Note
Antioxidative Activity of Microbial Metabolites of
(−)-Epigallocatechin Gallate Produced in Rat Intestines

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The antioxidative activities of (−)-epigallocatechin gallate (EGCg) metabolites degraded by rat intestinal flora were investigated by a flow injection analysis coupled to an on-line antioxidant detection system with the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation. All of the metabolites were found to have antioxidative activity, suggesting that the EGCg metabolites may show antioxidative activity in the body.

Key words: antioxidative activity; tea catechin; metabolite

Catechins, belonging to the flavan-3-ol group, are the major components of green tea. The principle catechins present in green tea are (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg), and (−)-epigallocatechin gallate (EGCg). They are well known to act as antioxidants and scavenge such free radicals as the superoxide anion, hydroxyl radical, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical.1,2) EGCg is the most potent antioxidant among the tea catechins. However, it is doubtful whether EGCg would show favorable antioxidative activity in the body, since this compound has been reported to be only slightly absorbed in the body.3,4) On the other hand, metabolites derived from EGCg by intestinal bacteria have been detected in abundance in mammalian urine and blood.3,4) These metabolites are therefore thought to be easily absorbed and circulated in the body, although information on their physiological activity is limited. Lambert et al.5) have reported that one of the EGCg metabolites, 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, could contribute to the anti-inflammatory and cancer-preventive activities of green tea. Unno et al.6) have reported the urinary metabolite, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, excreted in the urine following EC dosage as having half the EC antioxidative activity in Trolox equivalents of antioxidant capacity (TEAC). Information about the physiological activity of degradation products of tea catechin has been confined to these two reports to our knowledge.

We have already reported the transformation of EGCg by rat intestinal bacteria, and the purification and structural identification of the metabolites.7,8) We describe in this present report the antioxidative activities of EGCg metabolites, in addition to those of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 5-(3′-hydroxyphenyl)-γ-valerolactone which are metabolites produced from EC or EGCg.6,9) The radical scavenging ability was measured by an on-line flow injection system with the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation.

EGCg, EGC, ECg, EC, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (Tokyo, Japan), and 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was purchased from Wako Pure Chemical Industries (Osaka, Japan). The metabolites derived from EGCg by rat intestinal bacteria were purified according to the method reported in our previous paper.8) Chemically synthesized 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 5-(3′-hydroxyphenyl)-γ-valerolactone were kindly supplied by Dr. Nakajima of Toyama Prefectural University.10) All other reagents were of analytical or HPLC grade.

An on-line flow injection system was constructed by modifying the ABTS radical scavenging analysis reported by Stewart et al.11) for measuring the antioxidative activity. Each sample was injected and eluted with 0.03% phosphoric acid at a flow rate of 1 mL/min by an Alliance HPLC 2695 separation module (Nihon Waters, Tokyo, Japan) without connecting a column. Instead, the sample solution was directly put into a Waters 2998 photodiode array detector (PDA), before mixing with an ABTS radical solution through a peak mixing T-piece (Shimadzu, Tokyo, Japan). The ABTS solution was pumped into the T-piece at a flow rate of 0.5 mL/min with a PV-1580 pump (Jasco, Tokyo, Japan). The resulting mixture (pH 7.25) was continuously passed through a stainless steel loop (750 × 0.5 mm i.d.) maintained at 40 °C in a CH500 column oven (Eppendorf Co., Tokyo, Japan), and the absorbance at 700 nm was monitored by a Waters 2489 UV/VIS detector.

The ABTS radical solution was prepared by dissolving ABTS (4 mM) in 50 mL of 3.5 mM K2S2O8, which had been kept for 30 min at room temperature, and then diluting with 350 mL of 0.1 M potassium phosphate buffer (pH 8.0). After standing overnight at room temperature, the resulting solution was designated as the ABTS radical solution and was maintained on ice in the dark during analysis.
Each compound sample was dissolved in aqueous 30% methanol at final concentrations of 2–10 mM and was further diluted with the same solvent. Sample solutions at four or five different concentrations ranging from 100 to 300 µM were finally prepared. Catechin solutions were diluted with the 30% methanol in a series of concentrations from 10 to 200 µM. Trolox was also diluted with the same solvent to give final concentrations of 46, 185, 463, 616 and 740 µM. These samples (10 µL each) were analyzed by the on-line flow injection system, and the scavenging activity against the ABTS radical cation was measured at four or five different concentrations. The area of decolorization was plotted against the sample concentration, and the regression line for each sample was calculated by the least-squares method. The regression lines for Trolox, EGC, metabolite 6, 5-(3',4'-dihydroxyphenyl)-γ-valerolactone, 5-(3',4',5'-trihydroxyphenyl)-γ-valerolactone, and 3'-hydroxyphenyl group (5-(3'-hydroxyphenyl)-γ-valerolactone) are presented in Fig. 1. We defined the slope of the regression line for each compound as the ABTS radical scavenging ability. The ABTS radical scavenging ability of each compound is also expressed as an equivalent value for Trolox.

The relative radical scavenging abilities for the tea catechins and metabolites are summarized in Fig. 2. The results of our on-line flow injection method against the ABTS radical cation show an order of potency of the tea catechins and metabolites as radical scavengers of EGCg > ECg > EGC > metabolite 4 > EC > metabolite 13 ≈ metabolite 10 > metabolite 3 > metabolite 11 ≈ metabolite 6 > metabolite 5 > metabolite 9 > 5-(3',4'-dihydroxyphenyl)-γ-valerolactone > metabolite 12 > 5-(3'-hydroxyphenyl)-γ-valerolactone. The order of ABTS radical scavenging ability for the tea catechins is in good agreement with that obtained by Pannala et al.12)

The radical scavenging abilities of the metabolites tested were found to be stronger than or nearly equal to those of Trolox. The order of the radical scavenging ability of the 5-phenyl-γ-valerolactones, which have been mainly detected in mammalian urine, was stronger in the presence of the 3',4',5'-trihydroxyphenyl group (metabolite 10), 3',5'-dihydroxyphenyl group (metabolite 6), 3',4'-dihydroxyphenyl group (5-(3',4'-dihydroxyphenyl)-γ-valerolactone), and 3'-hydroxyphenyl group (5-(3'-hydroxyphenyl)-γ-valerolactone) in their structures. These results suggest that the number and arrangement of the hydroxyl group in the phenyl group of the γ-valerolactone were important to determine their radical scavenging potency. A similar relationship was apparent among the phenyl valeric acids (metabolites 13, 11 and 12). On the other hand, the radical scavenging ability of metabolite 5 having a 3,5-dihydroxyphenyl group was stronger than that of metabolite 9 possessing a 3,4,5-trihydroxyphenyl group in 4-hydroxy-5-phenyl valeric acid, and a similar relationship was also found between metabolites 3 and 4. The reason for the difference between the 5-phenyl-γ-valerolactones and 4-hydroxy-5-phenyl valeric acid is unclear, but one possible explanation is that the presence or absence of an alcoholic hydroxyl group (4-hydroxy group) could have affected their radical scavenging ability, in addition to the number and arrangement of the hydroxyl group in the phenyl moiety.

It is well known that oxidative disorder caused by radical oxygen produced in the body would involve the risk of lifestyle-related diseases including arteriosclerosis, carcinogenesis, coronary heart disease, liver injury and stroke. Tea catechins have been found to have strong antioxidative activity, and studies with many animal models have demonstrated the in vivo antioxidative effect following the consumption of tea catechins. Senthil et al.10) have reported that the administration of EGCg to rats reduced an age-related increase in oxidative stress. In addition, long-term dietary supplementation of tea polyphenol has been reported to decrease the thiobarbituric acid reactive substance (TBARS) in rat plasma.10) Nanjo et al. have examined the effect of dietary tea catechins on the levels of α-tocopherol and lipid peroxidation in both plasma and erythrocytes of rats fed on high palm oil and perilla oil diets.15) They demonstrated that supplementation with tea catechin prevented the decrease in the levels of rat plasma and erythrocyte α-tocopherol. These in vivo antioxidative effects by tea catechins are believed to contribute to lowering the risk of radical-mediated diseases. However tea catechins, especially EGCg, have been shown to be poorly absorbed and they may not, therefore, be the major contributors to in vivo antioxidative activity. In this respect, Kohri et al. have reported that the bioavailability of [4-14H]-EGCg after orally administering to rats was only 0.3% of the dose.3) It has also been reported that only 0.1% of ingested EGC appeared in human plasma after its oral dosage.16) The contribution of EGCg to in vivo antioxidative activity, if any, may therefore be limited. On the other hand, such ring-fission metabolites of tea catechins as 5-(3',4',5'-trihydroxyphenyl)-γ-valerolactone, 5-(3',4'-dihydroxyphenyl)-γ-valerolactone and 5-(3'-hydroxyphenyl)-γ-valerolactone have been detected in rat and human plasma and urine, and the cumulative urinary excretion of 5-(3',4',5'-trihydroxyphenyl)-γ-valerolactone and 5-(3',4'-dihydroxyphenyl)-γ-valerolactone, including their conjugated form, has been reported to account for 6–39% of ingested catechins (EGC and EC) in some human subjects.3,17,18) Furthermore, after orally administering EGCg, the amount of 5-(3',4'-dihydroxyphenyl)-γ-valerolactone, including its glucuronide conjugate, in rat urine was found to be 26% of its dose for 24 h.3) The bioavailability of the metabolites pro-
duced from tea catechins by intestinal microflora was thus found to be higher than that of the original catechins. Li et al. have reported that the human urinary cumulative excretion of the these microbial metabolites was as high as 8–25 times the levels of EGC and EC.

We have demonstrated in this study that the ring-fission metabolites of EGCg, including the \( \gamma \)-valerolactones, had antioxidative activities at least equivalent to Trolox, although their activities were one-seventh to two-thirds of EGCg. Taking into account the high bioavailability of the metabolites as already mentioned, it is likely that they may be the main contributors to the antioxidative effects, rather than the original catechins absorbed following the consumption of tea catechins. More studies are needed to elucidate the physiological properties of the microbial metabolites in order to better understand the beneficial health effects of tea catechins.

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References


Fig. 2. Scavenging Abilities of the Catechin Metabolites toward the ABTS Radical Cation. Each value is presented as the equivalent value for Trolox. *The configurations of metabolites 3, 4, 9 and 10 were not confirmed.