Note

Effect of Tea Catechins on Folate Analysis in Green Tea by Microbiological Assay

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Summary Green tea is thought to be a primary source of folate in the Japanese diet, based on folate content analyzed by a microbiological assay. Green tea also contains high amount of catechins, in particular, epigallocatechin gallate (EGCg), which was demonstrated to be able to inhibit the digestive enzyme activities and microbial growth in the folate assay. In the present study, we examined whether tea catechins interfered with components of the folate assay for green tea. A marked inhibitory effect of EGCg on microbial growth was observed at an inhibitory concentration of higher than 10 $\mu\text{g/mL}$. Tea catechins without the galloyl moiety did not show an inhibitory effect. EGCg inhibited the activity of the three enzymes used for assay sample preparation at an inhibitory concentration of higher than 750 $\mu\text{g/mL}$ for α -amylase, 1,000 $\mu\text{g/mL}$ for protease, and 50 $\mu\text{g/mL}$ for conjugase. However, with each step of the assay, the actual concentration of EGCg was decreased to below the inhibitory concentration of each analytical step. Lack of influence of EGCg on green tea folate assay was confirmed by an addition of folate standard in tea infusion. These results suggested that tea catechins have no practical impact on folate analysis in green tea, using the general microbiological assay.

Key Words green tea, food folate, epigallocatechin gallate, microbiological assay

Folate is an important vitamin, and its deficiency can lead to megaloblastic anemia (1). Low folate status leads to an increase in plasma homocysteine, which is a risk factor for cardiovascular disease (1). In addition, low folate status in women of childbearing age leads to an increased risk of fetal abnormalities, such as neural tube defects (1).

Folate in food exists in monoglutamate and polyglutamate forms. Folate naturally occurring in foods ("natural folate") exists mainly in the polyglutamated form, with different chemical structures in the pteridine ring, such as in tetrahydro-, dihydro-, formyl- and 5-methyl-tetrahydrofolates (2). Synthetic folic acid (pteroylm-onoglutamic acid) is the most oxidized and stable form, highly bioavailable, and used for dietary supplements and processed foods. Analysis of these folates in food is critical to estimate the intake amount. Various analytical methods have been presented, such as the microbiological assay, HPLC, and enzyme protein binding assay (3). Among these methods, the microbiological assay can analyze many forms of folates, which include mono-, di- and tri-glutamate derivatives existing in foods (4), and is adopted as the method of choice in the "Standard Tables of Food Composition in Japan". The microbiological assay procedure is divided into three steps: sample homogenization and dilution with buffer; treatment with digestive enzymes (α -amylase, protease and conju-

gase); and finally, addition to growth media for assessment of microbial growth rates (5, 6).

Green tea contains natural folate, and was regarded as one of the primary sources of folate in the Japanese diet (7), when folate consumption was estimated by the "Standard Tables of Food Composition in Japan". Additionally, green tea is known to contain catechins, which have antimicrobial activities (8, 9), and inhibit various enzymes (10–12). These actions of tea catechins may interfere with green tea folate analysis by the microbiological assay. However, there have been few data reported on the effect of tea catechins on green tea folate analysis by the microbiological assay.

In the present study, we examined whether tea catechins interfered with the microbiological assay for food folates, and investigated the inhibitory concentrations of tea catechins for the assay.

Materials and Methods

Chemicals. (–)-Epigallocatechin gallate (EGCg), (–)-epicatechin gallate (ECg), (–)-epigallocatechin (EGC), (+)-catechin (C), and (–)-epicatechin (EC) were purchased from Nagara Science Co., Ltd. (Gifu, Japan); pteroyl- γ -L-glutamic acid, pteroylhepta- γ -L-glutamic acid, 5-methyl-5,6,7,8-tetrahydropteroyl- γ -L-glutamic acid (5-MTHF₁) and 5-methyl-5,6,7,8-tetrahydro-pteroylpenta- γ -L-glutamic acid (5-MTHF₅) from Schircks Laboratories (Jona, Switzerland); α -amylase from *Aspergillus oryzae* and protease from *Streptomyces griseus*

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Fig. 1. Dose-dependent inhibition of tea catechins on microbial growth. *L. rhamnosus* was cultured in a complete folic acid casei medium with various concentrations of tea catechins (EGCg, ECg, EGC, C, EC). Values are mean $\pm$ SE, $n=3$. * Significant difference from the level without catechins.

from Sigma-Aldrich (St. Louis, MO); chloramphenicol-resistant *Lactobacillus rhamnosus* (ATCC 27773) from the American Type Culture Collection (Rockville, MD); folic acid casei medium from Becton Dickinson (Sparks, MD); and rat serum from Nippon Bio-Test Laboratories Inc., Tokyo. Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Alpha-amylase and rat serum were treated with charcoal to eliminate contaminating folate (6). Tea catechins were dissolved in a small amount of methanol and diluted with the buffer used in the respective assay.

Analytical Methods. The folate microbiological assay was performed using the tri-enzyme method described by Aiso and Tamura (5). In brief, samples were homogenized with 20 fold of 50 mmol/L potassium phosphate buffer (pH 6.2) that contained 0.2 mmol/L 2-mercaptoethanol and 114 mmol/L L-ascorbic acid. The sample (250 μ L) was mixed with 250 μ L of the phosphate buffer and 500 μ L of α -amylase (10 mg/mL), and incubated for 2 h at 37°C. After heating in boiling water for 10 min, the sample was treated with 500 μ L of protease (20 mg/mL) for 2 h at 37°C. The samples were heated in boiling water for 10 min, centrifuged at 8,000 $\times g$ for 10 min, and the resulting supernatant (250 μ L) was supplemented with 650 μ L of the phosphate buffer and 100 μ L of rat serum conjugase, and then incubated for 3 h at 37°C. After heating and centrifugation, the supernatant was diluted twice with 1% L-ascorbic acid, mixed with an equal volume of a complete folic acid casei medium containing *L. rhamnosus*, and cultured in a 96-well plate for 42 h at 37°C. Turbidity of the samples was measured at 600 nm. Folate concentration in a sample was expressed as the pteroyl- γ -L-glutamic acid equivalent.

The tea catechin effect on the growth of *L. rhamnosus* was determined by the addition of tea catechins (EGCg, ECg, EGC, C, EC) (up to 200 μ g/mL) to a complete folic acid casei medium, and cultured in a 96-well microplate.

The activity of α -amylase was determined by the Ber-

nfeld (13) procedure using soluble starch as a substrate. Briefly, the reaction mixture consisted of 50 μ L 1% (w/v) starch in 10 mmol/L phosphate buffer (pH 6.9) containing 3.35 mmol/L NaCl, and EGCg (up to 3 mg/mL), incubated for 5 min, and then treated with 50 μ L of α -amylase (20 mg/mL) for 10 min at room temperature. Blank samples for each EGCg concentration were prepared. The enzyme reaction was stopped by adding 50 μ L of 48 mmol/L 3,5-dinitrosalicylic acid in 1.06 mol/L sodium potassium tartrate and 0.4 mol/L NaOH, heated in boiling water for 15 min, followed by cooling to room temperature. After the addition of 500 μ L of water, absorbance of the sample was measured at 540 nm. Enzyme activity was calculated using maltose as the standard.

Protease activity was determined by the Anson (14) procedure using casein as a substrate. Casein was dissolved in 100 mmol/L phosphate buffer (pH 7.5) to make a 10% (w/v) substrate solution. To 400 μ L of the substrate, 100 μ L of EGCg (up to 9 mg/mL) was added and incubated for 5 min at room temperature, then treated with 100 μ L of protease (60 mg/mL) for 10 min at 37°C. The reaction was stopped by the addition of 500 μ L of 1.8% (w/v) trichloroacetic acid. The resulting sample was applied to a solid phase extraction column (15), because EGCg in the sample markedly interferes with the protein measurement assay. Casein-degraded product in the elute was determined by the Bradford Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA).

Conjugase activity was determined by the HPLC method previously described by Hasoun et al. (16). Briefly, the reaction mixture consisted of 100 μ L of 50 μ mol/L 5-MTHF₅, 700 μ L of 50 mmol/L phosphate buffer (pH 7.0) containing 114 mmol/L L-ascorbic acid and 0.2 mmol/L 2-mercaptoethanol, and 100 μ L of EGCg (0 to 15 mg/mL), incubated for 5 min, and then treated with 100 μ L of rat serum conjugase for 4 h at 37°C. The reaction was stopped by heating in boiling

Fig. 2. Dose-dependent inhibition of EGCg on α -amylase in a), protease in b), conjugase in c) and Lineweaver-Burk plot. The activities of the three enzymes used in the folate assay were determined with various concentrations of EGCg. In the Lineweaver-Burk plot, 1.5 mg/mL of EGCg was used. S and V indicate substrate concentration and reaction velocity. Values are mean $\pm$ SE, $n=4$. * Significant difference from the level without EGCg.

water for 10 min. After centrifugation at $10,000 \times g$ for 10 min, the supernatant was applied to a solid phase extraction column to eliminate the EGCg, as described in the protease assay section. The eluted samples containing 5-MTHF₁ were applied to a HPLC device equipped with a fluorescence detector (excitation 295 nm, emission 356 nm) (16).

For the estimation of the inhibitory action of EGCg on enzyme activities, α -amylase, protease, and conjugase were assayed with different concentrations of each substrate in the presence of EGCg (1.5 mg/mL) and a Lineweaver-Burk plot was calculated. Changes in actual concentration of EGCg during each of the analytical steps were determined by a HPLC device equipped with an ultraviolet detector at 230 nm. The initial content of EGCg in the green tea model sample was set at 670 mg/100 mL. HPLC was performed with a Shimadzu HPLC-VP system (Shimadzu Corporation, Kyoto, Japan). The sample was applied to an L-column ODS, 4.6×250 mm, $5 \mu\text{m}$ particle size (Chemical Inspection & Testing Institute, Tokyo, Japan), at 40°C , and eluted with a linear gradient of 5% acetonitrile containing 0.1% phosphoric acid (A) and 50% acetonitrile containing 0.1% phosphoric acid (B). The gradient protocol was 0–5.0 min, 5% B; 5.1–10 min, 5–10% B; 10.1–15 min,

10% B; 15.1–25 min, 10–20% B at a flow rate of 1.2 mL/min. Tea catechins in diluted samples were also analyzed by HPLC with electrochemical detection (17).

Green tea infusion sample was prepared from tea leaves (gyokuro) purchased from a local grocery store. Concentration of typical tea catechins in the green tea infusion was as follows: EGCg, 158 mg/100 mL; EGC, 158 mg/100 mL; ECg, 13 mg/100 mL; EC 36 mg/100 mL; C, 3 mg/100 mL. Folate analysis in the green tea infusion was performed with and without tri-enzyme treatment. Folate analysis in the green tea infusion was also performed by the addition of a known amount of folate standard: either pteroyl- γ -L-glutamic acid or pteroylhepta- γ -L-glutamic acid was added to the green tea infusion at concentration of $31.1 \mu\text{g}/100$ mL or $37.7 \mu\text{g}/100$ mL, respectively.

Statistical Analyses. Data are presented as the mean $\pm$ standard error (SE). Data were analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test. Values of $p < 0.05$ were considered significant.

Results

Effect of tea catechins on microbial growth

EGCg and ECg showed marked inhibition of micro-

Table 1. Changes in actual EGCg sample concentration during digestive enzyme treatments.

Treatment steps	EGCg concentration ($\mu\text{g/mL}$)	% of initial value
Homogenization	322	100%
α -Amylase	271 ± 46.2	84.1%
Protease	69 ± 0.9	21.3%
Conjugase	4.4 ± 0.4	1.4%

Initial green tea model sample (678 mg of EGCg/100 mL) was homogenized with 20 volumes of phosphate buffer, and sequentially treated with α -amylase, protease, and conjugase as in reference (4). EGCg concentrations in the treated samples were determined by HPLC. Values are mean $\pm$ SE, $n=5$.

bial growth at doses higher than 25–50 $\mu\text{g/mL}$, while C, EC and EGC did not show any inhibitory effect up to 200 $\mu\text{g/mL}$ (Fig. 1). The inhibitory effect of EGCg was higher than that of ECg, and complete inhibition was observed at 100 $\mu\text{g/mL}$ of EGCg. The inhibitory concentration of EGCg was estimated to be higher than 10 $\mu\text{g/mL}$.

Effect of EGCg on tri-enzyme activities

Because EGCg is the major catechin of green tea, and showed the highest inhibitory effect on microbial growth, the effects of green tea catechins on the tri-enzyme activities were investigated using EGCg. EGCg inhibited the tri-enzyme activities in a dose-dependent manner. Significant inhibition was detected at doses higher than 750 $\mu\text{g/mL}$ for α -amylase, 1,000 $\mu\text{g/mL}$ for protease, and 50 $\mu\text{g/mL}$ for conjugase (Fig. 2). The Lineweaver-Burk plot showed that the mode of the EGCg inhibitory effect was similar between α -amylase and protease, but slightly different for conjugase.

Decrease of EGCg during each analytical step

The green tea model sample (678 mg of EGCg/100 mL) was diluted 20 times as is done in the assay, and the actual concentration of EGCg after each assay step was determined (Table 1). The concentration of EGCg in the sample markedly decreased following treatment by each of the three enzymes. The highest decrease was observed in the conjugase treatment. Furthermore, the EGCg sample concentration decreased to 1.1 $\mu\text{g/mL}$ after addition to the culture medium in the microbial growth analysis step.

Folate analysis in green tea infusion with and without tri-enzyme treatments

Folate concentration in green tea infusion was increased by 4 fold by tri-enzyme treatment (Fig. 3). The folate concentration was increased by the addition of pteroyl- γ -L-glutamic acid, but not by pteroylhepta- γ -L-glutamic acid in green tea infusion without tri-enzyme treatment.

However, the green tea infusion with pteroylhepta- γ -L-glutamic acid showed an increase in folate concentration with tri-enzyme treatment. Recovery of pteroyl- γ -L-glutamic acid and pteroylhepta- γ -L-glutamic acid was 94% and 86%, respectively. The actual EGCg concentra-

Fig. 3. Folate analysis in green tea infusion with and without treatment of tri-enzyme and addition of folate standard. Green tea infusion was applied to microbiological folate analysis with and without tri-enzyme treatment. As an folate standard, pteroyl- γ -L-glutamic acid (PG1) or pteroylhepta- γ -L-glutamic acid (PG7) was added to the green tea infusion at concentration of 31.1 $\mu\text{g}/100\text{ mL}$ or 37.7 $\mu\text{g}/100\text{ mL}$. Values are mean $\pm$ SE, $n=5$. * Significant difference from the level without trienzyme treatment. # Significant difference from the level without addition of folate standard.

tion in the microbial growth analysis step was 0.65 $\mu\text{g/mL}$ in the non-treated sample, but an undetectable level in the tri-enzyme treated sample.

Discussion

Tea catechins, in particular EGCg, have been shown to inhibit various enzymes (10–12) as well as microbial growth (8). In the present study, EGCg inhibited three digestive enzymes and microbial growth, as demonstrated in the microbiological assay. A marked inhibitory effect of EGCg was observed on the growth of microorganism at a concentration of 10 $\mu\text{g/mL}$. However, measurement of EGCg concentration after each step of the assay revealed that the EGCg concentration of the green tea model sample contained high EGCg in the microbial culture medium decreased to 1.1 $\mu\text{g/mL}$, suggesting that EGCg is likely to show no practical interference on folate analysis in green tea. Lack of influence of EGCg on folate analysis in green tea was confirmed by the analysis of the actual concentration of EGCg and by the addition of folate standard in green tea infusion.

Among tea catechins, EGCg and ECg showed a significant inhibitory effect on microbial growth, while such an inhibitory effect was not observed in the other catechins that have no galloyl moiety. These results are consistent with a report by Sugita-Konishi et al. (8), who showed that among the tea catechins of green tea, EGCg and ECg inhibited the production and extracellular release of verotoxins from enterohemorrhagic *E. coli* EHEC at concentrations higher than 50 $\mu\text{g/mL}$. In the present study, we only investigated the inhibitory effect of EGCg on the tri-enzyme treatment, because EGCg was observed to have the highest concentration among the tea catechins in green tea. However, Makimura et al. (10) reported that an inhibitory effect on collagenase activity was observed with EGCg and ECg at a concentration of 100 $\mu\text{g/mL}$, but not with other tea catechins. Similar structure-associated inhibitory effects of tea cat-

echins were reported in studies of pancreatic lipase (12) and α -amylase (11). Therefore, it is highly unlikely that tea catechins without the galloyl moiety interfere with tri-enzyme digestion or microbial growth in the folate assay. It is well known that EGCg binds to casein and serum proteins, resulting in a decrease in its antioxidant (18) and anti-microbial activities (9). Proline groups in proteins are known to have a strong affinity for the hydroxyl group of EGCg (19). In the present study, a marked decrease in EGCg concentration was observed after the protease and conjugase treatments, which contained higher amounts of protein compared to the α -amylase treatment. Therefore, the decrease in EGCg concentration observed during the tri-enzyme treatment may be due to the binding of EGCg to protein. The mode of inhibitory action by EGCg on the three enzymes was similar between α -amylase and protease, but slightly different for the conjugase, which was a crude enzyme prepared from rat serum. Therefore, it is possible that the difference in the inhibitory mode of action may have been due to the protein content and possible impurities of the conjugase treatment.

Folate concentration in green tea infusion was increased to approximately 4 fold by tri-enzyme treatment, suggesting that folates in green tea exist as polyglutamate forms. The microorganism *L. rhamnosus* used in the folate assay mainly used the mono-glutamate form, but can use the di- and tri-glutamate form of folates (4). It has been shown that food folates exist in the 5-methyl polyglutamate form (2). Therefore, folate in green tea would exist in the 5-methyl polyglutamate form as in the other foods. Further detailed study is needed to clarify the exact chemical structure of folates in green tea.

In conclusion, the present study demonstrated that tea catechins, in particular EGCg, have no practical impact on the folate analysis of green tea. In the microbiological assay, the treatment order by the three enzymes was slightly changed compared to previous reports (5, 6). In addition, the initial dilution of the analytical sample may change due to the folate content of the food samples. Knowledge of the inhibitory concentrations of EGCg for each enzyme and for the microbial growth identified in the present study may be helpful in such cases.

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