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ARTICLE

Increase of Theaflavin Gallates and Thearubigins by Acceleration of Catechin Oxidation in a New Fermented Tea Product Obtained by the Tea-Rolling Processing of Loquat (*Eriobotrya japonica*) and Green Tea Leaves

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In a project to produce a new fermented tea product from non-used tea leaves harvested in the summer, we found that kneading tea leaves (*Camellia sinensis*) with fresh loquat leaves (*Eriobotrya japonica*) accelerated the enzymatic oxidation of tea catechins. The fermented tea obtained by tea-rolling processing of tea and loquat leaves had a strong, distinctive flavor and a plain aftertaste, which differed from usual black, green, and oolong teas. The phenolic constituents were similar to those of black tea. However, the concentrations of theaflavin 3-*O*-gallate, theaflavin 3,3′-di-*O*-gallate, and thearubigins were higher in the tea leaves kneaded with loquat leaves than in tea leaves kneaded without loquat leaves. The results from *in vitro* experiments suggested that acceleration of catechin oxidation was caused by the strong oxidation activity of loquat leaf enzymes and a coupled oxidation mechanism with caffeoyl quinic acids, which are the major phenolic constituents of loquat leaves.

KEYWORDS: Tea; loquat; catechin; oxidation; theaflavin; thearubigin; black tea

INTRODUCTION

Recently, polyphenol-rich beverages, especially tea and related products, have attracted more attention from food scientists, manufacturers, and consumers because of their health benefits (*1*−*3*). In Japan, however, there has been a reduction in the price of third-picked green tea, that is, the leaves that grow and develop during summer, and much of the green tea picked in summer and autumn is now discarded. The price of loquat (*Eriobotrya japonica*), a popular fruit cultivated in western Japan, has also been reduced, because of competition from low-cost imported fruits and an economic slump. In Japan, loquat leaves are traditionally used as folk medicine or as a tea, but this uses only a small proportion of the leaves. To use the unused tea and loquat leaves, a project was established to develop new tea manufacturing techniques. Previously, we reported that various plants, such as loquat and Japanese pear, have strong enzyme activity that oxidizes tea catechins (*4*). Therefore, we carried out a series of mixed fermentation experiments. After many trial studies, we found a promising method; that is, fresh tea leaves were mixed with loquat leaves (9:1, w/w) and then mechanically kneaded. After the mixture was dried, the tea product showed increased flavor and a plain aftertaste without astringency. We analyzed the extract using high-performance liquid chromatography (HPLC) and found that the addition of the loquat leaves had affected oxidation of catechin in the tea leaves. In this study, we investigated the chemical composition of the tea and the mechanisms by which loquat leaves accelerate catechin oxidation.

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MATERIALS AND METHODS

Materials. Fresh green tea leaves (*Camellia sinensis* var. *sinensis*, cv. Yabukita) were collected at the Higashisonogi Tea Branch, Nagasaki Agriculture and Forestry Experiment Station, Nagasaki, Japan. Fresh loquat leaves were collected at the Nagasaki Fruit Tree Experiment Station, Nagasaki, Japan. (−)-Epigallocatechin 3-*O*-gallate, (−)-epicatechin 3-*O*-gallate, (−)-epigallocatechin, and (−)-epicatechin were isolated from commercial green tea by column chromatography and purified by crystallization (*5*). Theaflavin, theaflavin 3-*O*-gallate, theaflavin 3′-*O*-gallate, theaflavin 3,3′-di-*O*-gallate, galloyl oolongtheanin, and theasinensins A and B were prepared by enzymatic oxidation of tea catechins, as described previously (*4*, *6*). Chlorogenic acid was isolated from *Vaccinium dunalianum* (*7*). All other chemicals were analytical-grade.

Analytical Procedures. We obtained UV/vis spectra with a JASCO V-560 UV/vis spectrophotometer (JASCO Co., Tokyo, Japan). The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded

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in a mixture of acetone-d_6 and D_2O (19:1, v/v) at 27 °C with a JEOL JNM-AL400 spectrometer operating at 400 MHz for ^1H and 100 MHz for ^{13}C (JEOL Ltd., Tokyo, Japan). Column chromatography was performed using 25−100 μm Sephadex LH-20 (GE Healthcare Bio-Science AB, Uppsala, Sweden), Diaion HP20SS (Mitsubishi Chemical, Tokyo, Japan), 75−150 μm MCI gel CHP 20P (Mitsubishi Chemical, Tokyo, Japan), 100−200 mesh Chromatorex ODS (Fuji Silysia Chemical, Kasugai, Japan), and TSK gel Toyopearl HW-40F (Tosoh, Tokyo, Japan). Thin-layer chromatography (TLC) was performed on precoated 0.2 mm thick Kieselgel 60 F_{254} plates (Merck, Darmstadt, Germany) with toluene/ethyl formate/formic acid (1:7:1, v/v/v) and $CHCl_3$/MeOH/water (14:6:1, v/v/v) and on 0.2 mm thick Cellulose F_{254} (Merck) with 2% AcOH. Spots were visualized using ultraviolet (UV) illumination and by spraying with 2% ethanolic $FeCl_3$ or 10% sulfuric acid reagent followed by heating. General HPLC analyses (conditions A) were conducted as follows: a 250 × 4.6 mm inner diameter, 5 μm, Cosmosil 5C_{18}-AR II (Nacalai Tesque, Inc., Kyoto, Japan) column with gradient elution from 4−30% (39 min) and 30−75% (15 min) of CH_3CN in 50 mM H_3PO_4; flow rate, 0.8 mL/min; and detection, JASCO photodiode array detector MD-910. Analyses of four theaflavins were carried out using HPLC conditions B, as follows: a 250 × 4.6 mm inner diameter, 5 μm, Cosmosil 5C_{18}-PAQ (Nacalai Tesque, Inc.) column with gradient elution from 10−25% (5 min) and 25−80% (40 min) of CH_3CN in 50 mM H_3PO_4; flow rate, 0.8 mL/min; and detection, UV 375 nm. Analyses of thearubigins were carried out using HPLC conditions C, as follows: a 250 × 4.6 mm inner diameter, 5 μm, Inertsil ODS-SP (GL Sciences, Inc., Tokyo, Japan) column with gradient elution from 10−23% (29 min) and 23−90% (1 min) and 90% (10 min) of CH_3CN in 50 mM H_3PO_4; column temperature, 35 °C; flow rate, 0.8 mL/min; and detection, UV 275 nm. HPLC conditions C were based on those used to analyze oolong tea polyphenols (*8*), with modifications.

Production of the Tea Kneaded with Loquat Leaves. The following procedure is not fully optimized for the production of the final tea product. Fresh tea leaves (27 kg) were partially dried by blowing air (70 °C) for 20 min in a primary tea-rolling dryer (60k-type, Kawasaki Co., Ltd., Shimada, Japan). The temperature of the leaves did not exceed 40 °C during this process. Then, the leaves were mixed with fresh loquat leaves (3 kg) and kneaded with a tea roller (60k-type, Kawasaki Co., Ltd.) at room temperature for 20 min. After a fermentation process for 0, 1, 2, 4, or 6 h at room temperature, the leaves were heated at 100 °C in a tea dryer (120k-type, Kawasaki Co., Ltd.) for 30 min to terminate enzymatic oxidation. The water content of the final tea leaves was less than 5%. Control tea products were prepared in a similar manner, except that loquat leaves were omitted.

Separation of Polyphenols of the Tea Kneaded with Loquat Leaves. The tea (125 g) was macerated with 70% acetone (3 L) in a Waring blender and extracted at room temperature. The mixture was filtered, and plant debris was extracted overnight at room temperature. The filtrates were combined and concentrated under reduced pressure until acetone had completely evaporated. The remaining insoluble precipitates, mainly composed of chlorophylls, were removed by filtration. The filtrate was partitioned with Et_2O to remove nonpolar substances, yielding the ether extract (8.7 g). The aqueous layer was concentrated and subjected to Sephadex LH-20 column chromatography (5 cm inner diameter × 35 cm) with water containing increasing proportions of MeOH (0−100%, 20% stepwise elution, each 500 mL) and finally 60% acetone (2 L) to give seven fractions: fraction 1 (18.7 g), fraction 2 (5.8 g), fraction 3 (3.1 g), fraction 4 (4.9 g), fraction 5 (6.0 g), fraction 6 (3.6 g), and fraction 7 (2.1 g). Analyses of the fractions using HPLC and TLC showed that fraction 1 mainly contained sugars, caffeine, and gallic acid. Fraction 2 contained caffeine and flavonol glucosides. Fraction 3 was a mixture of epicatechin and epigallocatechin, and fraction 4 contained theasinensin B, epicatechin, epigallocatechin, epigallocatechin 3-*O*-gallate, and epicatechin 3-*O*-gallate. Fraction 5 showed two major HPLC peaks attributable to epicatechin 3-*O*-gallate and epigallocatechin 3-*O*-gallate. Fraction 6 contained theasinensin A, theaflavin, and thearubigins, and fraction 7 was composed of theaflavin gallates and thearubigins. Fraction 6 was further separated by MCI-gel CHP20P column chromatography (3 cm inner diameter × 25 cm, 0−80% MeOH, 10% stepwise elution) to give theasinensin A (0.90 g), theaflavin (0.28 g) and fraction 6-2 (2.2 g). Separation of fraction 7 by MCI-gel CHP20P chromatography yielded a mixture of theaflavin gallates (0.6 g) and fraction 7-2 (1.2 g). Fractions 6-2 and 7-2, which contained mainly thearubigins, were combined and separated by a combination of column chromatography on Chromatorex ODS (H_2O/MeOH), Sephadex LH-20 (EtOH), TSK gel Toyopearl HW-40F (H_2O/MeOH), and Diaion HP20SS (H_2O/MeOH) to give oolongtheanin (39 mg) (*9*), (−)-epigallocatechin-3,3′(4′)-di-*O*-gallate (177 mg) (*5*), galloyl oolongtheanin (16 mg) (*9*), and thearubigins (1.27 g). The known compounds were identified by a comparison of ^1H NMR spectra to those of authentic samples.

Large-Scale Separation of Thearubigins, Theasinensin A, and Theaflavins. We extracted the tea kneaded with loquat leaves (1.93 kg) with H_2O (30 L) at room temperature overnight and then filtered the mixture. The filtrate was directly applied to a SephadexLH-20 column (10 cm inner diameter × 35 cm). The plant debris remaining on the filter paper was further extracted twice with 60% acetone (30 L), and the extract was concentrated under reduced pressure. Most of the phenolic compounds were extracted with aqueous acetone. We added MeOH to the resulting aqueous solution until the reddish brown precipitates (mainly composed of thearubigins, theaflavins, and caffeine) were dissolved. The final concentration of MeOH was approximately 20%. Insoluble precipitates, mainly composed of chlorophylls, were removed by filtration, and then the filtrate was applied to the aforementioned Sephadex LH-20 column. The column was washed with 20% MeOH (4 L), and then caffeine and flavonoid glycosides were eluted with 40% MeOH (4 L). Fractions containing epicatechin, epigallocatecin, epigallocatechin 3-*O*-gallate, epicatechin 3-*O*-gallate, and theasinensin B (52.3 g) were obtained by elution with 60−100% MeOH (20% stepwise, each 2 L). Acetone (60%, 6 L) was used to elute theasinensin A, theaflavins, and thearubigins (74.6 g). The last fraction was subjected to size-exclusion chromatography using Sephadex LH-20 (10 cm inner diameter × 35 cm) with acetone/7 M urea (3:2, v/v, adjusted to pH 2 with concentrated HCl) (*10*). The thearubigins with larger molecular weights eluted faster than theasinensins and theaflavins, and the fractions containing thearubigins (detected at origin on TLC) were combined. After evaporation of acetone, the aqueous solution was applied to a Diaion HP20SS column (5 cm inner diameter × 30 cm) and the column was washed with H_2O to remove urea and HCl. The polyphenols adsorbed on the gel were eluted using 80% MeOH to give thearubigins (21.4 g). Fractions of size-exclusion chromatography containing theasinensin A and theaflavins were concentrated, and the aqueous solution was applied to a Diaion HP20SS column (5 cm inner diameter × 30 cm) with H_2O containing increasing proportions of MeOH (10% stepwise) to yield theasinensin A (8.5 g) and a fraction mainly containing a mixture of theaflavins (30.1 g).

Thiol Degradation of Thearubigins. A solution comprised of the fractions containing thearubigins or thearubigins in 70% EtOH (25 mg/mL, 0.3 mL) was mixed with 5% mercaptoethanol in 60% EtOH containing 0.1% HCl (1.2 mL) and heated at 70 °C for 7 h. Seven small peaks were present in the HPLC analysis. The retention time and UV absorption of the peaks were comparable to those of standard samples of epicatechin (t_R = 24.1 min), epicatechin-4-hydroxyethylthioether (t_R = 28.9 min), epicatechin 3-*O*-gallate (t_R = 25.2 min), epigallocatechin 3-*O*-gallate-4-hydroxyethylthioether (t_R = 28.2 min), epicatechin 3-*O*-gallate (t_R = 31.8 min), epicatechin 3-*O*-gallate-4-hydroxyethylthioether (t_R = 34.9 min), and epiafzelechin 3-*O*-gallate-4-hydroxyethylthioether (t_R = 39.6 min) (*11*). The thearubigin fractions obtained from the control tea product showed similar peaks after thiol degradation; however, the peaks of epicatechin and epicatechin 4-hydroxyethylthioether were smaller than those observed for the thearubigin fraction of the tea kneaded with loquat leaves.

HPLC Analysis of Theasinensin A, Theaflavins, and Thearubigins. Pulverized tea leaves (2.0 g) (four samples of tea kneaded with loquat leaves and three samples of control tea produced in different trials) were extracted twice with 70% acetone (50 mL) at room temperature with occasional sonication. Acetone was removed with a rotary evaporator, and the volume of the resulting aqueous solution was adjusted to 50 mL. The solution was partitioned with EtOAc (50 mL) 3 times. The EtOAc solutions were combined and concentrated to dryness. The residue was dissolved in 70% EtOH (10 mL) and analyzed by HPLC (conditions A for catechins and theasinensin A and conditions B for theaflavins). The aqueous phase remaining after solvent partitioning was concentrated with a rotary evaporator to remove residual EtOAc. The resulting solution was acidified by the addition of a few drops of trifluoroacetic acid (pH < 4),

and the volume was adjusted to 100 mL. A portion (20 mL) was passed through a Sep-Pak Plus ODS cartridge (Waters, Milford, MA), and polyphenols adsorbed on the cartridge were eluted with 80% EtOH. The volume of the eluate was adjusted to 10.0 mL and analyzed by HPLC (conditions C for thearubigins). A single peak at 34.5 min was identified as thearubigins.

Separation of Loquat Leaf Constituents. Fresh loquat leaves (200 g) were homogenized with 70% MeOH (1 L) in a Waring blender and filtered. The plant debris was further extracted with 70% MeOH (1 L). The filtrate was combined and concentrated under reduced pressure below 40 °C. After removal of precipitates by filtration, the aqueous solution was subjected to Sephadex LH-20 column chromatography (4 cm inner diameter × 30 cm) with water containing increasing proportions of MeOH (0−100%, 10% stepwise elution) and then 60% acetone (1 L) to give three fractions. Fraction 1 (13.9 g) mainly contained sugars. A portion (1.0 g) of fraction 2 (2.17 g) was further separated by Chromatorex ODS column chromatography (2.5 cm inner diameter × 25 cm) with water containing increasing proportions of MeOH (0−100%, 10% stepwise elution) to yield 5-*O*-caffeoyl quinic acid (186.5 mg), chlorogenic acid (343.9 mg), and nerolidol 3-*O*-[α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-(α-L-rhamnopyranosyl-(1 → 6))]-β-D-glucopyranoside (125.4 mg) (*12*). A portion (1.5 g) of fraction 3 (3.07 g) was separated by Sephadex LH-20 column chromatography with EtOH to give EC (168 mg), dimeric procyanidins (193 mg), and trimeric procyanidins (299 mg), and elution of the column with 60% acetone yielded procyanidin oligomers (371 mg). Thiol degradation of fraction 3 yielded EC and epicatechin-4-hydroxyethylthioether. Our analyses indicated that the procyanidins were homogeneously composed of epicatechin units.

***In Vitro* Tea Fermentation with Loquat Leaves.** Fresh loquat leaves (5.0 g) were mixed with fresh tea leaves (45 g) and homogenized with H_2O (100 mL) in a Waring blender. The mixture was spread on a plastic dish (25 cm inner diameter) and stirred for 60 min at room temperature. Two additional experiments were carried out in the same conditions: one without loquat leaves and one with steamed loquat leaves (100 °C, 3 min). Each reaction mixture was mixed with EtOH, and the total volume was adjusted to 450 mL. After gentle stirring for 12 h, 2.5 μL of the extract was analyzed by HPLC.

***In Vitro* Tea Fermentation with Fractions of Loquat Leaf Extract.** Fresh tea leaves (45 g) were homogenized with H_2O (100 mL) at 0 °C in a Waring blender and filtered through four layers of gauze. A portion of the filtrate (2.0 mL, corresponding to 1.0 g of tea leaf) was added to an aqueous solution of fraction 1 obtained from fresh loquat leaves (0.10 mL, 70 mg/mL, corresponding to fraction 1 from 0.1 g of loquat leaf) and vigorously shaken (1500 rpm) for 90 min at room temperature. The mixture was heated in a microwave oven for 20 s and mixed with EtOH (2.9 mL). Three additional experiments were carried out under the same conditions: one with an aqueous solution of fraction 2 (0.10 mL, 14 mg/mL, corresponding to fraction 2 from 0.1 g of loquat leaf), one with fraction 3 (0.10 mL, 15 mg/mL, corresponding to fraction 3 from 0.1 g of loquat leaf), and one with H_2O instead of the loquat leaf extract. Each mixture was filtered though a membrane filter (0.45 μm) and analyzed by HPLC.

Oxidation of Epigallocatechin 3-*O*-Gallate and Epicatechin 3-*O*-Gallate with Japanese Pear Fruit Homogenate. Japanese pear fruits (50 g) were homogenized with H_2O (50 mL) and filtered through four layers of gauze. The filtrate (7.5 mL) was then mixed with an aqueous solution (2.5 mL) containing epigallocatechin 3-*O*-gallate (100 mg), epicatechin 3-*O*-gallate (20 mg), and chlorogenic acid (5 mg) and vigorously stirred at room temperature. Aliquots (0.2 mL) of the reaction mixture were taken at 15, 30, 60, 90, and 150 min and mixed with 0.8 mL of ethanolic *o*-phenylenediamine solution (0.5 mg/mL in 5% AcOH/EtOH). After filtration through membrane filters (0.45 μm), the mixtures were analyzed by HPLC (conditions A, detection at 280 nm). An experiment without chlorogenic acid was also performed under the same conditions.

Oxidation of Theaflavins with Loquat Leaf Homogenate. Loquat leaves (40 g) were homogenized with water (100 mL) and filtered through four layers of gauze. The homogenate (0.6 mL) was added to an aqueous solution (5 mg/1.0 mL) of a mixture of theaflavins obtained from the tea kneaded with loquat leaves by aforementioned large-scale separation, and the mixture was vigorously shaken (1500 rpm) for 5 min.

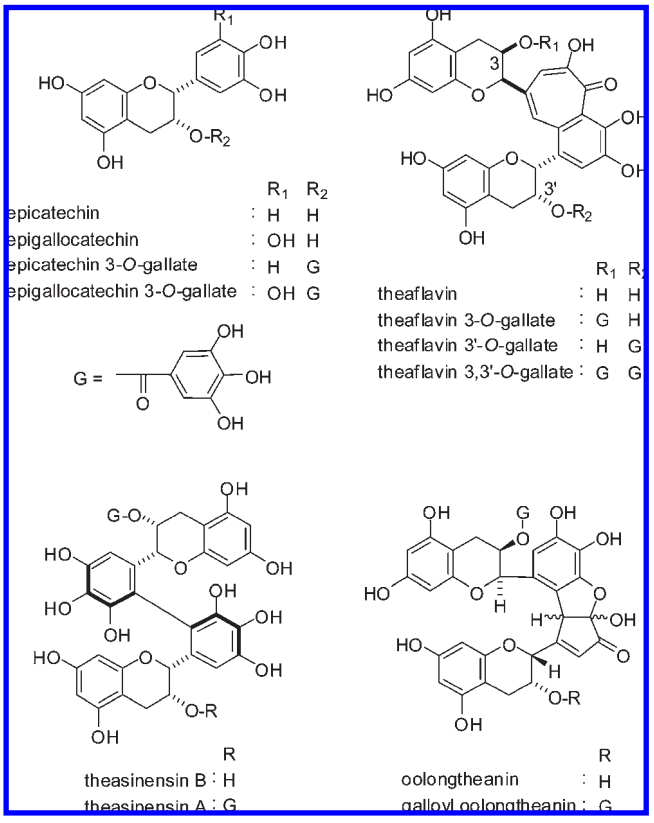

Figure 1. Structures of polyphenols in the fermented tea with loquat leaves.

An aliquot (0.1 mL) of the mixture was taken and mixed with EtOH (0.15 mL). After filtration through a membrane filter (0.45 μm), the filtrate was analyzed by HPLC (conditions B).

RESULTS AND DISCUSSION

Acceleration of Catechin Oxidation. In our research on the production of a polyphenol-rich functional tea, we found that kneading fresh tea leaves with a small amount of loquat leaves (10% of tea leaf weight) gave a fermented tea product with a characteristic flavor and a plain aftertaste, which differed from green, oolong, and black teas. HPLC analysis suggested that the phenolic composition of the tea kneaded with loquat leaves was similar to that of tea leaves kneaded without loquat leaves (control tea product). Major peaks were attributed to caffeine, epicatechin, epigallocatechin 3-*O*-gallate, epicatechin 3-*O*-gallate, theasinensin A, theasinensin B, and four theaflavins (theaflavin, theaflavin 3-*O*-gallate, theaflavin 3′-*O*-gallate, and theaflavin 3,3′-di-*O*-gallate). However, there was a lower concentration of catechins with *vic*-trihydroxyphenyl B rings (epigallocatechin and epigallocatechin 3-*O*-gallate) (**Figure 1**) in the tea kneaded with loquat leaf than in the control tea product (**Figure 2**). To confirm the difference in enzymatic catechin oxidation, the decrease in catechins were monitored during fermentation (**Figure 3**). The results clearly indicated that the addition of loquat leaves accelerated the oxidation of *vic*-trihydroxyphenyl-type tea catechins.

Polyphenol Composition of the Tea Kneaded with Loquat Leaves. We used a combination of chromatography with Sephadex LH-20, MCI-gel CHP20P, Toyopearl HW40F, and Chromatorex ODS to confirm the composition of phenolic constituents of the tea kneaded with loquat leaves. Besides the major compounds mentioned above, we isolated (−)-epigallocatechin-3,3′(4′)-di-*O*-gallate (*5*), oolongtheanin, and galloyl oolongtheanin (*9*) as minor

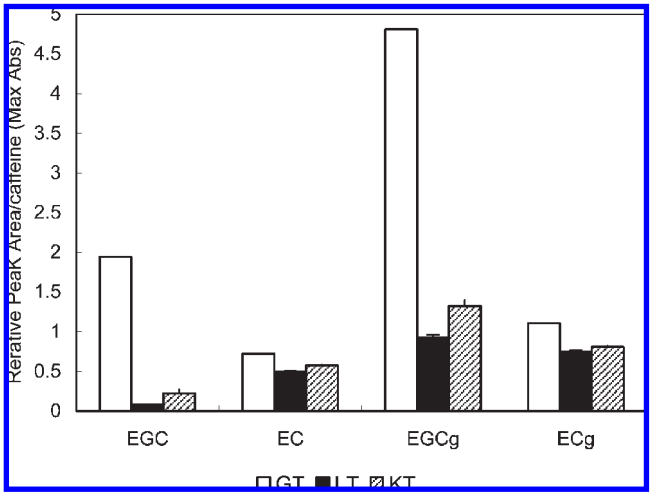

Figure 2. Decrease of tea catechins after 20 min of kneading in the presence (LT) or absence (KT) of loquat leaves. GT, dried fresh tea leaves used for production of the fermented tea; EGC, epigallocatechin; EC, epicatechin; EGCg, epigallocatechin 3-*O*-gallate; ECg, epicatechin 3-*O*-gallate.

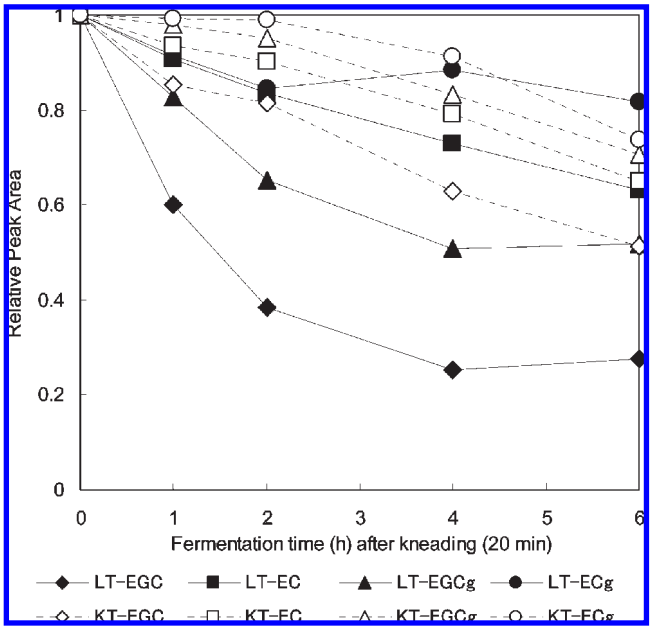

Figure 3. Decrease of tea catechins during fermentation after kneading. LT, catechins in leaves kneaded with loquat leaves; KT, catechins in leaves kneaded without loquat leaves. For the abbreviations of compounds, see **Figure 2**.

polyphenols. We did not detect compounds originating from loquat leaf in the HPLC analyses, except for epicatechin, which was one of the major polyphenols in tea leaves. In addition, a heterogeneous mixture of polyphenols comprised the major polyphenols of the tea kneaded with loquat leaf. These polyphenols were detected at the origin on TLC analysis and as a broad hump on the HPLC baseline (**Figure 4A**). Some of these polyphenols with relatively larger molecular weights were separated by size-exclusion column chromatography (*10*). The chromatographic behavior was similar to that of polymer-like polyphenols of black tea that possess lipase inhibitory activity (*13*). It is likely that these uncharacterized polyphenols are the same as or similar to the thearubigins, an ill-defined group of catechin oxidation products in black tea (*14*, *15*). In this paper, the

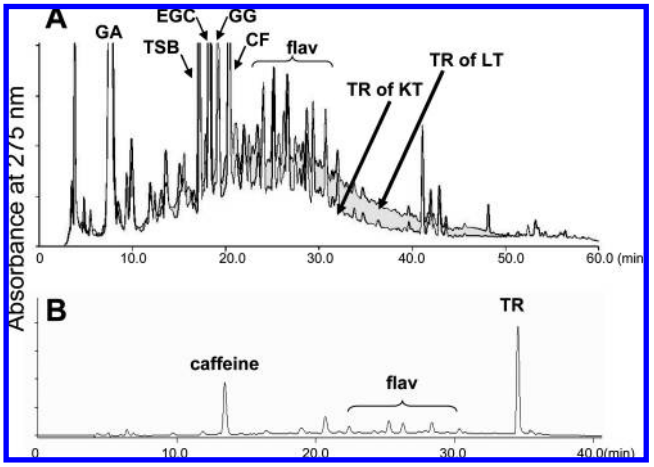

Figure 4. HPLC chromatograms of thearubigin-containing fractions. (A) HPLC (conditions A) of aqueous layers of the tea kneaded with loquat leaves and control tea product. (B) HPLC (conditions C) of aqueous layers of the tea kneaded with loquat leaves. TR, thearubigins; LT, tea knead with loquat leaves; KT, control tea product; TSB, theasinensin B; EGC, epigallocatechin; GG, digalloylglucose; CF, caffeine; flav, peaks of flavonol glycosides.

term thearubigins was provisionally used to represent these uncharacterized polyphenols. Loquat leaves contain procyanidins composed of epicatechin units. These were also detected as a broad hump on the baseline in the HPLC analysis. However, thiol degradation of the thearubigin fraction indicated that there was only a small amount of loquat procyanidin polymers among the polyphenols of the tea kneaded with loquat leaves. This was not unexpected, because the total polyphenol content of loquat leaves (about 3% of dried leaves, estimated from the weight of polyphenol-containing fractions of the loquat leaf extract and by the Folin−Ciocalteau method) is much lower than that of tea leaves (10−30%) (*16*, *17*). In addition, loquat leaves comprised only 10% of the mixture for the production of the tea product. Thus, polyphenols of the tea kneaded with loquat leaves were similar to those of black tea.

We compared the concentrations of major polyphenols of the tea kneaded to those of the control tea product (**Table 1**). In the analysis, theaflavins and theasinensin A were extracted with EtOAc by solvent partitioning and thearubigins remained in the aqueous layer. These components were estimated separately using different HPLC conditions. There were higher concentrations of theaflavin 3-*O*-gallate and theaflavin 3,3′-di-*O*-gallate in the fermented tea with loquat leaf than in the control tea product. These two theaflavins have strong α-glucosidase inhibition activities compared to other tea polyphenols (*18*). Both theaflavin 3-*O*-gallate and theaflavin 3,3′-di-*O*-gallate are derived from epigallocatechin 3-*O*-gallate, and the increase of these products may have resulted from accelerated epigallocatechin 3-*O*-gallate oxidation. However, oxidative degradation of theaflavin and theaflavin 3′-*O*-gallate may have occurred simultaneously (*19*).

Thearubigins in the aqueous layer of the tea kneaded with loquat leaves were detected as a broad hump on the baseline in HPLC (**Figure 4A**). A modified method originally used for analysis of oolong tea polyphenols (*8*) was applied to the analysis of thearubigins (**Figure 4B**), and the concentration of thearubigins was 24% higher in the tea kneaded with loquat leaves than in the control tea product (**Table 1**). However, we did not observe a change in the concentration of theasinensin A, a dimer of epigallocatechin 3-*O*-gallate found in oolong and black teas (*9*, *20*).

Table 1. Concentration of Polyphenols in the Fermented Tea with Loquat Leaf and Control Tea Product (mg/g of Dried Leaves)

	fermented tea with loquat leaf (*A*)	control tea product (*B*)	*A*/*B*
epigallocatechin 3-*O*-gallate	12.56 ± 5.91	22.55 ± 1.34	0.56
epicatechin 3-*O*-gallate	7.92 ± 2.90	11.12 ± 0.09	0.71
theasinensin A	5.74 ± 1.00	5.80 ± 0.43	0.99
theaflavin	0.65 ± 0.08	0.67 ± 0.04	0.96
theaflavin 3-*O*-gallate	0.90 ± 0.21	0.63 ± 0.04	1.43
theaflavin 3′-*O*-gallate	0.60 ± 0.06	0.58 ± 0.02	1.04
theaflavin 3,3′-di-*O*-gallate	1.25 ± 0.29	0.91 ± 0.06	1.38
thearubigins	20.31 ± 3.30	15.68 ± 1.25	1.24

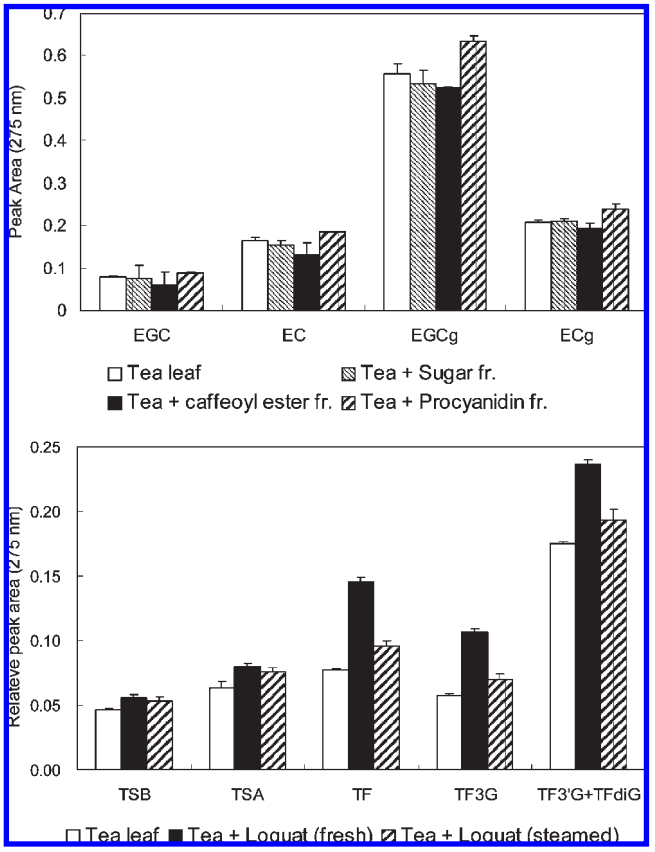

Figure 5. Effect of loquat leaf fractions on catechin oxidation in tea leaves. (A) Effect on oxidation products. (B) Effect on the decrease of tea catechins.

***In Vitro* Experiments.** When tea leaves were homogenized with fresh or steamed loquat leaves *in vitro*, the loquat leaves promoted the production of theaflavins and theasinensins (**Figure 5A**). This result clearly shows that loquat leaf enzymes play important roles in accelerating catechin oxidation (*4*). However, steamed leaves in which the enzymes were inactivated also increased the oxidation products but to a lesser extent. This result suggests that there is a heat-stable accelerating factor in loquat leaves. Next, we compared *in vitro* tea fermentation to three fractions of loquat leaf extract: a fraction containing mainly sugars, one containing caffeoyl esters, and one containing procyanidins (**Figure 5B**). For the experiments, we used fraction volumes that delivered an amount of compound similar to that in loquat leaves. The concentrations of epicatechin, epigallocatechin, and epigallocatechin 3-*O*-gallate in the fermented tea leaf treated with the caffeoyl ester fraction were lower than those treated with other fractions. The relatively rapid decrease of epigallocatechin and epigallocatechin 3-*O*-gallate compared to

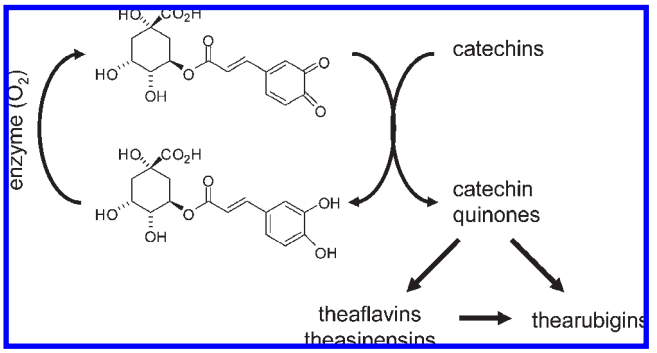

Figure 6. Coupling oxidation of tea catechins with chlorogenic acid.

epicatechin and epicatechin 3-*O*-gallate shown in **Figures 1** and **2** was due to a coupled oxidation reaction (*4*). In this reaction, enzymes preferentially oxidize *ortho*-dihydroxyphenyl-type catechins, such as epicatechin, and the resulting *o*-quinones oxidize the *vic*-trihydroxyphenyl-type catechins. This coupling oxidation mechanism was originally proposed for enzymatic oxidation of polyphenols in the presence of caffeoyl esters (*21*, *22*). Although the concentration of caffeoyl esters in the total mixture of tea and loquat leaves was relatively low (isolation yields of chlorogenic acid and 5-*O*-caffeoyl quinic acid from fresh loquat leaves were 0.04 and 0.02%, respectively), our results suggested that the caffeoyl esters take part in this coupling oxidation mechanism during the production of the tea kneaded with loquat leaves (**Figure 6**). The enzyme specificity for the *ortho*-dihydroxyphenyl ring of caffeoyl esters may be much higher than that for epicatechin. Interestingly, catechin oxidation was inhibited by the addition of the procyanidin fraction.

The effects of Japanese pear homogenate on the mixture provided further evidence for the coupling oxidation of tea catechins with chlorogenic acid. Japanese pear homogenate strongly oxidizes tea catechins, yielding oxidation products similar to those in fermented tea (*4*). In the present study, the enzymatic oxidation of a mixture of epigallocatechin 3-*O*-gallate and epicatechin 3-*O*-gallate was accelerated by the addition of Japanese pear homogenate. In our previous study, we showed that the oxidative coupling between epigallocatechin 3-*O*-gallate and epicatechin 3-*O*-gallate directly yields theaflavin 3,3′-di-*O*-gallate. In contrast, coupling between two molecules of epigallocatechin 3-*O*-gallate gives dehydrotheasinensin A (**Figure 7**), which was originally identified as a theasinensin A precursor (*6*). Subsequent decomposition of dehydrotheasinensin A upon heating or under weakly alkaline conditions produces theasinensin A, galloyl oolongtheanin, and a complex mixture of minor decomposition products. In addition, the presence of the epigallocatechin 3-*O*-gallate monomer quinone is also expected (*23*). Both dehydrotheasinensin A and quinone are unstable and cannot be detected as sharp HPLC peaks because of the presence of hydrated isomers. Thus, the reaction was monitored by HPLC after derivatization of the quinone metabolites by condensation with *o*-phenylenediamine (**Figure 7**). We observed accelerated consumption of epigallocatechin 3-*O*-gallate and epicatechin 3-*O*-gallate, accompanied by a slow decrease in the chlorogenic acid concentration (**Figure 8A**). The concentration of theaflavin 3,3′-di-*O*-gallate (**Figure 8B**) increased at the initial stage of the reaction (at 15 and 30 min). Chlorogenic acid also affected the production of dehydrotheasinensin A and EGCg-quinone; however, the increase was less than that of theaflavin 3,3′-di-*O*-gallate. This result is consistent with that observed for the production of the tea kneaded with loquat leaves (**Table 1**). The reason for the increase of theaflavin

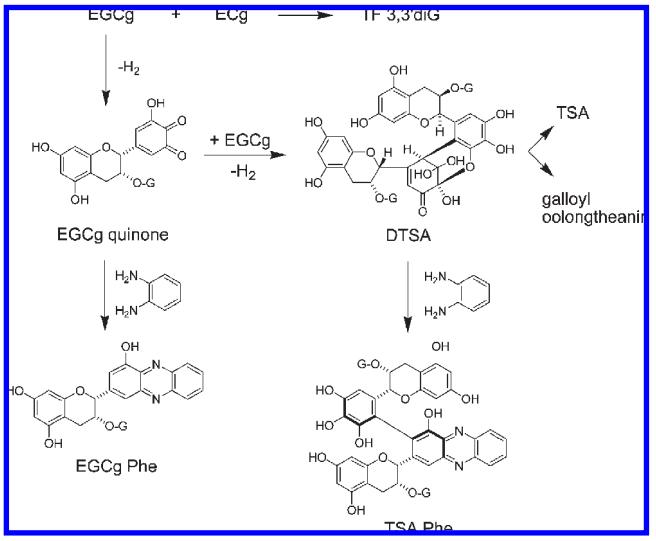

Figure 7. Oxidation of epigallocatechin 3-*O*-gallate and epicatechin 3-*O*-gallate and derivatization with *o*-phenylenediamine. +Ch, oxidation in the presence of chlorogenic acid; DTSA, dehydrotheasinensin A; TF3,3′diG, theaflavin 3,3′-di-*O*-gallate; Phe, phenazine derivatives.

3,3′-di-*O*-gallate is not clear. The coupling oxidation with chlorogenic acid may increase the oxidation of epicatechin 3-*O*-gallate as well as epigallocatechin 3-*O*-gallate.

To determine why theaflavins increased in the tea kneaded with loquat leaves, we compared the degradation of theaflavins *in vitro*. When a mixture of four theaflavins was treated with loquat leaf homogenate, theaflavin 3-*O*-gallate and theaflavin 3,3′-di-*O*-gallate decreased more slowly than theaflavin and theaflavin 3′-*O*-gallate (decreases after 5 min of reaction time: theaflavin, 35.9 ± 6.9%; theaflavin 3-*O*-gallate, 30.8 ± 4.4%; theaflavin 3′-*O*-gallate, 32.0±4.7%; theaflavin 3,3′-di-*O*-gallate, 30.8 ± 3.6%). Our previous results suggested that the oxidative degradation of the benzotropolone ring of theaflavin was promoted by the coupling oxidation mechanism with the catechol quinone (*4*). The relatively slow decrease of theaflavin 3-*O*-gallate and theaflavin 3,3′-di-*O*-gallate may be due to the stabilization of the benzotropolone moiety by stacking of the galloyl group located at the catechin C-3 position. Because galloyl groups are also oxidized (*24*−*26*), the balance between the production and degradation is important for theaflavin accumulation. It is likely to be the same for theasinensin A, and the complex cascade-type oxidation may result in thearubifgin production (*24*, *27*).

In the production of the new fermented tea, the most important effect of the loquat leaves was to accelerate catechin oxidation. The acceleration was caused by the strong enzyme activity of loquat leaves and the coupling oxidation with caffeoyl quinic acids. Our results suggest that a prolonged fermentation time may result in polyphenol composition similar to that of black tea. In fact, HPLC analysis showed that the products obtained by a 3 h fermentation after kneading were similar to those of commercial black tea. However, the tea produced after kneading leaves and with no fermentation process had a distinct flavor and a taste that was different from the usual black tea. Loquat leaves contain nerolidol glucoside (*12*), which is a characteristic sesquiterpene of *Citrus aurantium* flowers (neroli essential oil). The hydrolysis of the glycoside may have affected the flavor of the tea product. Detailed studies on volatile chemical constituents and the biological activities of this new tea product will be discussed elsewhere.

Supporting Information Available: HPLC chromatgrams of the tea knead with or without loquat leaves, including peak identification and a flowchart of chromatographic separation. This material is available free of charge via the Internet at http://pubs.acs.org.

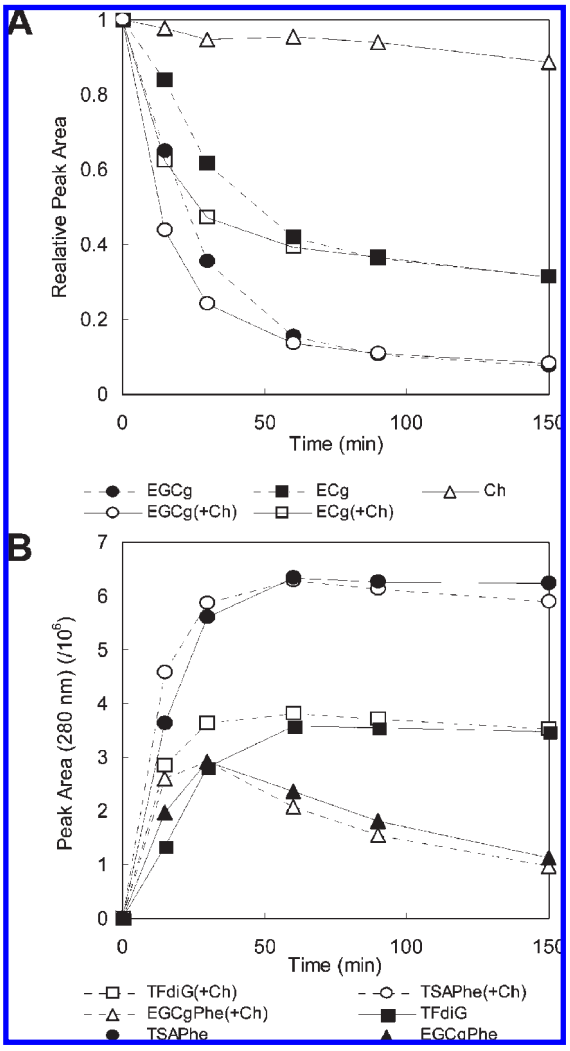

Figure 8. *In vitro* oxidation of a mixture of epigallocatechin 3-*O*-gallate and epicatechin 3-*O*-gallate in the presence of chlorogenic acid. (A) Decrease of epigallocatechin 3-*O*-gallate (EGCg), epicatechin 3-*O*-gallate (ECg), and chlorogenic acid (Ch). (B) Production of oxidation products. For the abbreviations of compounds, see **Figure 7**.

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