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Laccase-catalyzed conversion of green tea catechins in the presence of gallic acid to epitheaflagallin and epitheaflagallin 3-*O*-gallate

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Abstract—Green tea extract containing catechin mixtures was treated with gallic acid using laccase (EC 1.10.3.2) to improve its function as a food material. After enzymatic oxidation, two new products were detected on an HPLC chromatogram. These products were purified by extraction with ethyl acetate, sequential column chromatographies, and crystallization. On the basis of $^{1}H/^{13}C$ NMR, MALDI-TOF/high resolution MS, and UV–visible absorption spectral analyses, they were confirmed to be epitheaflagallin (**5**) and epitheaflagallin 3-*O*-gallate (**6**). This enzymatic process allows us to preferentially convert catechin derivatives in a crude mixture of green tea into different compounds.

1. Introduction

Tea is one of the most widely consumed beverages in the world. More than 300 different kinds of tea are produced from the leaves of *Camellia sinensis* by different fermentation processes, and these can be divided into three general types: green tea (non-fermented), oolong tea (semi-fermented), and black tea (fermented). World-wide tea production has reached 2.97×10^{6} metric tons/year, and more than 75% of tea products are black tea. In recent years, green tea containing catechin derivatives such as (−)-epicatechin (EC) (**1**), (−)-epicatechin gallate (ECG) (**2**), (−)-epigallocatechin (EGC) (**3**), (−)-epigallocatechin gallate (EGCG) (**4**) have been recognized as a useful functional food material;[1–3] this tea is popular in Japan, China, and Korea. To date, there have been many epidemiological or physiological studies of green tea, including those investigating a reduction in the risk of cardiovascular diseases, diabetes, and cancer. Recently many beneficial effects of green tea have been attributed to its abundant catechin, EGCG (**4**).[4]

In this paper, we describe the application of fungal laccase (benzenediol/oxygen oxidoreductase, EC 1.10.3.2),[5] which belongs to a group of polyphenol oxidase containing copper atoms in the catalytic center, to the bioconversion of green tea extracts. We confirm that epitheaflagallin (**5**) and epitheaflagallin 3-*O*-gallate (**6**) are preferentially produced from **3** and **4** in the presence of gallic acid. We also found that these two products are very minor components of orange-red pigments in black tea.

2. Results and discussion

2.1. Conversion of green tea catechins by laccase in the presence of gallic acid

Laccase was used to synthesize two benzotropolone derivatives (Fig. 1) from crude tea catechins in the presence of oxygen and gallic acid; one product was **5** and the other was **6**, which were previously synthesized from **3/4** with catechol/pyrogallol by Takino and Imagawa.[6] Using authentic compounds including **1**–**4**, we found that **5** and **6** were synthesized from **3** and **4**, respectively, by the oxidation reaction of laccase with gallic acid. The reaction involves the preferential oxidation of gallic acid and the pyrogalloyl group (B ring) in **3** and **4** to the quinone intermediate (galloquinone),[7] followed by the Michael addition of the oxidized pyrogalloyl group in **3/4** prior to a carbonyl addition across the ring and subsequent decarboxylation (Fig. 2). Recently, Sang et al. reported the enzymatic synthesis of tea theaflavin derivatives

Keywords: Laccase-catalyzed oxidation; Gallic acid; Epitheaflagallin; Epitheaflagallin 3-*O*-gallate; Green tea catechins.
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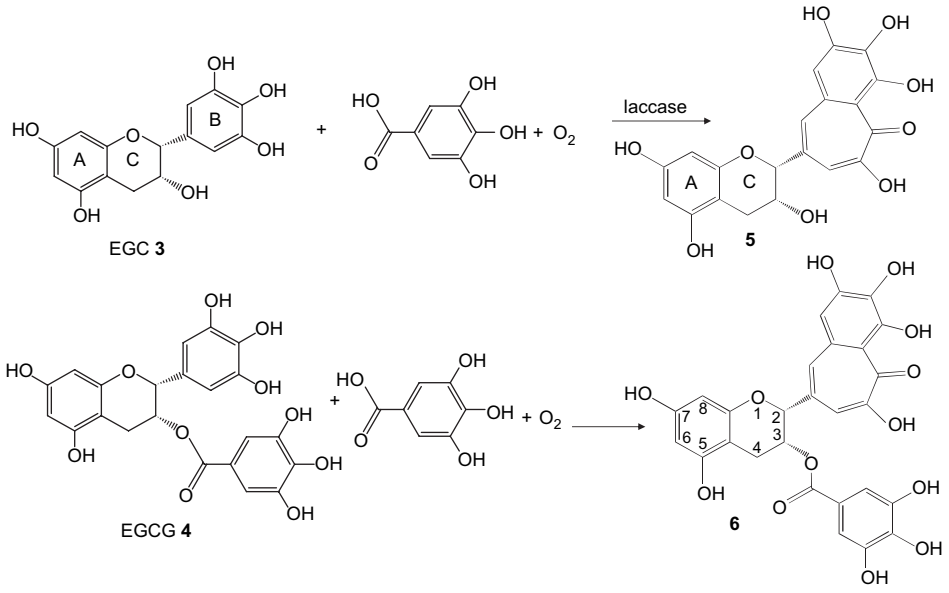

Figure 1. Reaction of EGC (**3**) and EGCG (**4**) with gallic acid by laccase-catalyzed oxidation.

from catechin derivatives and hydrogen peroxide using peroxidase.[8,9] In the case of peroxidase oxidation, the catechoyl/pyrogalloyl/galloyl group in EC (**1**)/ECG (**2**)/EGC (**3**)/EGCG (**4**) is easily oxidized to form the quinone intermediates to give various theaflavin-related compounds. In our oxidation system, preferential conversion of **3** and **4** was possible, and the EC (**1**) and ECG (**2**) hardly reacted with galloquinone intermediate to be left in the reaction mixture (Fig. 3a and c). Laccase-dependent preferential oxidation of gallic acid and the pyrogalloyl group in **3** and **4** to galloquinone intermediate made it possible to convert **3** and **4** in the crude tea catechins to **5** and **6**. The proposed mechanism for this reaction was supported by the observation that the addition of gallic acid to the reaction mixture apparently accelerated the productions of **5** and **6** in the HPLC chromatogram (Fig. 3a and b). We presumed that the low level productions of **5** and **6** without gallic acid should be due to the free gallic acid in green tea extracts. We also confirmed that a laccase reaction gradually oxidized authentic **3/4** without gallic acid to give unknown compounds, however, this reaction should be suppressed in crude green tea extracts when gallic acid was used as an external additive, because the reaction would rapidly occur between oxidized gallic acid and **3/4**. Matuo et al. reported that **3** was oxidized with a Japanese pear homogenate containing polyphenol oxidase to give quinone dimers, one of which was converted to theaflavin-related compounds, and the other minor one was converted to **5** and hydroxytheaflavin.[10]

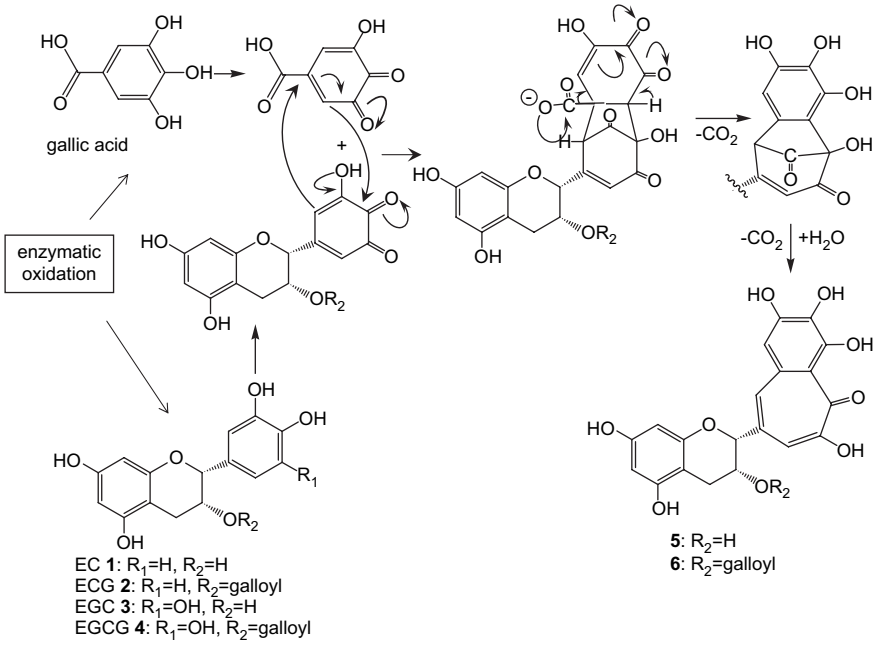

Figure 2. The proposed mechanism of epitheaflagallin (**5**) and epitheaflagallin 3-*O*-gallate (**6**) formation.

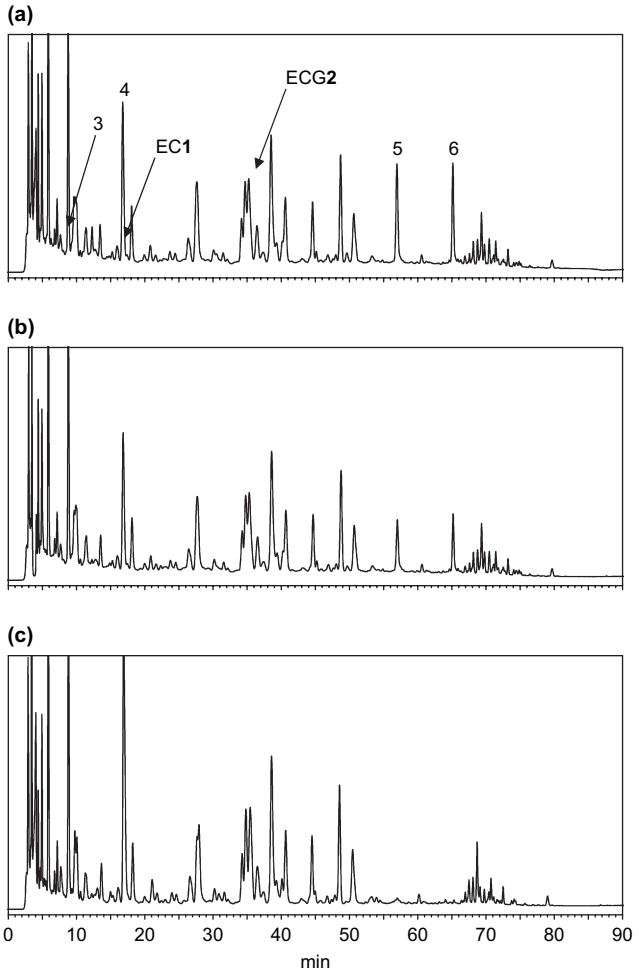

Figure 3. HPLC analysis of products from green tea extract. (a) With laccase in the presence of gallic acid. (b) With laccase in the absence of gallic acid. (c) Not treated.

Therefore, laccase oxidation of **3/4** without gallic acid might form such compounds.

Although the reaction was not optimized for each substrate in the crude tea catechins, conversion yields of **5/6** were less than 20%. This suggested that the reaction did not stoichiometrically proceed, in spite of the observation that **3/4** in the reaction mixture was almost consumed. We have not yet determined, which products were synthesized by the reaction besides **5** and **6**. We speculate that highly polymerized compounds of **3/4** were formed by the oxidation with gallic acid, since there were no peaks observed on an HPLC chromatogram under general conditions.

This biooxidation process converting **3** and/or **4** to epitheaflagallin derivatives could be applicable to the production of useful functional food materials, since **6** is known to induce apoptosis of cancer cells.[11]

2.2. Purification of products and determination of epitheaflagallin (5) and epitheaflagallin 3-*O*-gallate (6)

In order to elucidate the chemical structure of products **5** and **6**, they were purified from the reaction mixture as described in Section 4. The products were extracted with ethyl acetate from the reaction mixture (2 l), which contained 250 g of green tea extract, 20 g of gallic acid monohydrate, and 200,000 units of laccase. After evaporation, about 90 g of the residual substances was subjected to sequential reverse-phase and oxalic acid-treated silica gel column chromatographies. We observed that the latter chromatography was very effective in eliminating minor impurities from the products. Finally we obtained 10 mg of an orange crystal of **5** from an ethyl acetate/*n*-hexane solution, and 14 mg of orange-red crystal of **6** from an ethanol/water solution.

The structures of the products **5** and **6** have been confirmed by MS and NMR spectroscopic analyses. The molecular formula of product **5** was determined to be $C_{20}H_{16}O_9$ by ESI-MS ($[M+H]^+$ at *m/z* 401.0869) as well as by the 20 carbon signals of its ^{13}C NMR spectral data, and MALDI-TOF MS data ($[M+H]^+$ and $[M+Na]^+$ at *m/z* 401 and 423). The ^1H and ^{13}C NMR spectral data for **5** were similar to those of EGC (**3**), and its ^1H NMR spectrum indicated the existence of protons conserved in the A and C rings in the flavan-3-ol nucleus. These observations indicated that the A and C rings of **5** did not undergo any change during oxidation. In the ^{13}C NMR spectrum of **5**, besides the signals of carbons in A and C rings, there were 11 signals involving one carbonyl (δ_c 183.4 ppm) and 10 olefinic carbons. The UV–visible absorption spectrum of **5** indicated the characteristic peaks at 281, 306, and 430 nm. These data, together with the data from the heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond coherence (HMBC) experiments, supported the presence of a benzotropolone group, and coincided with the data from the chemically synthesized epitheaflagallin reported by Takino et al.[12] and Nonaka et al.[13]

The molecular formula of product **6** was determined to be $C_{27}H_{20}O_{13}$ by ESI-MS ($[M+H]^+$ at *m/z* 553.0993), which was in agreement with the carbon signals from the ^{13}C NMR spectrum, and MALDI-TOF MS data ($[M+H]^+$ and $[M+Na]^+$ at *m/z* 553 and 575). In the ^1H NMR spectrum of **6**, the signals of C-2 (δ_H 4.81 ppm) and C-3 (δ_H 4.27 ppm) hydrogens observed in the flavan-3-ol moiety of **5** were shifted to a lower magnetic field and two carbonyl carbon signals in ^{13}C NMR spectrum (δ_c 183.4 ppm for benzotropolone ring and 167.4 ppm for ester) were observed, suggesting the ester formation of a C-3 hydroxyl group. It was confirmed that product **6** was a gallate ester of product **5** at the C-3 position, which had originated from EGCG (**4**). Other spectroscopic data including those obtained in the HMQC and HMBC experiments, asserted that product **6** is epitheaflagallin 3-*O*-gallate, and these data coincided with those reported by Nonaka et al.[13] and Sang et al.[9]

2.3. Detection of epitheaflagallin (5) and epitheaflagallin 3-*O*-gallate (6) in black tea extracts

To detect the presence of **5** and **6** in natural tea products, four kinds of black tea extracts (A–D) were analyzed. Figure 4 displays the HPLC chromatogram for sample A. We found that epitheaflagallin derivatives were minor components of all of the black tea extracts tested, with the exception of sample C (Table 1). The black tea extracts contained a mean of approximately 0.1% (w/w) of **5** and **6**. The content of **6** was approximately 1.8 times higher than that of **5** in three

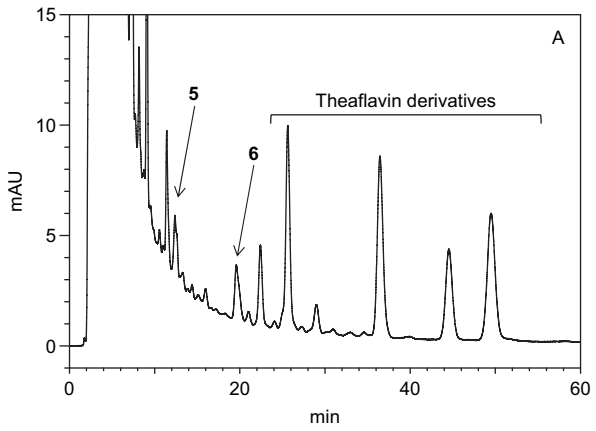

Figure 4. HPLC analysis of epitheaflagallin (**5**) and epitheaflagallin 3-*O*-gallate (**6**) in black tea extract. The HPLC conditions given in 'Section 4' were modified: detection was at 280 nm and operation at 30 °C.

Table 1. Contents of epitheaflagallin (**5**) and epitheaflagallin 3-*O*-gallate (**6**) in black tea extracts

Content[b] (mg/g extract)	Sample[a]			
	A	B	C	D
Epitheaflagallin **5**	0.35	0.46	N.D.[c]	0.25
Epitheaflagallin 3-*O*-gallate **6**	0.57	0.84	N.D.	0.47

[a] FD/SD black tea extracts with different lot numbers were purchased from San-Ei Gen F.F.I., Inc., Osaka, Japan.
[b] The contents of **5** and **6** were calculated from each area on the HPLC chromatogram using the calibration curves made for purified **5** and **6**.
[c] N.D.: not detected.

samples. These results indicate that epitheaflagallin derivatives are naturally synthesized as minor components in the general tea fermentation process.[14]

3. Conclusion

Epitheaflagallin (**5**) and epitheaflagallin 3-*O*-gallate (**6**), which are minor components of black tea, were preferentially synthesized from EGC (**3**) and EGCG (**4**) in green tea extracts using laccase and gallic acid. This biooxidation process could be applicable to the production of epitheaflagallin derivatives to improve the functionality of green tea.

4. Experimental

4.1. Reaction components and enzyme assay

Laccase from *Trametes* sp. (Daiwa Y120, 120,000 U/g) was obtained from Amano Enzyme Co., Ltd., Nagoya, Japan. The enzymatic activity was spectrophotometrically assayed at 30 °C by measuring the formation of quinonimine dye at 505 nm from phenol and 4-aminoantipyrine (pH 4.5), according to the manufacturer's protocol. The reaction mixture consisted of 83.3 μmol phenol, 3 μmol 4-aminoantipyrine, and 167 μmol acetate buffer (pH 4.5), and a diluted enzyme solution in a total volume of 1.0 ml. One unit of the enzyme was defined as the amount that increased the absorbance at 505 nm by 0.1 optical density per min.

The reaction mixture used to isolate the products consisted of 250 g of green tea extract (SD green tea extract, San-Ei Gen F.F.I., Inc., Osaka, Japan), 20 g of gallic acid monohydrate, 1.66 g of laccase (200,000 U), and 50 mM sodium phosphate buffer (pH 2.4) in a total volume of 2 l. The reaction proceeded for 10 h at 55 °C. Analytical HPLC was performed with a Shimadzu LC-10AT$_{VP}$ system using a YMC-Pack ODS-A312 (6.0×150 mm, YMC Co., Ltd., Kyoto, Japan) at 40 °C under the following conditions: solvent A: 50 mM NaH_2PO_4/acetonitrile (20:1) (mobile phase), solvent B: 50 mM NaH_2PO_4/acetonitrile (1:2) [from 10% (0 min) to 90% (100 min) using gradient curve 3 (Shimadzu LC-system)], a flow rate of 1.0 ml/min, and detection at 320 nm.

4.2. Extraction and isolation

After the reaction, the mixture was extracted twice with 2.0 l of ethyl acetate. The combined ethyl acetate extracts were washed twice with 100 ml of 5% $NaHCO_3$ and water, and then dried with Na_2SO_4. Following filtration with a cotton plug, the ethyl acetate layer was evaporated to a solid (90 g). The products were dissolved in 50 mM NaH_2PO_4/acetonitrile (17:3) (mobile phase solution), and one fifth of the product was subjected to a reverse-phase column (Lobar LiChroprep RP-18, 37×440 mm) and eluted in a step-wise manner using 15 and 20% acetonitrile solutions (500 ml). The combined fractions containing products were separately extracted with ethyl acetate, and then each ethyl acetate layer was condensed by evaporation to give 730 mg of product **5** and 770 mg of product **6** mixture. Each product was further purified by oxalic acid-treated silica gel chromatography. The product was applied to a column (15 mm diameter, 10 g of silica gel) and eluted with increasing concentrations of ethyl acetate (from 50 to 67%) in *n*-hexane. Product **5** was crystallized from an ethyl acetate solution by the addition of *n*-hexane, resulting in an orange crystal (10 mg). Product **6** was crystallized from an ethanol (99.5%)/water solution to give a deep-orange crystal (14 mg).

4.3. Instrumental analyses of the purified products

The purified product was analyzed by ^{1}H NMR (400 MHz, CD_3OD) with tetramethylsilane (TMS) as a reference, ^{13}C NMR (100 MHz, CD_3OD), HPLC-electrospray ionization (ESI)-time-of-flight (TOF)-MS (HPLC-ESI-TOF-MS) (Agilent LC1100, ABI QSTAR XL), and matrix-assisted laser desorption/ionization (MALDI)-TOF-MS (Shimadzu-KRATOS, AXIMA-CFR plus) using 2,5-dihydroxybenzoic acid (2,5-DHB) or 2,5-DHB and NaCl as a matrix solution. Optical absorption was measured with a Shimadzu UV-250 spectrophotometer in a 50% ethanol solution.

4.3.1. Epitheaflagallin (5). Orange solid (10 mg); ^{1}H NMR (CD_3OD) δ_H (ppm) 2.82, 2.93 (2H, m), 4.27 (1H, m), 4.81 (1H, s), 5.97, 5.98 (each 1H, d, J=2.4 Hz), 6.89 (1H, s), 7.33 (1H, d, J=1.0 Hz), 7.50 (1H, s); ^{13}C NMR (CD_3OD) δ_C (ppm) 29.7, 67.0, 81.5, 95.9, 96.7, 99.8, 111.9, 116.2, 117.1, 133.4, 134.6, 135.6, 135.7, 152.60, 152.65, 155.0, 156.9, 157.8, 158.1, 183.4; ESI-TOF-MS m/z 401.0869 $[M+H]^+$ for $C_{20}H_{16}O_9$; MALDI-TOF-MS m/z 401 $[M+H]^+$, 423 $[M+Na]^+$, $E_{1\,cm}^{1\%}$ 371 (281 nm), 446 (306 nm).

4.3.2. Epitheaflagallin 3-*O*-gallate (6). Deep-orange solid (14 mg); ^{1}H NMR (CD_3OD) δ_H (ppm) 2.91, 3.07 (2H, m), 5.04 (1H, s), 5.67 (1H, m), 5.99 (1H, d, J=2.2 Hz), 6.02 (1H, d, J=2.2 Hz), 6.64 (1H, s), 6.89 (2H, s), 7.33 (1H, d, J=1.2 Hz), 7.46 (1H, s), ^{13}C NMR (CD_3OD) δ_C (ppm) 27.1, 69.3, 80.4, 95.9, 96.9, 99.2, 110.2, 112.0, 116.1, 116.5, 121.1, 133.5, 134.3, 134.5, 135.8, 139.9, 146.4, 152.5, 152.7, 155.1, 156.8, 158.0, 158.1, 167.4, 183.4; ESI-TOF-MS m/z 553.0993 $[M+H]^+$ for $C_{27}H_{20}O_{13}$; MALDI-TOF-MS m/z 553 $[M+H]^+$, 575 $[M+Na]^+$, $E^{1\%}_{1\,cm}$ 494 (280 nm), 469 (302 nm).

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