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Specificity of tyrosinase-catalyzed synthesis of theaflavins

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ABSTRACT

This study kinetically characterized the mechanism of the enzymatic synthesis of theaflavins (TFs) from catechins by mushroom tyrosinase (EC 1.14.18.1). In reactions containing one of four catechins, (-)-epicatechin (EC), (-)-epigallocatechin (EGC), and their galloylated forms (ECg and EGCg), they were oxidized by tyrosinase with apparent K_M values of 3.78, 5.55, 0.80, and 3.05 mM, respectively, and with different consumption rates, of which EC was more than four times higher than those of the others. In reactions with binary combinations of catechins with tyrosinase, the synthesis of TF1 from EC and EGC occurred most efficiently, while the yields of mono- and di-galloylated TFs, TF2A, TF2B, and TF3, were low. Time-dependent changes in concentrations of the reactants suggested that the enzymatic oxidation of catechins and the subsequent non-enzymatic coupling redox reaction between the quinone derived from EC or ECg and the intact pyrogallol-type catechin (EGC or EGCg) proceeded concurrently. The latter reaction induced the rapid decrease of EGC and EGCg and it was remarkable for EGCg. So the efficiency of condensation of a pair of quinones from catechol- and pyrogallol-type catechins is restricted, critically influencing the yield of TFs. Using green tea extracts as mixtures of the four substrate catechins, tyrosinase produced TF1 most abundantly. Furthermore, a remarkable enhancement of production of TF2A and TF2B as well as TF1 was observed, when the initial concentration of EGCg was low. These results suggest that the catechin composition has an impact on yields of TFs.

1. Introduction

Theaflavins (TFs), the characteristic pigments in black tea leaves and their exudates, are known to contribute to the orange or orange-red color and astringency of various kinds of brewed tea. Recently, TFs have attracted attention due to their various bioactivities, such as antioxidant activity against LDL oxidation [1], radical-scavenging activity [2], anticancer activity [3,4], antidiabetic effects [5], and inhibitory activities against the cholesterol and fat absorption [6–8] and the bone loss in models of osteoporosis [9]. However, the amount of TFs in black tea is fairly low, about 8–20 mg/100 mL tea brewed from a teabag [10]. In addition, TFs are not stable in aqueous solution, especially under neutral or alkaline conditions [11–13]. Therefore, research on the bioactivities of TFs as well as their application into foods and pharmaceutical products has been restricted.

TFs are known to be produced during the process of fermentation of the leaves of *Camellia sinensis*, in which endogenous polyphenol oxidase (PPO) is involved. Condensation of two oxidized catechins and decarboxylation afford TF production [14,15]. Condensation of different pairs of catechins, one with a di-hydroxylated B-ring (catechol-type) and the other with a tri-hydroxylated B-ring (pyrogallol-type), results in formation of four kinds of TFs, theaflavin (TF1), theaflavin-3-O-gallate (TF2A), theaflavin-3′-O-gallate (TF2B), and theaflavin-3,3′-O-digallate (TF3) (Fig. 1). TF synthesis by plant PPOs has been investigated [14,15], and a possible mechanism has been proposed as shown in Fig. 1. Some reasons why the yield of TFs is generally low have been suggested as follows: 1) the plant PPOs preferentially catalyze catechols rather than pyrogallols [16,17], 2) pyrogallols are susceptible to oxidation by quinone, reducing it to catechol, 3) the quinone, an oxidative product of catechin, is so highly reactive that it binds to other molecules possessing nucleophilic amino- and thiol groups, such as proteins [18], and it also attacks TFs leading to their degradation [15,19] and 4) further uncontrolled reactions pro-

Abbreviations: EA, ethyl acetate; EC, (-)-epicatechin; EGC, (-)-epigallocatechin; ECg, (-)-epicatechin gallate; EGCg, (-)-epigallocatechin gallate; GLB, green tea leaves etracted with the buffer; GPE, green tea powder extracted with ethyl acetate; PPO, polyphenol oxidase; TF, theaflavin.

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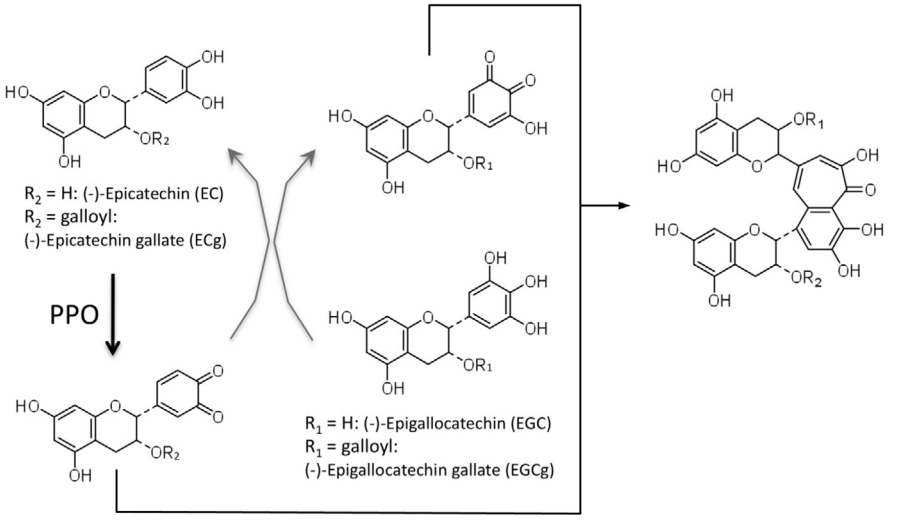

Theaflavins

	R_1	R_2	Parent flavonols
TF1	H	H	EC + EGC
TF2A	galloyl	H	EC + EGCg
TF2B	H	galloyl	ECg + EGC
TF3	galloyl	galloyl	ECg + EGCg

Fig. 1. Structures of tea polyphenols and prospective mechanism of polyphenol oxidase- catalyzed synthesis of theaflavins from catechins.

ceed providing polyphenol oligomers, for example, theasinensins, theanaphtoquinones, dehydrotheaflavins, bistheaflavins, and so on [20]. In addition, in vitro reactions using PPO and peroxidase (POD) purified from tea leaves have recently demonstrated that once synthesized TFs are converted to the reddish-brown thearubigins, which are detected as a broad hump on RP-HPLC chromatograms, probably through the POD-catalyzed reaction [21,22]. Since the enzymatic and non-enzymatic reactions proceed concurrently and their sub-products interfere with the synthesis of TFs, it is difficult to understand the overall reaction mechanisms.

Tyrosinase (EC 1.14.18.1) from the mushroom *Agaricus bisporus*, a copper-containing oxidase with both monophenol monooxygenase- and diphenol oxidase activities, has been characterized and used in various studies [23–27]. This mushroom tyrosinase is able to oxidize pyrogallols in addition to catechols [28]. If it reacts with catechol- and pyrogallol-type catechins concurrently producing both-types of quinones, condensation of these two oxidative products could be accelerated, increasing TF production. Thus, tyrosinase may be a promising enzyme as a catalyst for the efficient synthesis of TFs. Indeed, it has been used for in vitro TF synthesis from catechins in place of PPO [29,30]. However, to our knowledge, the optimum reaction conditions and substrate specificities as well as TF yields have not been fully investigated yet. In this study, we compared the tyrosinase-catalyzed reactions not only with single catechin but also with binary combinations of catechins. We found that the enzymatic specificities for catechins were not directly responsible for the yields of the corresponding TFs. Furthermore, the addition of tyrosinase into green tea extracts differing in catechin composition indicated that TF1 was preferentially produced and that a higher concentration of EGCg suppressed tyrosinase-catalyzed TF synthesis.

2. Materials and methods

2.1. Materials

EC, EGC, ECg, and EGCg were kindly provided by Mitsui Norin Co., Ltd. (Shizuoka, Japan). Tyrosinase from mushroom was obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Instant green tea powder (AVT Natural Products Ltd., Chennai, India) was provided by Tea Solutions, Hara Office Inc. (Tokyo, Japan) and green tea leaves were commercially obtained. All other reagents used were of analytical grade.

2.2. Tyrosinase-catalyzed oxidation of catechins

Unless otherwise specified, enzymatic reactions were performed using tyrosinase at 0.05 mg/mL (156 mU/mL) with each catechin and 0.1 mg/mL (313 mU/mL) for TF synthesis in 50 mM Na-phosphate buffer, pH 6.0, at 25 °C without pH control. Respective catechin solutions (10 mM) were prepared using 20% ethanol/40 mM Na-phosphate buffer, pH 6.0. After certain incubation periods, an aliquot (100 μL) of the reaction mixture was collected and added into 1 mL of 25 mM citric acid solution (pH 2.4) to stop the reaction. These samples were cooled in an autosampler (L-2200, Hitachi, Tokyo) at 5–8 °C and analyzed by RP-HPLC, which was carried out using a Hitachi HPLC system (L-2130 pump, L-2400 UV detector) equipped with a SUS line filter (GL Science, Tokyo, Japan) and a Phenomenex Synergi™ 4 μm Polar-RP 80 Å (4.6 mm × 150 mm) column (Shimadzu GLC, Tokyo, Japan). Catechins were eluted using an aqueous 20% MeCN solution containing 0.05% phosphoric acid at flow rate of 1 mL/min, and detected at 280 nm. For TF1, TF2A, TF2B, and TF3, aqueous 32% MeCN solutions containing 0.05% phosphoric acid were used as eluents, and each retention time (t_R) was identified using the TF standards, which were obtained as described previously [31]. The analysis of chromatograms was performed with the data processing software Chromato-PRO (Run Time Corporation, Tokyo, Japan).

2.3. Preparation of catechin mixtures from green tea

In order to investigate the effects of the differences of catechin composition on the product yields in tyrosinase-catalyzed TF synthesis, green tea powder and a type of green tea leaf were used for the extraction of catechins. For the green tea powder, 3 mL of 0.1 M citric acid solution was added to 170 mg of powder. After agitation for 3 min, it was centrifuged at 8400 × *g* for 15 min at 25 °C. Its supernatant was mixed well with a 1.5-fold volume of ethyl

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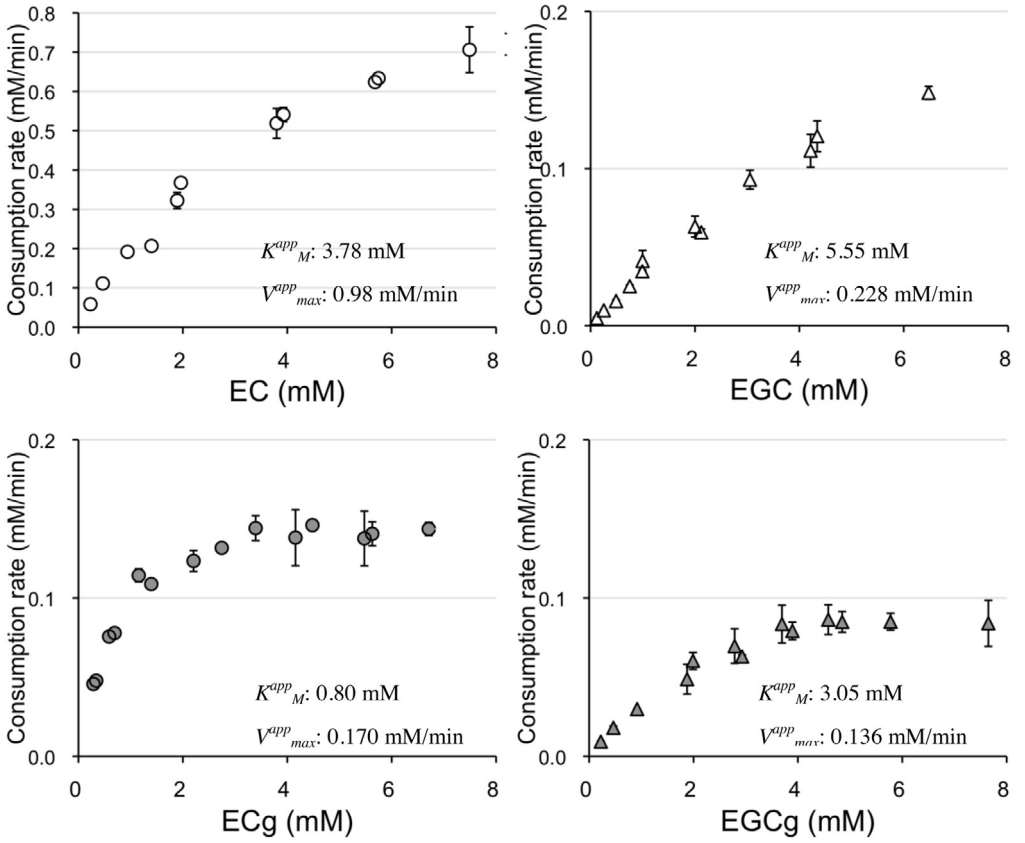


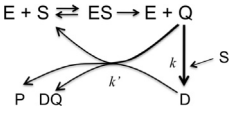
Fig. 2. Kinetic analysis of the tyrosinase-catalyzed reaction with each catechin.
Each of four catechins, EC (open circle), EGC (open triangle), ECg (closed circle), and EGCg (closed triangle), was reacted with 0.05 mg/mL tyrosinase in 50 mM Na-phosphate buffer, pH 6.0, for 3 min at 25 °C. Data of consumption rates are indicated as means ± SD (n = 3). Apparent values of K_M (mM) and V_{max} (mM/min) are also described.

acetate (EA), and then left to stand for about 10 min. The upper fraction of 2.4 mL was collected and evaporated. The concentrated mixture was solubilized in 4.8 mL of 50 mM Na-phosphate buffer, pH 6.0, just before use as the green tea powder extracted with EA (GPE) sample. For green tea leaves, 390 mg of leaves were infused in 6 mL of 50 mM Na-phosphate buffer, pH 6.0, at room temperature for 5 min. After the infusion was agitated and centrifuged similarly to the green tea powder, the supernatant was collected and used within a day as the green tea leaves extracted with buffer (GLB) sample for the tyrosinase-catalyzed reaction. Each sample of the two green tea extracts, GPE and GLB, were reacted with tyrosinase at 25 °C, and then diluted by an eight-fold volume of 25 mM citric acid solution to stop the reactions. Aliquots of 50 μL were analyzed by RP-HPLC which was carried out as described above, except for the eluting conditions: 0–10–40–42–47 min, aqueous 8–20–32–80–80% MeCN solution containing 0.05% phosphoric acid.

3. Results and discussion

3.1. Tyrosinase-catalyzed reaction with respective catechins

Since the optimum pH for tyrosinase from *A. bisporus* is around 6–7 [23,28] and the stabilities of catachins and TFs are low under neutral and alkaline conditions [11–13], the reaction was performed at pH 6.0 in this study. We confirmed that EC, EGC, ECg, EGCg, and TFs were relatively stable and almost all of them remained during a 60-min incubation in the absence of tyrosinase in 50 mM Na-phosphate buffer, pH 6.0, at 25 °C (data not shown). During the tyrosinase-catalyzed reaction with catechins, the quinones derived from substrates cause non-enzymatic oxidations converting substrates into self-dimer or polymer, of which rate constants are likely to be very high [32,33] (Scheme 1).



Scheme 1. Tyrosinase-catalyzed and quinone-induced oxidation of catechins.
Meanings of the symbols are as follows: S: catechin substrate, E: enzyme, ES: enzymesubstrate complex, Q: quinone produced by enzymatic reaction with substrate, D: dimer of substrate, DQ: dimer quinone, P: polymer produced by non-enzymatic reaction related to quinone, k and k': rate constants corresponding to quinoneinduced chemical reactions.

The direct measurement of substrate cannot give accurate kinetic constants, such as K_M, because the quinone is highly reactive and unstable, resulting in large k and k' [32]. However, when the enzymatic oxidation of substrate was very slow together with [S] > > [Q], this rate-limiting step would realize [Q] ≈ 0 in the early stage of reaction. Under such a condition, the rate of substrate consumption could be expressed as follows:

$$\frac{dS}{dt} = V_{enz} + k[Q][S] - k'[Q][D] \approx V_{enz}, \quad (1)$$

where $V_{enz} = V_{max}[S]/(K_M + [S])$. So we performed the direct analyses of each of the four catechins incubated for a short-term reaction (for 3 min) with 0.05 mg/mL of tyrosinase. The consumption rates of catechins showed Michaelis-Menten saturation curves (Fig. 2). We confirmed the proportional relationship between $|dS/dt|$ and [E] at below 0.05 mg/mL (data not shown), which suggested the condition permitting Eq. (1). Therefore, the apparent K_M and V_{max} values for EC, EGC, ECg, and EGCg, as indicated in Fig. 2, were tentatively determined by Lineweaver-Burk plots. The differ-

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ences between values of the apparent K_M in this study and the $K^{ppo}{}_M$ determined by Munoz-Munoz et al. [33] might be due to the differences in assays as well as reaction pHs. However, the highest affinity of tyrosinase for ECg is agreement with their result determined by the chronometric method based on a spectrophotometric measurement [33]. Furthermore, the order of V_{max}/K_M of four catechins, EC > > ECg > EGCg ≈ EGC, is also similar to that reported in the literature [33]. Since the galloylated catechins (ECg, EGCg) are known to bind to proteins with higher affinity than non-galloylated ones (EC, EGC) [34–37], the former could also bind to tyrosinase non-specifically via hydrophilic and hydrophobic interactions, making it possible for the enzyme to accommodate them nearby its active site.

To investigate effects of the subsequent non-enzymatic oxidation of catechins on the tyrosinase-catalyzed reaction, 5 mM of each catechin and 0.1 mg/mL tyrosinase were incubated for 60 min. The order of overall substrate consumption was EC > EGCg > EGC > ECg (Fig. 3), which was inconsistent with the results shown in Fig. 2. This suggests that the pyrogallol-type catechins, in particular EGCg, are highly reactive with their quinones possibly providing self-dimers and polymers effectively. For ECg, in spite of the lowest K_M, its consumption became slower after 30 min (Fig. 3). This might be due to the product inhibition, on which further studies are undertaken.

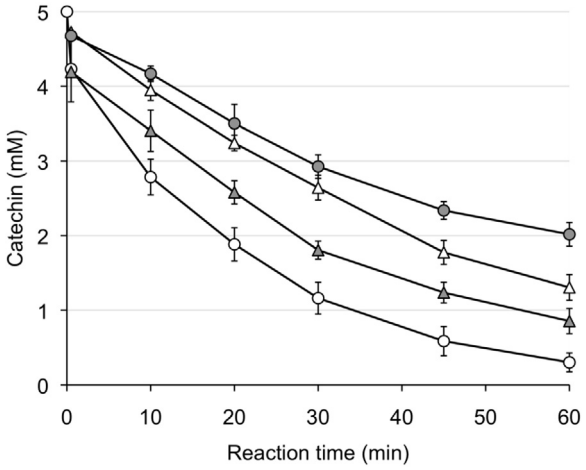

Fig. 3. Time-dependent changes of catechins during the tyrosinase-catalyzed reaction with respective catechins.
Each of four catechins, EC (open circle), EGC (open triangle), ECg (closed circle), and EGCg (closed triangle), at 5 mM was reacted with 0.1 mg/mL tyrosinase in 50 mM Na-phosphate buffer, pH 6.0, for 60 min at 25 °C. Data are indicated as means ± SD (n = 3).

3.2. Tyrosinase-catalyzed reaction with binary combinations of catechins

In the tyrosinase-catalyzed reactions with different binary combinations of 2 mM catechins, the respective reactions produced the corresponding TFs (Fig. 4A – D). Pyrogallol-type catechins (EGC, EGCg) were consumed faster than catechol-type ones (EC, ECg) (Fig. 4A – D), suggesting that the former catechins were oxidized by not only tyrosinase but also quinones derived from the latter ones. As tyrosinase preferentially reacted with EC about 5.8-times faster than ECg (Fig. 2), the rates of the pyrogallol oxidation by EC-quinone (Fig. 4A&B) seemed to be higher than those by ECg-quinone (Fig. 4C&D). Meanwhile, the consumption rates of EC and ECg were suppressed especially at the early stage of the reaction

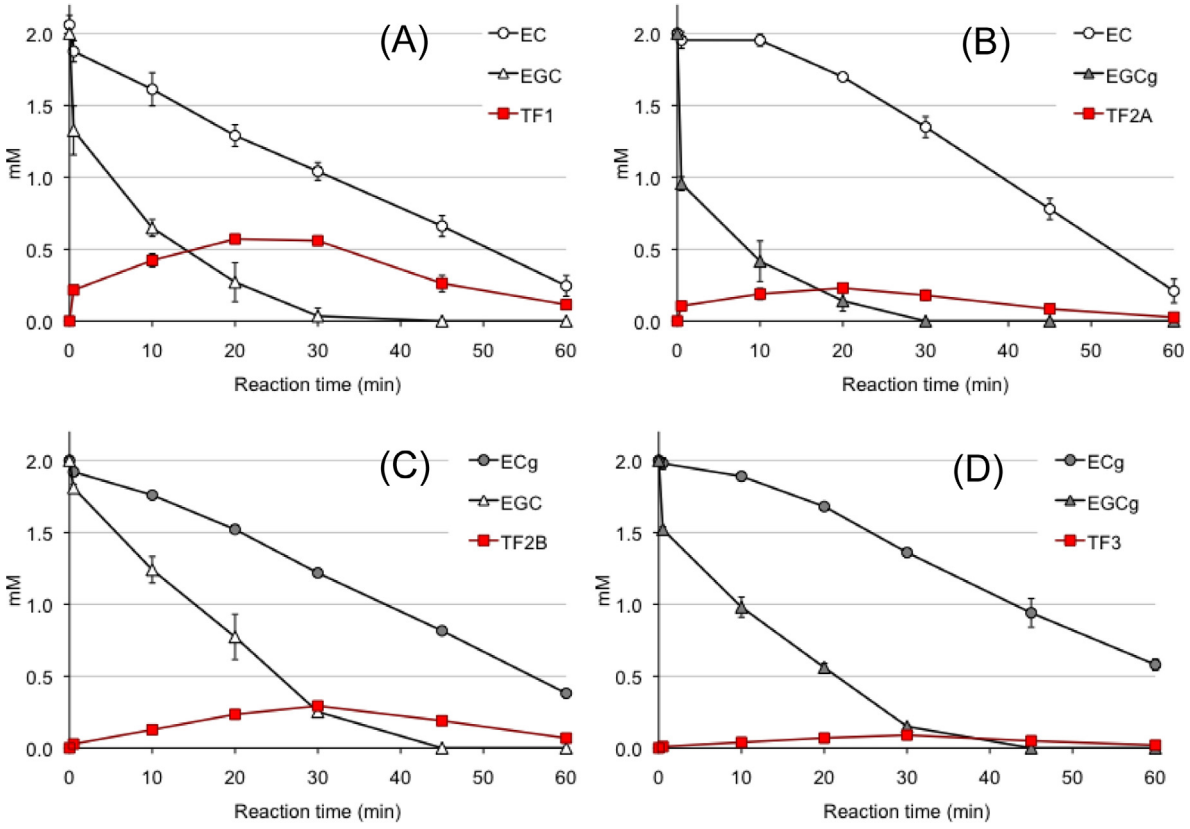

Fig. 4. Time-dependent changes of catechins and TFs during the tyrosinase-catalyzed synthesis of TF1 (A), TF2A (B), TF2B (C), and TF3 (D).
Initial substrate concentrations were 2 mM. Temporal changes in concentrations of EC (open circles), EGC (open triangles), ECg (closed circles), EGCg (closed triangles), and corresponding TFs (closed red squares) are indicated. Enzymatic reaction was conducted with 0.1 mg/mL tyrosinase in 50 mM Na-phosphate buffer, pH 6.0, at 25 °C. Data are indicated as means ± SD (n = 3).

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Table 1
Maximum yields of TFs in tyrosinase-catalyzed synthetic reactions (Fig. 3). Each reaction time (min) when we observed the highest TF yield is shown.

TF synthesis from catechins		min	TF yields (%) based on consumed catechins with different B-ring types	
			Catechol (EC or ECg)	Pyrogallol (EGC or EGCg)
TF1	2 mM EC: 2 mM EGC	20	75.3 ± 4.7	34.5 ± 3.5
TF2A	2 mM EC: 2 mM EGCg	20	65.3 ± 4.0	12.4 ± 0.9
TF2B	2 mM ECg: 2 mM EGC	30	48.9 ± 3.4	19.2 ± 1.2
TF3	2 mM ECg: 2 mM EGCg	30	13.9 ± 0.7	4.8 ± 0.1

Data are indicated as means ± SD (n = 3).

combined with EGCg (Fig. 4B&D) more than EGC (Fig. 4A&C). This represents that EGCg is more susceptible to the quinone-induced oxidation of pyrogallol than EGC. Although the molecular mobility of EGCg may be also restricted by its non-specific binding to tyrosinase, this property would allow EGCg to contact effectively with quinones released from the catalyst. Taking into account the susceptibility of EGCg against ECg- and self-quinones as described above, the non-enzymatic condensation between ECg- and EGCg-quinones rarely occurred, resulting in the slow production of TF3 (Fig. 4D). The faster production of TF1 than TF2A also reflected the lower susceptibility of EGC than EGCg (Fig. 4A&B).

TFs continued to increase until the complete consumption of pyrogallols, and later, markedly decreased (Fig. 4). Since we confirmed that TFs were not directly consumed by tyrosinase (data not shown), quinones and/or other products synthesized by the tyrosinase-catalyzed oxidation of catechols probably caused electrophilic attacks on TFs, leading to their breakdown [15,20]. High amounts of EGC and EGCg in the reaction mixture could inhibit the quinone-induced TF breakdown through the coupled reduction of catechol quinones with their pyrogallol groups. However, even if the pyrogallols remained, both synthesis and breakdown of TFs would occur concurrently in the reaction. The ratio between catechol- and pyrogallol-catechins is therefore an important factor for the yield of TFs.

TF yields based on the consumed pyrogallol-type catechins were lower than those based on catechol-type catechins (Table 1). EGC and EGCg were susceptible to the quinone-induced oxidation, reducing quinones to the original catechols. The resulting EGC- and EGCg-quinones were likely to provide dehydrotheasinensins through the subsequent non-enzymatic coupling reaction [38,39]. A comparison of maximum TF yields (Table 1) indicated that TF1 was most efficiently synthesized. In contrast, the yield of TF3 was extremely low. For example, ratios of the TF yields were as follows: TF2A/TF1 was 0.87 on the EC base (reflecting the effect of substrate exchanging EGC for EGCg), TF3/TF2B was 0.28 on the ECg base (exchanging EGC for EGCg), TF2B/TF1 was 0.56 on the EGC base (exchanging EC for ECg), and TF3/TF2A was 0.39 on the EGCg base (exchanging EC for ECg). These differences between TF3 and the others seemed to be beyond the properties of the tyrosinase-catalyzed catechin oxidation as shown in Figs. 2 and 3. Thus, further studies are currently undertaken to investigate whether the lower efficiency of condensation between ECg- and EGCg-quinones resulted from a certain regioselective control or not.

3.3. Tyrosinase-catalyzed TF synthesis using green tea extracts

We prepared two green tea extracts to investigate the tyrosinase-catalyzed TF synthesis from available catechin mixtures. One was extracted from a commercially obtained instant green tea powder with an acidic solution, which was used to prevent catechin degradation. As the fine powder provided a green solution rich in not only catechins but also other water-soluble components such as caffeine, amino acids, and proteins (data not shown), catechins were subsequently extracted from the solution with ethyl acetate (EA). The EA fraction recovered about 25% EGC, 40–50% EC, 30% caffeine, 50% EGCg, and 60% ECg, in order of retention time in RP-HPLC (Fig. 5). Although hydrophilic EGC was lost to a remarkable extent through EA extraction, other catechins were more effectively extracted. The twice-diluted green tea powder-EA extract was used in this study as a GPE sample. On the other hand, the green tea leaves were immersed and agitated in phosphate buffer, pH 6.0, giving the green tea leaves-buffer extract (GLB). The overall extraction efficiency of the leaves was low compared to the powder, however, hydrophilic EGC was more abundant than EGCg in GLB (Fig. 5, Table 2).

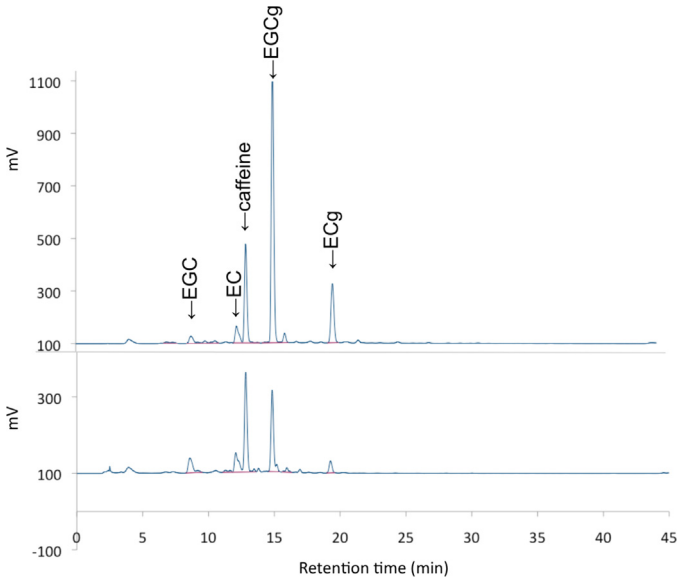

Fig. 5. Chromatograms of green tea extracts (upper: GPE, lower: GLB).

Table 2
Catechin compositions in green tea extracts.

(mM)	EC	EGC	ECg	EGCg
GPE	1.2	2.7	0.95	4.3
GLB	1.0	3.9	0.12	0.86

Table 3
Concentrations of TFs in reaction mixtures after a 48-min reaction with 0.1 mg/mL tyrosinase at 25 °C.

(μM)	TF1	TF2A	TF2B	TF3
GPE	42 ± 1.5	6.6 ± 0.8	7.8 ± 0.7	5.9 ± 0.7
GLB	174 ± 8.9	21 ± 1.6	30 ± 1.4	2.1 ± 0.3

Data are indicated as means ± SD (n = 3).

During the incubation of GPE or GLB with tyrosinase at 25 °C, TFs increased linearly over time for 48 min (data not shown). TF1 was predominantly synthesized in both samples (Fig. 6, Table 3). TF3 was less produced in the GLB sample (Fig. 6B, Table 3) due to lower concentrations of both ECg and EGCg (Table 2). The GPE sample showed slow TF production (Fig. 6A), probably because a large

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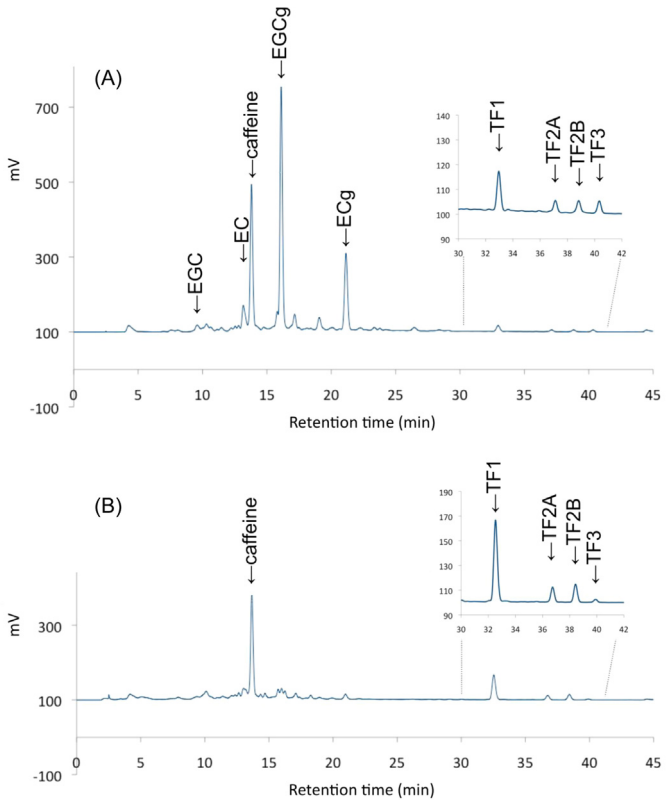

Fig. 6. Chromatograms of green tea extracts (A: GPE, B: GLB) after a 48-min reaction with 0.1 mg/mL tyrosinase at 25 °C.

amount of EGCg competitively inhibited the tyrosinase-catalyzed oxidation of the other catechins and/or concurrently reduced EC- and ECg-quinones. In contrast, much higher levels of TF1, TF2A, and TF2B accumulated in GLB samples (Fig. 6B, Table 3) in spite of the low doses of ECg and EGCg (Table 2). A little higher (1.4-fold) EGC concentration in GLB compared to GPE might have contributed to the enhancement of production of TF1 (4.1-fold) and TF2B (3.8-fold). The production of TF2A was also effectively increased by 3.2-fold. This might have been achieved through a mechanism in which the low concentration of EGCg allowed for the rapid, excessive synthesis of EC-quinone by the enzyme over the rate of its reduction coupled with EGCg oxidation, and then, the subsequent condensation between EC- and EGCg-quinones provided TF2A.

4. Conclusion

Our kinetic analyses with respect to catechin substrates indicated that *A. bisporus* tyrosinase oxidized respective four catechins with the apparent kinetic constants of K_M: EGC > EC > EGCg > ECg and V_{max}/K_M: EC > > ECg > EGCg ≥ EGC (Fig. 2). On the other hand, the long-term reactions involving the non-enzymatic reactions showed the rapid catechin consumption in the order of EC > EGCg > EGC > ECg (Fig. 3). The discrepancy between Figs. 2 and 3 suggests the properties of EGC and EGCg that are susceptible to the quinone-induced oxidation. In addition to the substrate specificities of the enzyme, we found that the catechin composition in the reaction mixture influenced the yield of TFs to a great extent, because the tyrosinase-catalyzed TF synthesis was drastically affected by non-enzymatic redox reactions between catechins and their oxidative products. This study also demonstrated that the green tea extract containing relatively high EGC and low EGCg could significantly enhance the production of TF1, TF2A, and TF2B. Since TF monogallate isomers, TF2A and TF2B, are known to show specific bioactivities [6,7,40,41], their selective syntheses are desired for their use in in vitro and in vivo studies, and their application to foods, beverages, and pharmaceutical products. Further investigation focusing on the effects of catechin composition on TF yields would suggest the proper conditions for tyrosinase-catalyzed TF synthesis. When using available green tea leaves as a source of catechins, the catechin composition could be customized by the extraction procedures, such as by the solvents used.

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