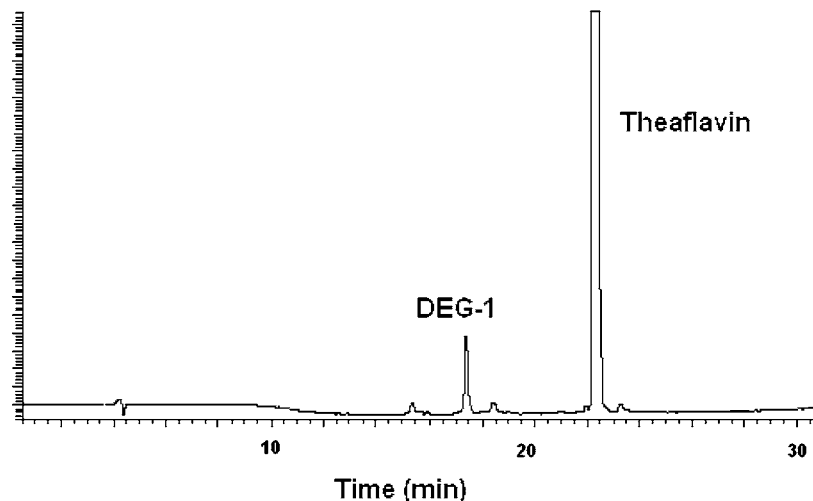

Figure 4. Theaflavin degradation product at alkaline condition.

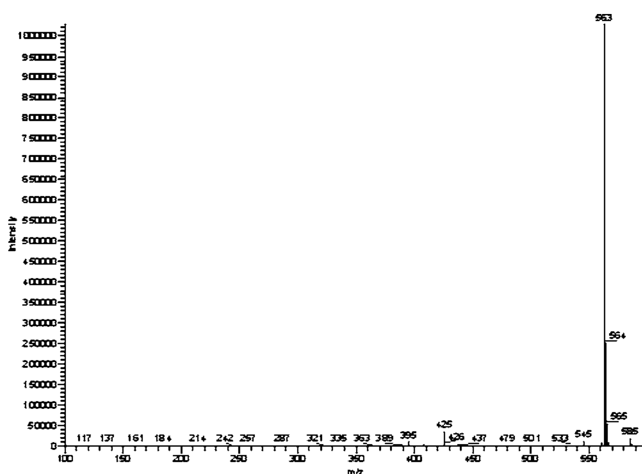

Figure 5. ESI spectrum of theaflavin.

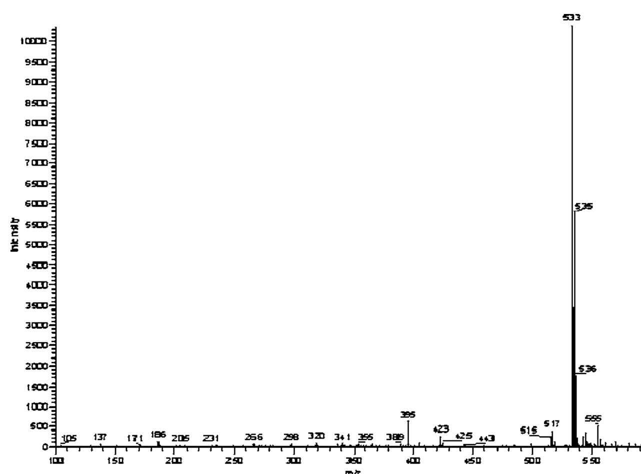

Figure 6. ESI spectrum of DEG-1.

pH 7.4, and pH 8.5, were chosen to examine the stability of theaflavin in acidic and alkaline conditions. It has been reported that plant flavonoids are vulnerable in alkaline condition, and several studies have demonstrated that tea catechins are unstable in neutral and alkaline pH (*15*−*17*). For example, the tea catechins, EGC and EGCG, are completely degraded in pH 7.4 phosphate buffer solution within 3 h (*17*). Interestingly, EGC and EGCG, which contain pyrogallol in the B ring, were much less stable than tea catechins EC and ECG, which have a catechol B ring. One reason for the difference is that pyrogallol-containing catechins form semiquinones more readily than those containing the catechol moiety (*17*).

When theaflavin was incubated in the acidic solutions, pH 5.5 buffer solution, and simulated gastric juice, as shown in **Figure 3**, this compound exhibited a high degree of stability during a 24 h incubation period. In contrast, theaflavin was unstable in alkaline conditions. At pH 7.4 buffer solution, 34.8% of theaflavin was degraded after 8 h of incubation. Moreover, when theaflavin was added into pH 8.5 buffer solution, it rapidly changed to a dark brown color, and 78.4% of theaflavin was degraded after 2 h of incubation.

During the course of this stability study, it was noteworthy that a major degradation product of theaflavin was identified during HPLC analysis in alkaline conditions: pH 7.4 and pH 8.5 (**Figure 4**). To identify the degradation product, the solution was partitioned with ethyl acetate and the organic part was analyzed with LC/ESI. **Figure 5** represents the negative ion ESI spectrum of theaflavin (m/z 563 $[M - H]^-$). The LC/ESI experiment revealed that the DEG-1 has a molecular ion at m/z 533 $[M - H]^-$ (**Figure 6**). In **Figure 6**, the pseudo-molecular ion peak at m/z 535 $[M - H]^-$ was also identified as a result of the reduction product. This result indicated that the spectral character of DEG-1 was the same as theanaphthoquinone reported by Tanaka et al. (*12*). After comparison of HPLC analysis of an isolated theanaphthoquinone standard and DEG-1, we concluded that DEG-1 is theanaphthoquinone. This observation might be important to the black tea beverage industry. It is possible that theaflavin in black tea beverage undergoes a change to theanaphthoquinone in aqueous solution. To date, little scientific information is available about the biological activities of theanaphthoquinone. It might be interesting to understand its biological activity and to examine its presence in commercial black tea beverage products.

In conclusion, theanaphthoquinone was identified as an oxidation product of theaflavin using DPPH and peroxidase/hydrogen peroxide oxidant model systems. This indicated that the benzotropolone moiety in theaflavin may play an important role in its antioxidant activity. The stability of theaflavin was examined in solutions of varying pH. The results confirmed that theaflavin is unstable in alkaline conditions in agreement with a recent report of Su et al. (*18*). In contrast, theaflavin was stable in acidic condition. Theanaphthoquinone was identified as an autoxidation product of theaflavin during its stability study in alkaline conditions. As shown in **Figure 2**, the formation of theanaphthoquinone from theaflavin can be facilitated in alkaline conditions.

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