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Transformation of tea catechins and flavonoid glycosides by treatment with Japanese post-fermented tea acetone powder

Takashi Tanaka*, Hirotaka Umeki, Sachi Nagai, Takuya Shii, Yosuke Matsuo, Isao Kouno

Department of Natural Product Chemistry, Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

* Corresponding author at: Department of Natural Product Chemistry, Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan, Tel: +81 95 8192433, E-mail address: t-tanaka@nagasaki-u.ac.jp (T. Tanaka)

Abstract: Japanese post-fermented teas are produced by a combination of aerobic and anaerobic microbial fermentation of the leaves of tea plant. Recently, we revealed that tea products contain characteristic polyphenols identical to the tea catechin metabolites produced by mammalian intestinal bacteria, such as (2*S*)-1-(3',4',5'-trihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)-propan-2-ol (EGC-M1). In the present study, degradation of epigallocatechin-3-*O*-gallate (EGCg) and epigallocatechin (EGC) with acetone powder prepared from Japanese post-fermented tea was examined. Under aerobic conditions, EGCg was hydrolyzed to EGC and gallic acid, which were further converted to gallocatechin (GC) and pyrogallol, respectively. Under anaerobic conditions, EGCg was hydrolyzed to EGC, which was further metabolized to GC, EGC-M1 and (4*R*)-5-(3,4,5-trihydroxyphenyl)-4-hydroxypentanoic acid (EGC-M2). Gallic acid was degraded to pyrogallol and then further decomposed. Anaerobic treatment of EGC with the acetone powder yielded EGC-M1, EGC-M2, (4*R*)-5-(3,4,5-trihydroxyphenyl)-γ-valerolactone, and (4*R*)-5-(3,4 -dihydroxyphenyl)-γ-valerolactone. Furthermore, similar anaerobic treatment of rutin and hesperidin yielded 3,4-dihydroxyphenylacetic acid and 3-(3,4-dihydroxyphenyl)propanoic acid, respectively.

Keywords: Post-fermented tea; anaerobic fermentation; acetone powder; catechin metabolites; polyphenol

1. Introduction

Teas are produced from the leaves of *Camellia sinensis* (L.) O. Kuntze (Theaceae), and are generally classified by their manufacturing process into four categories: unfermented (green), fermented (black), semi-fermented (oolong) and post-fermented (dark tea, ripe Pu-erh tea, and related products). Black and oolong tea production includes a process called “tea fermentation”, in which constituents of fresh tea leaves are chemically altered by enzymes originally contained in the leaves (Roberts, 1962; Tanaka, Matsuo, & Kouno, 2010). In contrast, the post-fermented teas (PFT) are produced by aerobic or anaerobic microbial fermentation of heat-processed green tea leaves (Gong, Watanabe, Yagi, Etoh, Sakata, Ina, & Liu, 1993; Okada, Takahashi, Ohara, Uchimura, & Kozaki, 1996b). In a previous study, we looked at the chemical constituents of Chinese PFT produced by aerobic microbial fermentation of green tea, and demonstrated that the major characteristic constituents are polymeric polyphenols derived from tea catechins (Jiang, Shii, Matsuo, Tanaka, Jiang, & Kouno, 2011). On the other hand, Japanese PFT produced by anaerobic microbial fermentation of green tea contains (2*S*)-1-(3',4'-dihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)-propan-2-ol (EC-M), and (2*S*)-1-(3',4',5'-trihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)-propan-2-ol (EGC-M1), which are identical to the tea catechin metabolites formed by mammalian intestinal bacteria (Takagaki & Nanjo, 2010; Wang, Meselhy, Li, Nakamura, Min, Qin, & Hattori, 2001; Meselhy, Nakamura, & Hattori, 1997; Tanaka, Nagai, Shii, Matsuo, & Kouno, 2011). Japanese PFT is mainly produced in Tokushima, Kochi and Ehime prefectures in the Shikoku region. The PFT in Tokushima prefecture is produced by anaerobic fermentation of green tea, and it was demonstrated that *Lactobacillus plantarum* and

Klebsiella pneumoniae are the dominant microorganisms involved in the fermentation (Okada, Takahashi, Ohara, Uchimura, & Kozaki, 1996a). The PFT products in Kochi and Ehime prefectures are produced by both aerobic and anaerobic fermentation processes, and many aerobic fungi and anaerobic bacteria have been identified (Tamura, Kato, Omori, Nanba, & Miyagawa, 1994). The HPLC profiles of these two types of Japanese PFT are quite different: Tokushima-type contains catechin galloyl esters along with the tea catechin metabolites. In the Ehime and Kochi-type, the galloyl esters are completely hydrolyzed, and the resulting gallic acid is further converted to pyrogallol (Tanaka et al., 2011). The aims of this study were 1) to confirm the constituents of PFT produced in Ehime-prefecture, and 2) to examine degradation of tea catechins and flavonoids by treatment with an acetone powder prepared from the PFT.

2. Materials and methods

2.1. Materials

The PFT produced in Ehime prefecture, Japan, was purchased in October, 2010. Standard samples of tea catechins were separated from green tea and purified by crystallization from water. (2*S*)-1-(3',4'-Dihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)-propan-2-ol and (2*S*)-1-(3',4',5'-trihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)-propan-2-ol were isolated from PFT produced in Tokushima prefecture, Japan (Tanaka et al., 2011).

2.2. Analytical Procedures

UV spectra were obtained using a JASCO V-560 UV/VIS spectrophotometer (Jasco Co., Tokyo, Japan). ^{1}H and ^{13}C NMR spectra were recorded in acetone-d_6 + D_2O

(9:1, v/v), CD_3OD, or DMSO-d_6 at 27°C with a JEOL JNM-AL400 spectrometer (JEOL Ltd., Tokyo, Japan) operating at a ^1H frequency of 400 MHz. Coupling constants are expressed in Hz and chemical shifts are presented on a δ (ppm) scale. Column chromatography was performed using Sephadex LH-20 (25–100 μm, GE Healthcare Bio-Science AB, Uppsala), Diaion HP20SS (Mitsubishi Chemical, Japan), MCI gel CHP 20P (75–150 μm; Mitsubishi Chemical, Tokyo, Japan) and Chromatorex ODS (100–200 mesh; Fuji Silysia Chemical, Kasugai, Japan) columns. Thin layer chromatography was performed on precoated Kieselgel 60 F_{254} plates (0.2 mm thick, Merck KGaA, Darmstadt, Germany) with toluene-ethyl formate-formic acid (1:7:1, v/v) and $CHCl_3$-MeOH-water (14:6:1, v/v). Spots were detected using ultraviolet (UV) illumination and by spraying with 2% ethanolic $FeCl_3$ or a 10% sulfuric acid reagent followed by heating. Analytical HPLC was performed using a Cosmosil 5C_{18}-AR II (Nacalai Tesque Inc., Kyoto, Japan) column (4.6 mm i.d. × 250 mm) with gradient elution from 4–30% (39 min) and 30–75% (15 min) of CH_3CN in 50 mM H_3PO_4; the flow rate was 0.8 mL/min, and detection was achieved using a Jasco photodiode array detector MD-910.

2.3. Extraction and separation

PFT (64 g) was pulverized using a blender and extracted with 60% acetone in H_2O (2 L) at rt for 18 h. After filtration, the filtrate was concentrated until acetone was completely removed. The resulting precipitates were removed by filtration, and the filtrate was fractionated by Diaion HP20SS column chromatography (5 cm i.d. × 25 cm) with water containing increasing proportions of MeOH (0 – 100%, 10% stepwise, each 300 ml) to give 11 fractions: Fr. 1 (7.2 g), Fr. 2 (0.6 g), Fr. 3 (0.9 g), Fr. 4 (1.6 g), Fr. 5

(3.0 g), Fr. 6 (0.5 g), Fr. 7 (0.8 g), Fr. 8 (1.6 g), Fr. 9 (1.8 g), and Fr. 10 (6.3 g). A portion (0.57 g) of Fr. 1 was separated by silica gel with $CHCl_3$-MeOH-H_2O, 40:10:1 and then 14:6:1, (v/v) to give lactic acid (107 mg). Fr. 2 and 3 were identified to be gallic acid and pyrogallol, respectively, by comparison of ^1H NMR data and HPLC retention time with those of authentic samples. Fr. 4 was separated by Sephadex LH-20 column chromatography (3 cm i.d. × 20 cm) with EtOH to give (-)-gallocatechin (619 mg) as colorless needles from H_2O, $[\alpha]_D^{22}$ +0.03 (*c* = 1.0, acetone), and pyrogallol (130 mg). Fr. 5 was found to be a mixture of (-)-epigallocatechin and (2*S*)-1-(3',4',5'-trihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)-propan-2-ol by HPLC and TLC comparison with the authentic samples. Fr. 6 was applied to a Sephadex LH-20 column (3 cm i.d. × 16 cm) with 60% MeOH to yield catechin-(4α→8)-epigallocatechin (41 mg). Fr. 7 was subjected to Sephadex LH-20 column chromatography (3 cm i.d. × 20 cm) with EtOH to give 3-*O*-methylgallic acid (43 mg) and (-)-catechin (146 mg) as colorless needles from H_2O, $[\alpha]_D$ -6.59 (*c* 1.0, acetone). HPLC analysis of Fr. 8 revealed the presence of (-)-epicatechin and (2*S*)-1-(3',4'-dihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)-propan-2-ol. Fr. 9 was subjected to Sephadex LH-20 column chromatography (3 cm i.d. × 20 cm) with EtOH to give 3-*O*-methyl pyrogallol (12.4 mg), (-)-epigallocatechin-3-*O*-gallate (85 mg) and dihydromyricetin (8.6 mg). HPLC analysis showed that Fr. 10 contained caffeine, quercetin, and kaempferol.

2.4. Determination of absolute configuration of lactic acid

Lactic acid (20 mg) isolated from the PFT was dissolved in MeOH (1.0 mL) and treated with an ether solution of diazomethane in an ice bath for 30 min. After

removal of solvent and excess reagent by evaporation, the residue was dissolved in pyridine (0.5 mL) containing 4-methoxybenzoyl chloride (50 mg) at rt for 2 h. The reaction mixture was partitioned between H_2O and diethyl ether, and the organic layer was dried over Na_2SO_4. After filtration, the filtrate was concentrated and subjected to silica gel chromatography with hexane-acetone (9:1, v/v) to give methyl 2-(4-methoxybenzoyl)-lactate (2 mg). The product was analyzed by CHIRALCEL OJ-RH (4.6 mm × 150 mm, Daicel Chemical Industries, Ltd.) with 40% CH_3CN in 100 mM KPF_6 (pH 2), at 35°C, flow rate 0.8 mL/min. Retention times of the derivatives of D- and L-lactic acid was obtained using the derivatives prepared from racemic methyl lactate and L-methyl lactate in a similar manner (L-derivative: 11.6 min, D-derivative: 13.6 min).

2.5. Determination of absolute configuration of gallocatechin.

Gallocatechin (100 mg) isolated from PFT was methylated with dimethylsulfate (1.0 mL), K_2CO_3 (1.0 g) in dry acetone (30 mL) under reflux for 1 h. After removal of inorganic salts by filtration, the filtrate was concentrated and separated by silica gel chromatography with toluene containing increasing proportions of acetone (0 – 10%, 2% stepwise) to yield pentamethylate (16 mg) as colorless needles from MeOH, mp 165-166°C, $[\alpha]_D^{22}$ +15.0 (*c* = 0.1, $CHCl_3$). The methylate (3.0 mg) was treated with a mixture of dicyclohexylcarbodiimide (10.0 mg), 4-dimethylaminopyridine (5.0 mg) and (*R*)-(+)-α-methoxy-α-trifluoromethylphenylacetic acid (5.0 mg) in CH_2Cl_2 (1.0 mL) at rt for 12 h. The resulting mixture was separated by silica gel chromatography with hexane-acetone (85:15, v/v) to give the (*R*)-MTPA ester (5.6 mg). The use of

(*S*)-(-)-α-methoxy-α-trifluoromethylphenylacetic acid gave the (*S*)-MTPA ester (4.5 mg). $\Delta\delta$ (δ_S-δ_R): H-2 (-0.059), B-ring H-2, 6 (-0.116), H-4eq (-0.076), and H-4ax (+0.127).

2.6. Preparation of acetone powder

The PFT (20 g) was crushed using a mechanical blender and further pulverized with a mortar and pestle. The powder was mixed with H_2O (40 mL) and placed in an incubator (30°C) for 2 days. The wet powder was cooled in an ice bath, mixed with acetone (0°C, 260 mL), and gently stirred at 0-4°C for 2 h. The resulting tea leaf powder was collected by vacuum filtration, washed repeatedly with cooled acetone, and dried under reduced pressure (14.1 g). The powder was stored at -20°C.

2.7. Aerobic and anaerobic treatment of tea catechins with acetone powder

For aerobic treatment, the acetone powder (250 mg) was added to an aqueous solution (5 mL) of (-)-epigallocatechin (25 mg) and incubated at 30°C. After 24 h, 48 h, and 72 h, aliquots (300 μL) were taken and mixed with EtOH (700 μL). The solution was filtered through a membrane filter (0.45 μm) and analyzed by HPLC. (-)-Epigallocatechin was examined in a similar manner. For anaerobic treatment, the acetone powder (100 mg) was added to an aqueous solution (1 mL) of (-)-epigallocatechin-3-*O*-gallate (5 mg) and incubated at 37°C in a nitrogen atmosphere for 10 days. After addition of EtOH (1.5 mL), the mixture was filtered through a membrane filter (0.45 μm) and analyzed by HPLC.

2.8. Anaerobic treatment of epigallocatechin with acetone powder

In a 50 mL vial, the acetone powder (1.0 g) was mixed with an aqueous

178 solution (8 mL) of (-)-epigallocatechin (50 mg). The vial was placed in a vacuum
179 desiccator and the air was completely replaced by nitrogen gas. The desiccator was
180 placed in an incubator (35°C) for 10 days. Then the solution was mixed with 80% EtOH
181 (22 mL) and stirred at rt for 4 h. After filtration, the filtrate was concentrated under
182 reduced pressure and separated by Diaion HP20SS (2 cm i.d. × 20 cm) with water
183 containing increasing proportions of MeOH (0 – 100%, 10% stepwise, each 50 mL) to
184 yield EGC-M2 (13 mg), EGC-M3 (8.4 mg), and a fraction containing EGC-M1 (10 mg).
185 The EGC-M2 (13 mg) was dissolved in 2% trifluoroacetic acid (0.5 ml) and applied to a
186 Sephadex LH-20 column (1 cm i.d. × 15 cm) equilibrated with water, and eluted with
187 MeOH to give EGC-M3 (8.2 mg). The fraction containing EGC-M1 was separated by
188 Sephadex LH-20 column chromatography (1 cm i.d. × 15 cm) with water containing
189 increasing proportions of MeOH (0 – 100%, 10% stepwise, each 10 mL) to afford
190 EGC-M1 (1.0 mg), EGC-M4 (1.2 mg), and a mixture of epigallocatechin and
191 gallocatechin (0.9 mg). EGC-M1: ^{1}H-NMR (acetone-d_6 + D_2O) δ: 6.25 (2H, s), 5.91
192 (2H, s), 3.99 (1H, m), 2.93 (1H, dd, *J* = 3, 14 Hz), 2.64 (1H, dd, *J* = 7, 14 Hz), 2.65 (1H,
193 dd, *J* = 4, 14 Hz), 2.47 (1H, dd, *J* = 8, 14 Hz). EGC-M2: ^{1}H-NMR (acetone-d_6 + D_2O) δ:
194 6.22 (2H, s, H-2',6'), 3.71 (1H, m, H-4), 2.53 (1H, dd, *J* = 7, 13 Hz, H-5), 2.42 (1H, dd,
195 *J* = 7, 13 Hz, H-5), 2.41, 2.29 (each 1H, m, H-2), 1.71, 1.54 (each 1H, m, H-3).
196 EGC-M3: ^{1}H-NMR (acetone-d_6 + D_2O) δ:6.29 (2H, s, H-2',6'), 4.63 (1H, br quintet, *J* =
197 6 Hz, H-4), 2.79 (1H, dd, *J* = 6, 14 Hz, H-5), 2.66 (1H, dd, *J* = 7, 14 Hz, H-5), 2.44 (1H,
198 ddd, *J* = 8, 9, 18 Hz, H-2), 2.33 (1H, ddd, *J* = 5, 98, 18 Hz, H-2), 2.22, 1.93 (each 1H, m,
199 H-2). EGC-M4: ^{1}H-NMR (acetone-d_6 + D_2O) δ:6.73 (1H, br s, H-2'), 6.71 (1H, d, *J* = 8
200 Hz, H-5'), 6.54 (1H, br d, *J* = 8 Hz, H-6'), 4.65 (1H, br quintet, *J* = 6 Hz, H-4), 2.83 (1H,
201 dd, *J* = 6, 14 Hz, H-5), 2.73 (1H, dd, *J* = 7, 14 Hz, H-5), 2.44 (1H, ddd, *J* = 8, 9, 18 Hz,

H-2), 2.32 (1H, ddd, J = 5, 9, 18 Hz, H-2), 2.21, 1.91 (each 1H, m, H-2).

2.8. Anaerobic treatment of rutin with acetone powder

Rutin (50 mg) was dissolved in H_2O (20 mL) by heating. After cooling, the acetone powder (2.0 g) was added to the solution and placed in the vacuum desiccator. After the air was replaced by nitrogen gas, the desiccator was placed in the incubator (37°C) for 7 days. 80% EtOH (40 mL) was then added to the mixture and stirred at rt for 5 h. Insoluble materials were removed by filtration, and the filtrate was concentrated by rotary evaporation. The residue was separated by Diaion HP20SS (2 cm i.d. × 20 cm) with 0.1% trifluoroacetic acid containing increasing proportions of MeOH (0 – 100%, 10% stepwise, each 50 mL) to yield 3,4-dihydroxyphenylacetic acid (6.7 mg) as an amorphous powder, ^{1}H NMR (CD_3OD) δ: 6.74 (2H, br s, H-2', H-6'), 6.59 (1H, d, J = 8 Hz, H-5'), 2.38 (2H, s, H-2), ^{13}C NMR (CD_3OD) δ:172.9 (C-1), 145.9, 145.0 (C-3', C-4'), 127.4 (C-1'), 121.9 (C-6'), 117.5, 116.5 (C-2', C-5'), 41.3 (C-2).

2.9. Anaerobic treatment of hesperidin with acetone powder

Hesperidin (100 mg) was dissolved in H_2O (20 mL) by heating. After cooling, the acetone powder (2.0 g) was added to the solution and placed in the vacuum desiccator. After the air was replaced by nitrogen gas, the desiccator was placed in the incubator (37°C) for 7 days. Then, the product was separated in a manner similar to that described for rutin to yield 3-(3,4-dihydroxyphenyl)propanoic acid (41.6 mg) as an amorphous powder: ^{1}H NMR (acetone-d_6) δ: 6.67 (1H, d, J = 2 Hz, H-2'), 6.63 (1H, d, J = 8 Hz, H-5'), 6.48 (1H, dd, J = 2, 8 Hz, H-6'), 2.63, 2.30 (each 2H, br t, J = 6 Hz, H-2, H-3), ^{13}C NMR (acetone-d_6) δ:179.7 (C-1), 145.0, 143.2 (C-3', C-4'), 134.6 (C-1'), 120.0

(C-6'), 116.0, 115.8 (C-2', C-5'), 39.6, 31.9 (C-2, C-3).

3. Results and discussion

Previously we concluded that the major constituents of the PFT produced in the Ehime prefecture are EGC-M1, EC-M, (-)-epigallocatechin (EGC), (-)-gallocatechin (GC), pyrogallol, caffeine, (-)-catechin (Cat) and (-)-epicatechin (EC) (Fig. 1). In addition, lactic acid, gallic acid, catechin-(4α→8)-epigallocatechin (Hashimoto, Nonaka, & Nishioka, 1989), 3-*O*-methylgallic acid, 3-*O*-methylpyrogallol, dihydromyricetin (Jeon, Chun, Choi, & Kwon, 2008), quercetin, and kaempferol have also been identified in PFT from the Ehime prefecture. The lactic acid was a mixture of the D and L-forms (2: 3), which was determined by chiral HPLC after methylation and 4-methoxybenzoylation. Configuration of (-)-catechin was confirmed by its optical rotation, and that of (-)-gallocatechin was determined by comparison of the optical rotation of the pentamethyl ether (Delle Monache, D'Albuquerque, De Andrade Chiappeta, De Mello, 1992) and application of advanced Mosher's method (Kusumi, Ohtani, Inoue, & Kakisawa, 1988). The significantly high concentration of (2*S*,3*R*)-catechins compared with that in green tea strongly suggested occurrence of epimerization at the benzylic C-2 positions of the original (2*R*,3*R*)-catechins during fermentation. Production of EGC-M1 and EC-M apparently indicated occurrence of reductive cleavage of the catechin C-rings. Furthermore, the absence of galloylated catechin and the presence of a large amount of pyrogallol suggested occurrence of hydrolysis of the galloyl esters and subsequent decarboxylation of gallic acid.

To confirm the chemical conversions of tea catechins, we prepared an acetone powder of the PFT and examined its ability to metabolize epigallocatechin-3-*O*-gallate

(EGCg), which is the most abundant tea catechin in fresh tea leaf. In the PFT production at Ehime prefecture, the heated tea leaves are first fermented under aerobic conditions; therefore, EGCg was first treated with the acetone powder under aerobic conditions at 30°C, and the products were analyzed by reversed-phase HPLC (Fig. 2A). EGCg was first hydrolyzed to give epigallocatechin (EGC) and gallic acid. This is probably owing to the activity of tannase, which is typically isolated from *Aspergillus* sp (Tamura et al., 1994; Sharma, Bhat, & Dawra, 1999; Murugan, Saravanababu, & Arunachalam, 2007). Further aerobic fermentation for 2 days yielded pyrogallol and gallocatechin (GC). Production of pyrogallol from gallic acid was confirmed by treatment of gallic acid with the acetone powder under similar conditions. Production of GC from EGC was slower than hydrolysis (Fig. 2B) and production of EGC-M1 was not observed under aerobic conditions.

After the aerobic fermentation process during the PFT production, the leaves next undergo anaerobic fermentation. Under aerobic conditions, EGCg was converted to EGC; therefore, degradation of EGC under anaerobic conditions was examined (Fig. 4). Treatment of EGC with PFT acetone powder in a nitrogen atmosphere at 35°C for 10 days afforded EGC-M1 and several unknown products. Separation by column chromatography using Diaion HP20SS and Sephadex LH-20 afforded EGC-M2, EGC-M3 and EGC-M4 along with a mixture of EGC and GC. Comparison of the ^{1}H NMR spectra revealed that the products were (4*R*)-5-(3,4,5-trihydroxyphenyl)-4-hydroxypentanoic acid, (4*R*)-5-(3,4,5-trihydroxyphenyl)-γ-valerolactone (Takagaki, et al., 2010; Kohri, Matsumoto, Yamakawa, Suzuki, Nanjo, Hara, & Oku, 2001), and (4*R*)-5-(3,4-dihydroxyphenyl)-γ-valerolactone (Li, Lee, Sheng, Meng, Prabhu, Winnik,

Huang, Chung, Yan, Ho, & Yang, 2000), respectively (Fig. 5). The structure of EGC-M1 was also confirmed by treatment of EGC-M1 with trifluoroacetic acid which afforded EGC-M2. Although only EGC-M1 was isolated from the PFT products, the results indicated that the PFT acetone powder is capable of producing EGC-M2, M3 and M4, as the intestinal bacteria degrade EGCg (Takagaki, et al., 2010; Li, et al., 2000). Direct anaerobic treatment of EGCg with PFT acetone powder afforded mainly EGC and GC, and small peaks attributable to EGC-M1 and M2 were also detected upon HPLC analysis (Fig. 3). However, peaks of gallic acid and pyrogallol were not detected. Analysis of the total peak area of the HPLC suggested that a large part of the initial EGCg decomposed to uncharacterized products, which were not detected under the present HPLC conditions.

In addition to the tea catechins, reaction of rutin and hesperidin, which were selected as the important flavonoids in vegetables and fruits, were examined. Treatment of the flavonoids with the PFT acetone powder under anaerobic conditions afforded 3,4-dihydroxyphenylacetic acid (Booth, Murray, Jones, & DeEds, 1956) and 3-(3,4-dihydroxyphenyl)propanoic acid (Honohan, Hale, Brown, & Wingard, 1976), respectively (Fig. 6). The degradation products were the same as those observed from bacterial metabolism in the mammalian intestine. The results suggested that the PFT acetone powder has the ability to metabolize the flavonoids, though the metabolites were not detected from the original PFT.

4. Conclusions

In this study, we showed that the acetone powder of Japanese PFT has the

ability to produce compounds identical to the metabolites of polyphenols produced by mammalian intestinal bacteria, though there are some differences between the constituents of the original tea and the products obtained in our experiments. As mentioned previously, the PFT of Ehime prefecture, which was used in this study, is produced by a combination of aerobic and anaerobic fermentation. In the PFT, galloyl esters are hydrolyzed and pyrogallol was produced from gallic acid. In contrast, lack of an aerobic fermentation step in the production in the PFT from Tokushima prefecture is reflected by the presence of catechin galloyl esters and the absence of pyrogallol. The acetone powder produced from the Tokushima PFT did not show activity. It is well known that pyrogallol and pyrogallol-type tea catechins, EGC and EGCg, have strong antioxidative activity; however, the reactivity is reflected by its instability especially under neutral and alkaline conditions. The oxidation-sensitive polyphenols are probably stabilized by lactic acid which is produced by the anaerobic fermentation and is responsible for the sour taste of the PFT. Lactic acid keeps the pH low and suppresses the dissociation of the phenolic hydroxyl groups of the pyrogallol rings. The Japanese PFT produced by anaerobic fermentation has been used for drinking in a local area of Shikoku Island of Japan, and it is very interesting to note that some related tea products are also known in Myanmar (lepet-so), Thailand (miang), and China (zhu-tong-cha) (Namba, Nyein, Win, & Miyagawa, 1999; Tanasupawat, Pakdeeto, Thawai, Yukphan, & Okada, 2007). It is well recognized that tea catechins have various health benefits; however, it is also known that the direct absorption of catechins from the digestive tract is not very high (Miyazawa, 2000; Zhu, Chen, & Li, 2000). Therefore, absorption of gut bacterial metabolites with smaller molecular sizes is important, and this may partly be responsible for the biological activities of tea catechins. Thus, the presence of EGC-M1

322 and EC-M in the Japanese PFT as the major polyphenols may be significant from the
323 viewpoint of nutritional science.
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405 **Figure Captions**

406 Fig. 1 HPLC profile of 60% EtOH extract of Japanese post-fermented tea.

407 Detection at 220 nm, Caf: caffeine, EC: epicatechin, EC-M:
408 (2*S*)-1-(3',4'-dihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)-propan-2-ol, EGC:
409 epigallocatechin, EGC-M1: (2*S*)-1-(3',4',5'-trihydroxyphenyl)-3-
410 (2",4",6"-trihydroxyphenyl)-propan-2-ol, EGCg: epigallocatechin-3-*O*-gallate, Fl:
411 quercetin monoglycoside, GA: gallic acid, GC: gallocatechin, K: kaempferol, PG:
412 pyrogallol, Q: quercetin.

413

414 Fig. 2 HPLC profiles of aerobic treatment of epigallocatechin-3-*O*-gallate (A) and
415 epigallocatechin (B) with acetone powder.

416

417 Fig. 3 HPLC profile of products obtained by anaerobic treatment of
418 epigallocatechin-3-*O*-gallate.

419 EGC: epigallocatechin, EGCg: epigallocatechin-3-*O*-gallate, GC: gallocatechin,
420 EGC-M1: (2*S*)-1-(3',4',5'-trihydroxyphenyl)-3- (2",4",6"-trihydroxyphenyl)-propan-2-ol,
421 EGC-M2: (4*R*)-5-(3,4,5-trihydroxyphenyl)-4-hydroxypentanoic acid.

422

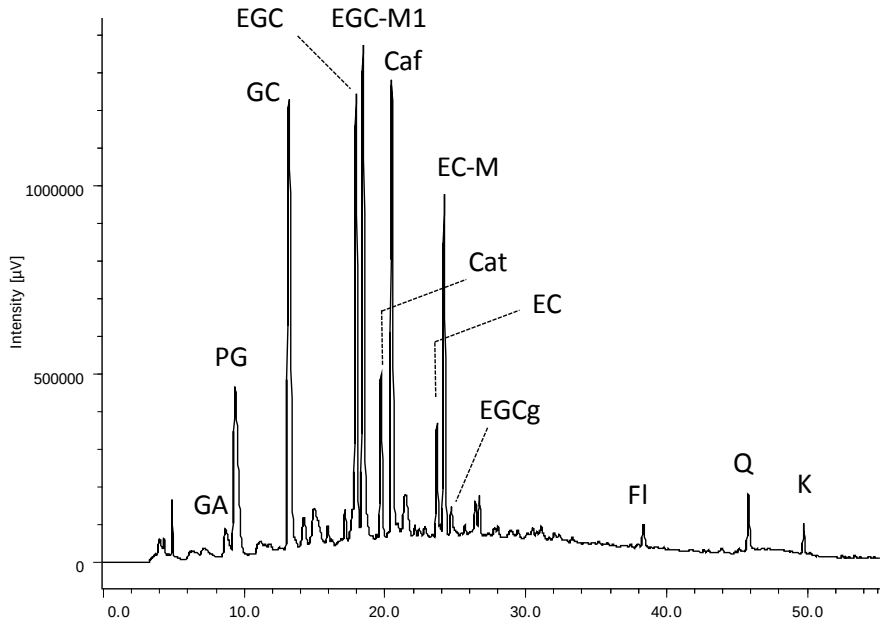
423

424 Fig. 4 HPLC profile of products obtained by anaerobic treatment of epigallocatechin.

425 EGC-M1: (2*S*)-1-(3',4',5'-trihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)-propan-2-ol,
426 EGC-M2: (4*R*)-5-(3,4,5-trihydroxyphenyl)-4-hydroxypentanoic acid, EGC-M3:
427 (4*R*)-5-(3,4,5-trihydroxyphenyl)-γ-valerolactone, EGC-M4: (4*R*)-5-(3,4
428 -dihydroxyphenyl)-γ-valerolactone.

Fig. 5 Degradation of epigallocatechin-3-*O*-gallate on treatment with the Japanese post-fermented tea acetone powder.

Fig. 6 Degradation of rutin and hesperidin on treatment with Japanese post-fermented tea acetone powder.


EGC
EGC-M1
Caf
GC
EC-M
Cat
EC
PG
EGCg
GA
Fl
Q
K
Intensity [µV]
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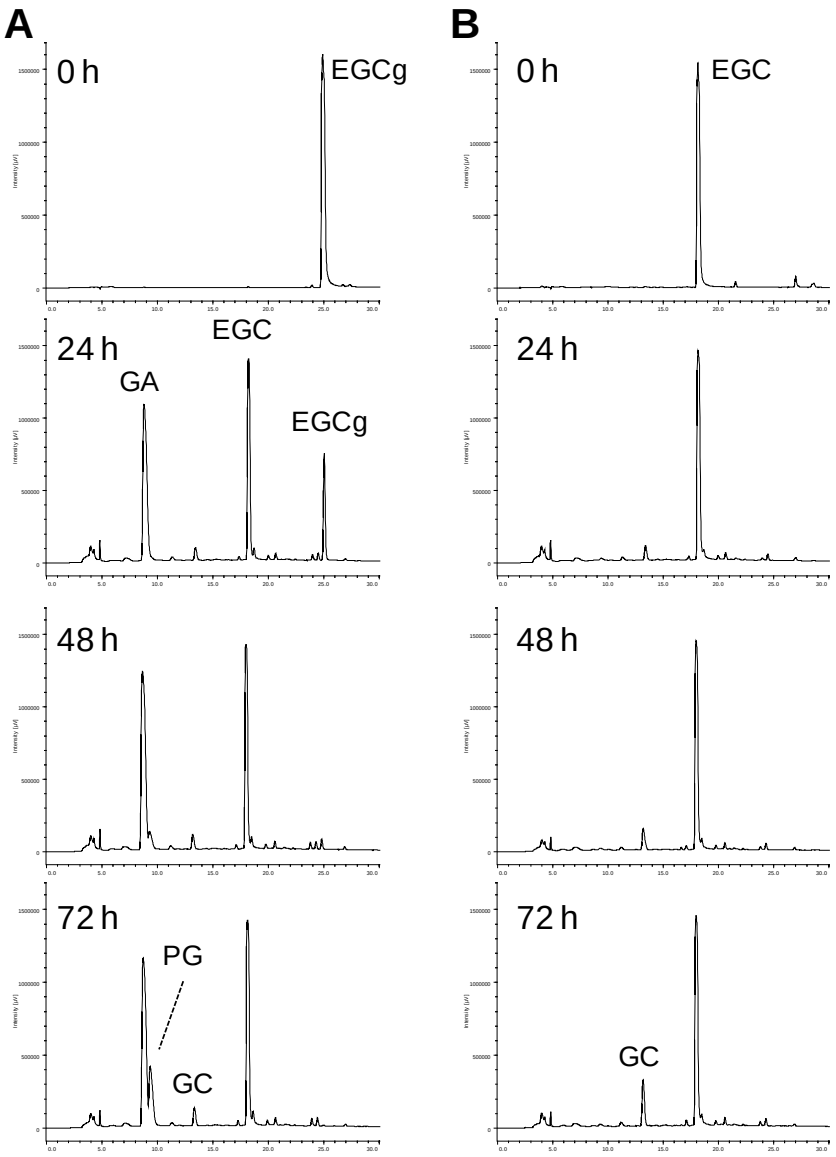
500000
0
0.0
10.0
20.0
30.0
40.0
50.0

Fig. 1

443



444

445

446

447 Fig. 2

448

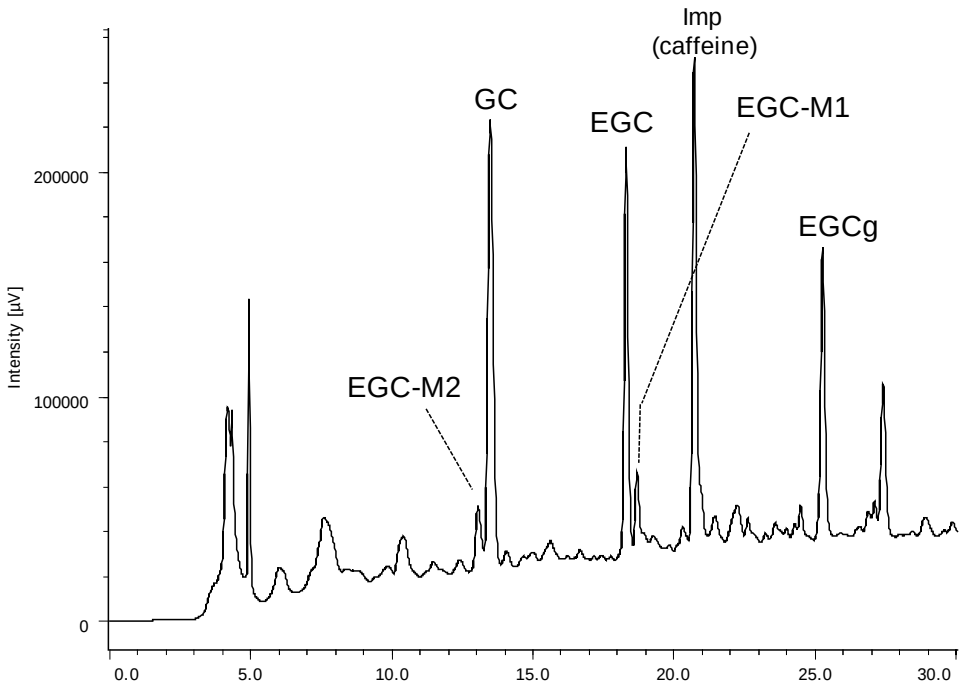

Imp
(caffeine)
GC
EGC
EGC-M1
EGCg
EGC-M2
Intensity [µV]
200000
100000
0
0.0
5.0
10.0
15.0
20.0
25.0
30.0

Fig. 3

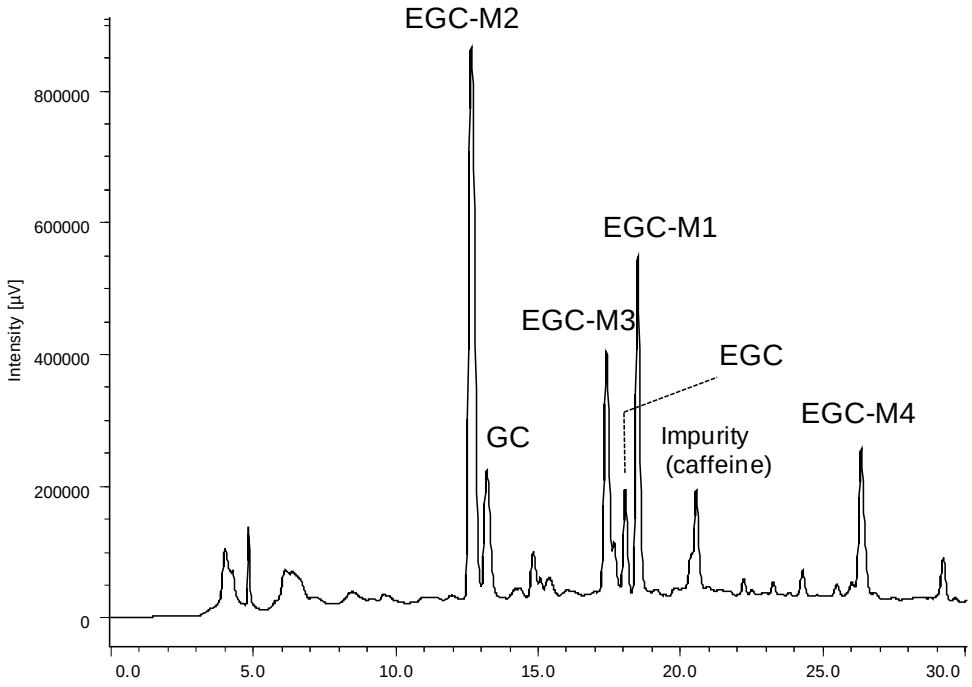

EGC-M2
EGC-M1
EGC-M3
EGC
EGC-M4
GC
Impurity
(caffeine)
Intensity [µV]
800000
600000
400000
200000
0
0.0
5.0
10.0
15.0
20.0
25.0
30.0

Fig. 4

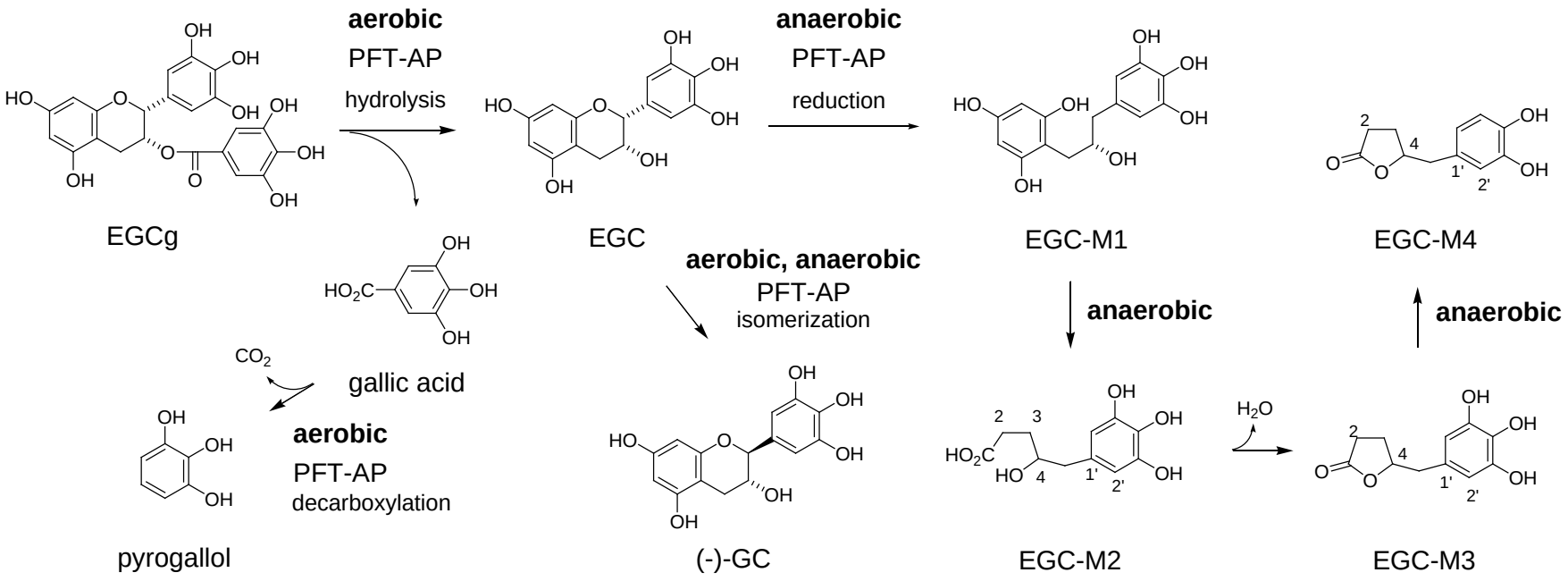
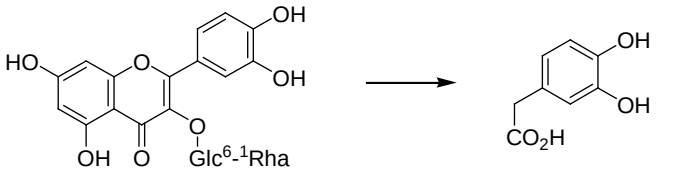
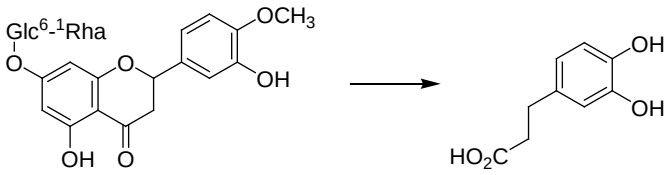

aerobic
PFT-AP
hydrolysis
anaerobic
PFT-AP
reduction
EGCg
EGC
EGC-M1
EGC-M4
gallic acid
aerobic, anaerobic
PFT-AP
isomerization
anaerobic
anaerobic
CO2
aerobic
PFT-AP
decarboxylation
H2O
pyrogallol
(-)-GC
EGC-M2
EGC-M3

Fig. 5



rutin



hesperidin

Fig. 6