Synthesis of theaflavins with *Camellia sinensis* cell culture and inhibition of increase in blood sugar values in high-fat diet mice subjected to sucrose or glucose loading

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Abstract

Theaflavin and its galloyl esters are major polyphenolic pigments of black tea. We compared the efficiency of a variety of oxidizing enzyme systems to synthesize theaflavin and its galloyl esters. *Camellia sinensis* cell culture efficiently synthesized theaflavin from epicatechin and epigallocatechin with 70% yield and 100% conversion in 4 min. In an administration experiment performed in mice, theaflavin inhibited the increase blood glucose levels in mice that were fed a high-fat diet and subjected to glucose or sucrose loading in mice.

Key words: theaflavin, theaflavin galloyl esters, *Camellia sinensis* cell cultures, attenuation of blood sugar level increase

Theaflavin (TF) and its galloyl esters are the main red pigments in black tea, and have recently been shown to exhibit various anti-obesity¹⁾, glucose-lowering²⁾ and anticancer³⁾ effects. Therefore, it has become recognized as potentially useful not only as a natural coloring agent but also as a bioactive substance. However, further research on the compound is required. TF has no galloyl esters. Theaflavin's galloyl esters have three different galloyl esters: theaflavin-3-*O*-gallate (TF3G), theaflavin-3'-*O*-gallate (TF3'G), and theaflavin-3,3'-di-*O*-gallate (TFDG). Their chemical structures are shown below in Scheme 1.

Scheme 1. The chemical structures of TF, TF3G, TF3'G, and TFDG

RR'

HH Theaflavin (TF) : EC + EGC (0.08 %)

GH Theaflavin 3-*O*-gallate (TF3G) : EC + EGCG (0.3%)

HG Theaflavin 3'-*O*-gallate (TF3'G) : ECG + EGCG (0.2 %)

GG Theaflavin 3,3'-di-*O*-gallate (TFDG) : ECG + EGCG (0.4 %)

TF and its galloyl esters (TF3G, TF3'G, TFDG) are produced from their parent catechins [epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin-3-*O*-gallate (EGCG)] by polyphenol oxidase or peroxidase in tea leaves during production of black tea leaves or in tea fermentation, and are obtained by extraction from black tea^{4),5)}. However, the content in black tea is extremely low as shown in Scheme 1⁶⁾. Therefore, it has

been difficult to obtain a sufficiently usable amounts of TF and its galloyl esters simply by extraction from black tea leaves.

TF is biosynthesized from EC and EGC. TF3G is biosynthesized from EC and EGCG. TF3'G is biosynthesized from ECG and EGC and TFDG is biosynthesized from ECG and EGCG (Scheme 1)^{4),5)}. Development of the use of enzymes for oxidative reactions, which is a green chemistry approach, has been increasing in recent years. Horseradish peroxidase (HRP) is a commercially available metalloporphyrin enzyme. We observed that *Camellia* (*C.*) *sinensis* cell culture is a rich source of peroxidase (POD) enzymes⁷⁾. Therefore, we applied them to the oxidative coupling of 2-naphthol derivatives⁸⁾ and production of a variety of biologically active compounds⁷⁾. In this study, we report a method using *C. sinensis* cell culture for efficient synthesis of TF from EC and EGC with high yield⁹⁾.

To investigate the synthesis of TF by oxidation of EC and EGC, we surveyed a variety of oxidizing agents [commercial polyphenol oxidase (PPO) (Funakoshi Co., Ltd), commercial HRP¹⁰⁾ (Wako Co., Ltd), *C. sinensis* cell culture, *Nicotiana(N.) tabacum* cell culture⁷⁾, and *Daucus (D.) carota* cell culture⁷⁾] to optimize conditions as shown in Table 1. Synthesis of TF from EC (20mg) and EGC (21mg) was performed **with stirrer**. A) 10mg PPO was added to a mixture of EC and EGC in 0.1 M H₃PO₄ buffer (pH 6, 20 ml), B) 10 mg HRP was added to a mixture of EC and EGC in 0.1 M H₃PO₄ buffer (pH 6, 20 ml), acetone (2 ml), and 3% H₂O₂ (0.5 ml), C) 10 ml of plant cell culture was added to a mixture of EC and EGC in 0.1M H₃PO₄ buffer (pH 6, 20 ml), acetone (2 ml), and 3% H₂O₂ (0.5ml).

As shown in Table 1 as oxidizing agent entry 3, EC and EGC were converted into TF in 15 min with 48% yield as the sole product, and the residual starting materials (EC, EGC) were absent. For the other oxidizing agents (entries 1, 2, 4, and 5), yields of TF were low, and the starting materials (EC, EGC) were recovered. For *C. sinensis* cell culture (entry 6), a shortened reaction time led to the highest chemical yield of TF. We succeeded in quantitative synthesis of TF as the sole product with 70% yield and 100% conversion from EC and EGC in *C. sinensis* cell culture with a reaction time of 4 min. **Fig 1 shows HPLC chromatogram of**

Table 1. Synthesis of TF¹¹⁾ with oxidizing agents

No	Oxidizing agents	Time (min)	Y. (%)	Recovered EC (%)	No	Oxidizing agents	Time (min)	Y. (%)	Recovered EC (%)
1	PPO	18	29	49	4	<i>N. tabacum</i>	15	25	45
2	HRP	60	30	28	5	<i>D. carota</i>	15	19	51
3	<i>C. sinensis</i>	15	48	0	6	<i>C. sinensis</i>	4	70	0

TF synthesis (entry 6 at reaction time 1 min). Peak at 3.6 min is EGC and peak at 4.4 min is EC. Fig 2 shows HPLC chromatogram of TF synthesis (entry 6 at reaction time 4 min). Peak at 14.2 min is TF. These HPLC chromatogram shows that all EC and EGC were converted to TF. Fig 3 illustrates the time course of entry 3. TF increased with reaction times, reaching maximum and decreased. On the other hand, EC and EGC decreased in 4 min, reaching 0%. For a typical experiment, TF was separated from the reaction mixture and purified after the synthetic reaction was completed. TF-containing extraction residues may be separated and

Fig1

Fig2

Fig 3

purified using techniques such as column chromatography. However, column chromatography of TF mixtures containing EC and EGC led to the decomposition of TF which lowered its yield. Because the method shown in entry 6 produced TF alone, it was possible to obtain the target substance with high purity by solvent extraction of the reaction mixture. In a typical experiment, EC (290 mg) and EGC (306 mg) were added to a mixture of acetone and phosphate buffer (pH 6.0) (1:10 v/v, 100 ml), *C. sinensis* cell culture (50ml including 10.2 g cells), and 3% H₂O₂ (0.8 ml). The mixture was stirred for 4 min and then extracted using CH₃COOEt. Further, the organic layer was dried over anhydrous MgSO₄ and concentrated *in vacuo* to provide TF (395 mg) with 70% yield and 100% conversion. In the HPLC analysis¹²⁾, peaks for EC, and EGC were virtually absent, but a peak for TF was observed.

According to the established procedure described above, we used HRP, PPO, or *C. sinensis* cell culture to synthesize TF3G from EC and EGC, TF3'G from EGC and EGC, and TFDG from EGC and EGC. These results are summarized in Table 2. With respect to the yields of all TF3G, TF3'G, and TFDG, *C. sinensis* cell culture was not superior to PPO or HRP. These results (Tables 1 and 2) suggest that *C. sinensis* cell culture contains a specific enzyme for synthesis of TF from EC and EGC.

Table 2. Synthesis using different oxidizing agents to produce TF 3G, TF3'G, and TFDG¹¹⁾

No	TFs	Oxidizing agents	Time (min)	Y. (%)	No	TFs	Oxidizing agents	Time (min)	Y. (%)
1	TF3G	HRP	90	10	6	TF3'G	<i>C. sinensis</i>	120	6
2	TF3G	PPO	50	19	7	TFDG	HRP	120	5
3	TF3G	<i>C. sinensis</i>	12	15	8	TFDG	PPO	120	10
4	TF3'G	HRP	120	14	9	TFDG	<i>C. sinensis</i>	120	5
5	TF3'G	PPO	90	10					

The effect of TF on inhibition of increases in blood sugar values in mice was evaluated¹³⁾. Oral administration of TF (30.6 mg/kg) to 5-week-old C57BL6 mice (n=6) significantly inhibited increases in blood glucose in a sucrose loading test (2 g/kg) relative to the effect of administration of distilled water in control mice (maximum blood sucrose levels 20 min after administration of TF: 195.0±22.4*, control: 220.0±8.2). Five-week-old C57BL6 mice were provided with TF *ad libitum* (5 mg/15 ml water) and a high-fat diet, Quick Fat. When the

mice (n=7) were 16-weeks-old, sucrose (2 g/kg) or glucose (2 g/kg) was administered. Increases in blood glucose levels were significantly inhibited relative to those in the control group of mice that received distilled water (blood sucrose levels 40min after sucrose administration of TF: $234.0 \pm 19.8^{**}$, control: 309.3 ± 27.0 ; blood glucose levels 20 min after glucose administration of TF: $407.6 \pm 35.6^{*}$, control: 482.6 ± 17.2). The fasting blood sugar level was used as a baseline to calculate the area under the blood sugar elevation curve (Δ AUC). The sugar data Δ AUC was tabulated relative to the water Δ AUC set as 100 to obtain relative values (sucrose: control, 100; TF, $82.6 \pm 3.1^{**}$; glucose: control, 100; TF, $79.5 \pm 10.9^{*}$). The Δ AUC of TF was significantly suppressed relative to that of the Δ AUC of the control. These results indicated that TF significantly inhibited increases in blood sugar levels when glucose or sucrose was orally administered to mice. The mechanism underlying inhibition of elevated blood sugar levels was not investigated in this experiment. Further studies are in progress.

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11. The structures of TF, TF3G, TF3'G, and TFDG were confirmed by a comparison of the ^1H -NMR, FAB-MS data, and HPLC with authentic samples.
12. A JASCO HPLC system (PU-980, UV-970) and a 4.6 mm $\times$ 250 mm ODS120A column (TOSO) were used with the following HPLC analytical conditions: solvent: acetonitrile : CH_3COOEt : 0.05% H_3PO_4 (21:3:76); flow rate, 1.0 ml/min; temperature, 25°C; with UV detection at UV 280 nm.

13. All data are expressed as means $\pm$ SEs. The statistical significance of differences between groups were analyzed as follows: Student's *t*-test was used after the *F*-test to analyze differences between groups in the experiments involving two experimental groups. *P*-values less than 0.05 were considered to indicate statistical significance.