Biomimetic One-Pot Preparation of a Black Tea Polyphenol Theasinensin A from Epigallocatechin Gallate by Treatment with Copper(II) Chloride and Ascorbic Acid

Takuya Shii, Makoto Miyamoto, Yosuke Matsuo, Takashi Tanaka,* and Isao Kouno

Graduate School of Biomedical Sciences, Nagasaki University; 1–14 Bunkyo-machi, Nagasaki 852–8521, Japan.
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Chromatographic separation of black tea polyphenols is too difficult to supply sufficient quantities of pure compounds for biological experiments. Thus, facile methods to prepare black tea constituents were desired. Treatment of epigallocatechin gallate with copper(II) chloride efficiently afforded an unstable quinone dimer, dehydrotheasinensin A, and subsequent treatment with ascorbic acid stereoselectively yielded theasinensin A. The latter is a dimer with an *R*-biphenyl bond, one of the major polyphenols found in black tea. The method is simpler and more effective than enzymatic preparation.

Key words theasinensin A; black tea; epigallocatechin gallate; dehydrotheasinensin A; copper(II) chloride

Black tea is one of the most popular beverages in the world, accounting for 70% of global tea production.[1] It is produced by mechanical distortion and bruising of fresh tea leaves and contains abundant polyphenols, generated by enzymatic oxidation of tea leaf catechins. Theasinensin A (**3**)[2—4] is a biologically active black tea polyphenol,[5—7] produced by enzymatic oxidation of epigallocatechin gallate (**1**), the most abundant polyphenol found in fresh tea leaves.[1] Experiments using fresh tea leaves proved that the production mechanism of **3** is not simple oxidative coupling.[8] *In vitro* model experiments to mimic black tea production revealed that the enzymes oxidize **1** to *ortho*-quinone and subsequent dimerization of the quinone affords an unstable dimer, named dehydrotheasinensin A (**2**).[9] On heating and drying of the tea leaves in the final stage of black tea production, **2** undergoes redox dismutation to give reduction products **3** and its atropisomer, along with a complex mixture of oxidation products, including compounds produced by oxidative cleavage of aromatic rings[9] (Chart 1). It is also known that reduction of **2** with reducing agents, such as mercaptoethanol and ascorbic acid, afford only **3**, with an *R*-biphenyl bond. Stereoselective formation of **2** from **1** was accounted for by the presence of a chiral center at the benzylic position of the pyrogallol ring.[10,11] Recently various health benefits of black tea polyphenols have been reported.[12,13] However, biological activities of each polyphenol component have not been studied sufficiently, compared with green tea polyphenols. Because black tea polyphenols are a complex mixture of catechin oxidation products, including polymeric substances,[14] chromatographic separation of each polyphenol component is too difficult to supply sufficient quantities of pure compounds for biological experiments. Therefore, development of methods for selective preparation of each black tea polyphenol is desired. Theaflavins,[15,16] which are characteristic pigments of black tea with the benzotropolone moiety, can be prepared by *in vitro* enzymatic and chemical oxidation of readily available green tea catechins.[17,18] As for theasinensins, the yields of chemical and enzymatic preparations from tea catechins are low.[9,19] Therefore, in this study we developed a facile and biomimetic method to prepare **3** and its analogs.

Results and Discussion

The oxidative enzymes, tyrosinase and catecholoxidase, are ubiquitous plant enzymes containing a binuclear copper center.[20,21] They oxidize *o*-diphenols to the corresponding *o*-quinones, coupled with the reduction of molecular oxygen to water. To mimic the oxidation of catechins in tea leaves, **1** was treated with various copper salts ($CuSO_4$, $Cu(CH_3COO)_2$, $CuCO_3$, $CuCl_2$) and ferrous salts ($FeSO_4$, $K_3[Fe(CN)_6]$, $FeCl_3$) in aqueous MeOH and vigorously stirred to mix with oxygen from the air. HPLC comparison of the reaction mixtures indicated that $CuCl_2$ is the most effective for oxidation of **1** to **2**. The amount of $CuCl_2$ was shown to be at least 0.5 molar equivalents, and the amount affected the reaction rate. Mixing of air (oxygen) into the reaction mixture regenerates $CuCl_2$. The optimum concentration of MeOH in the reaction solvent was 30%. A higher concentration of MeOH increased uncharacterized byproducts, while a lower concentration slowed the rate of oxidation. Other solvents, such as aqueous acetonitrile, afforded complex mixtures. The optimum pH of the reaction mixture was shown to be 4 to 5 and addition of buffer agents was unnecessary. At higher pH, decomposition

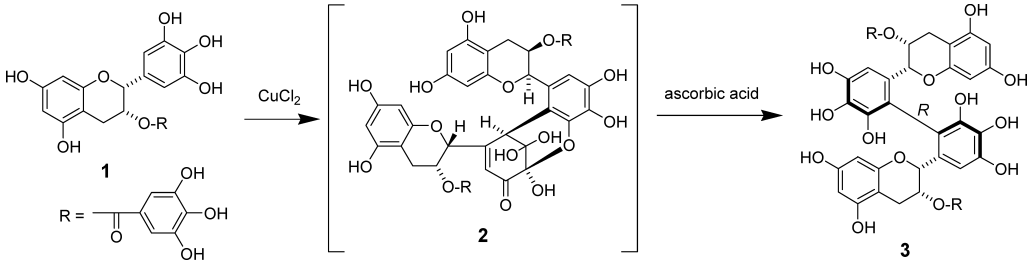

Chart 1. Production of Theasinensin A (**3**) from Epigallocatechin Gallate (**1**) *via* Dehydrotheasinensin A (**2**)

* To whom correspondence should be addressed. e-mail: t-tanaka@nagasaki-u.ac.jp

of product **2** was observed.[9] Addition of imidazole, ethylenediamine, and oxalic acid as ligands was not effective. The initial oxidation step of the reaction was carried out at room temperature and elevation of the reaction temperature increased byproducts.

The oxidation product **2** is unstable and gradually decomposes during isolation.[9] Therefore, **2** was reduced without separation and the reduction was successfully achieved by heating with ascorbic acid. On the heating, increase of byproducts was not observed, probably because reduction proceeded preferentially and the metal ions were removed by complexation with ascorbic acid. Reduction with mercaptoethanol instead of ascorbic acid reduced the yield of **3**.

A reaction under optimized conditions is as follows: **1** (2.2 mmol) and $CuCl_2$ (2.00 mmol) were dissolved in 30% MeOH and vigorously stirred to mix air into the solution at room temperature for 24 h. The HPLC profile at this stage is shown in Fig. 1B. The reaction mixture was heated with an excess amount of ascorbic acid and separated by Diaion HP20 column chromatography to afford **3** (0.58 mmol, 53%). The yield was much higher than that by enzymatic preparation of **3** (usually less than 30%[19]). Further elution of the column gave a complex mixture of dimeric and oligomeric products. A large part of black tea polyphenols remain to be chemically identified due to difficulty in purification.[22,23] These byproducts are probably related to the unknown black tea polyphenols and chemical characterization is now in progress.

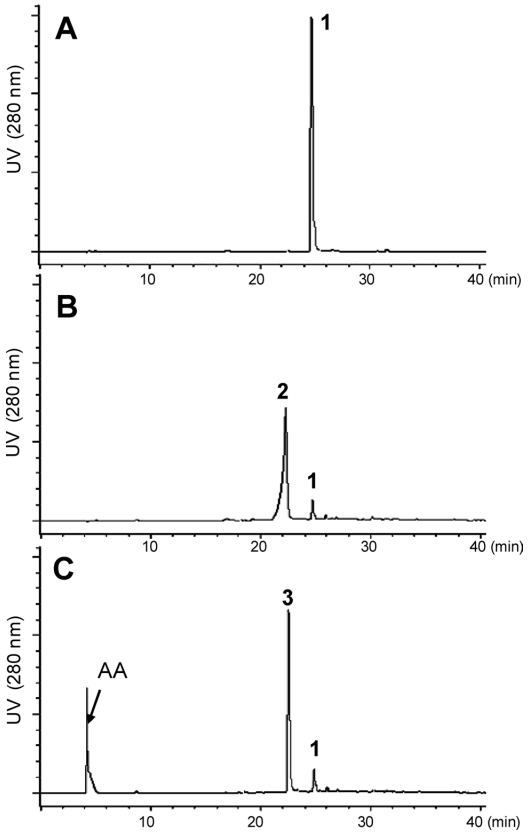

Fig. 1. HPLC Profiles of the Reaction Mixture of **1** with $CuCl_2$ and Ascorbic Acid

(A) Starting material, (B) after treatment with $CuCl_2$ (24 h), (C) after treatment with ascorbic acid (AA) (85 °C, 15 min). Peak **2** was broadened due to equilibrium between hydrated quinone structures, detection: 280 nm.

Animal experiments normally require pure samples at gram scale. Considering practicality and cost, the price of **1** as a starting material is too high and therefore the reaction was subsequently applied to commercially available green tea polyphenol mixtures, which have recently become less expensive. The extract contained mainly **1**, epicatechin gallate, epigallocatechin, epicatechin, and their isomers (Fig. 2A). After treatment of the mixture with $CuCl_2$, HPLC analysis (Fig. 2B) revealed that **1** and epigallocatechin were selectively oxidized to dehydrotheasinensins (**2**, **4**—**6**)[8] (Fig. 3). Catechol-type catechins were not oxidized because the redox potential is higher than that of pyrogallol-type catechins.[24,25]

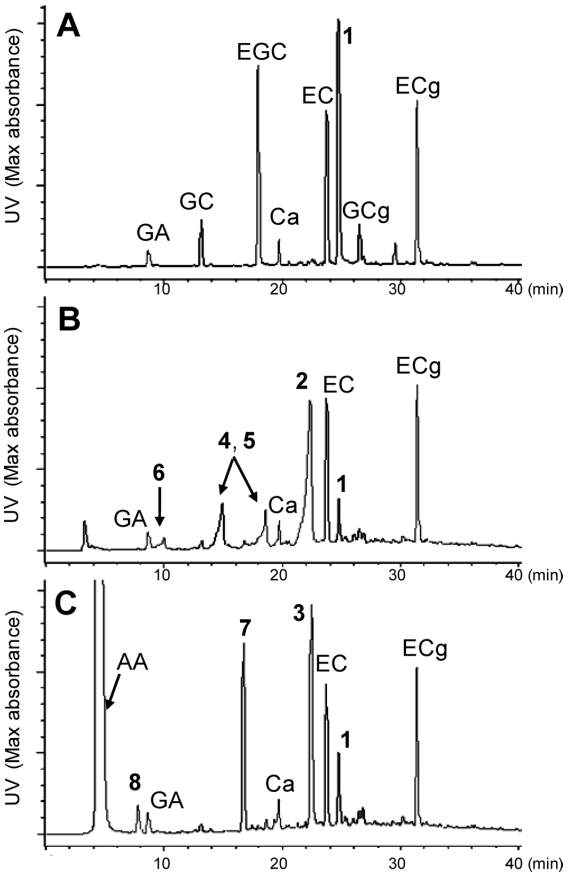

Fig. 2. HPLC Profiles of the Reaction Mixture of Tea Polyphenols with $CuCl_2$ and Ascorbic Acid

(A) A commercial tea catechin mixture, (B) after treatment with $CuCl_2$ (24 h), (C) after treatment with ascorbic acid (85 °C, 15 min). GA: gallic acid, GC: gallocatechin, EGC: epigallocatechin, Ca: catechin, EC: epicatechin, ECg: epicatechin gallate, GCg: gallocatechin gallate, AA: ascorbic acid.

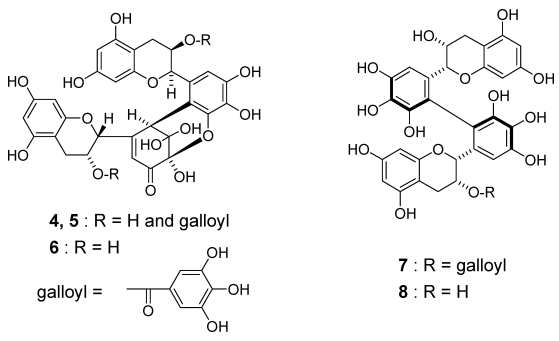

Fig. 3. Structures of **4**—**8**

Subsequent reduction with ascorbic acid yielded theasinensins B (**7**) and C (**8**) as well as **3**, which are easily separable by Diaion HP20 and Sephadex LH-20 column chromatography.

The concentration of theasinensins in black tea is comparable to that of theaflavins.[26,27] However, their biological activities have not been fully evaluated because of difficulties with supply of pure compounds, due to the complexity of black tea polyphenols. Our results provide a facile and efficient method for preparation of pure theasinensins. In addition, it is important that the reactions mimic the oxidation of catechins in tea leaves during black tea production. Dehydrotheasinensins (**2**, **4**—**6**) are key intermediates of oxidation of epigallocatechin and its gallate, which in total account for over 70% of tea catechins. Production of dehydrotheasinensins during black tea production has been confirmed[8] but they are not detected in the final products. Understanding the degradation of dehydrotheasinensins is essential for characterization of unknown black tea polyphenols. Detailed analysis of the minor reaction products will contribute to black tea chemistry. The difference between enzymatic oxidation in the tea leaves and the *in vitro* reaction with $CuCl_2$ is that the tea leaf enzymes preferentially oxidize catechol-type catechins rather than pyrogallol-type catechins, which are chemically more sensitive to oxidation.[18] Oxidative couplings between the quinones produced from catechol-type and pyrogallol type catechins generate theaflavins.[15—17,28] In the present experiments, the catechol-type catechins were not oxidized and thus theaflavins were not detected in the reaction products. Enzymatic oxidation of a commercially available mixture of green tea catechins produces theaflavins as well as theasinensins. However, the reactions are accompanied by production of many other, uncharacterized oxidation products, produced by complex quinone coupling reactions as well as further degradation of theaflavins.[29,30] Chromatographic separation of theaflavins and theasinensins from the resulting complex mixture is problematic. The method for preparing theasinensins from tea catechins described here outperforms the enzymatic preparation in terms of selectivity, cost, and purification of the products.

Experimental

General Column chromatography was performed using Diaion HP20SS (Mitsubishi Chemical Co., Japan). Thin-layer chromatography (TLC) was performed on 0.2-mm-thick precoated Kieselgel 60 F_{254} plates (Merck) using toluene–ethyl formate–formic acid (1 : 7 : 1, v/v) or cellulose F_{254} (Merck) uisng 2% AcOH. Spots were detected by UV illumination, sprayed with 2% methanolic $FeCl_3$. Analytical reverse-phase HPLC was performed on a Cosmosil $5C_{18}$-AR II column (Nacalai Tesque Inc., Japan; 4.6 mm i.d.×250 mm) using an elution gradient of 4—30% (39 min) and 30—75% (15 min) CH_3CN in 50 mM H_3PO_4 (flow rate 0.8 ml/min; detection uisng a JASCO photodiode array detector MD-910). Tea catechin mixture was purchased from Guilin Layn Natural Ingredients Corp. (Guilin, China). Standard samples of theasinensins A—C were synthesized from **1** and epigallocatechin by enzymatic oxidation.[9,10]

Oxidation of 1 in Small Scale Tea catechin mixture (5 mg) or **1** (5 mg, 0.01 mmol) was added to a 30% MeOH (2.0 ml) solution containing $CuCl_2$ or other metal salts (0.01 mmol) and shaken vigorously at room temperature for 24 h. was ascorbic acid (50 mg) was added to the solution and heated at 80—85 °C for 15 min.

Oxidation of 1 and Separation of 3 A solution of **1** (1.0 g, 2.18 mmol) and $CuCl_2$ (269 mg, 2.00 mmol) in 30% MeOH (400 ml) was vigorously stirred to mix air into the solution at room temperature for 24 h. The HPLC profile at this stage is shown in Fig. 1B. To the reaction mixture, an excess amount of ascorbic acid (10 g) was added and heated at 85 °C for 15 min. After cooling, the mixture was concentrated to evaporate MeOH and the resulting aqueous solution was applied to a Diaion HP20 column (3.0 cm i.d.×25 cm) with water. After washing the column with water to remove reagents, gradient elution with water containing MeOH (5% stepwise gradient, each 100 ml) afforded **3** (0.533 g, 0.58 mmol, 53%).

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