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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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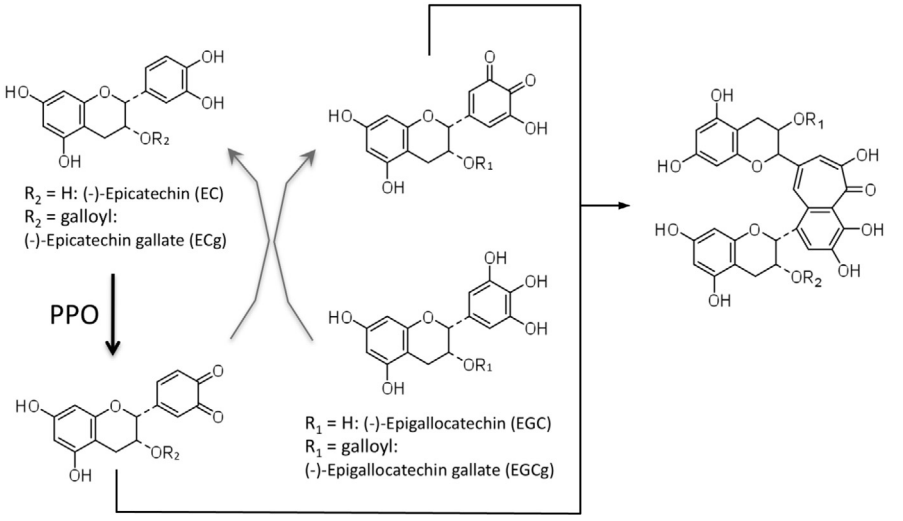

Theaflavins

	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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