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Differential activities of fungi-derived tannases on biotransformation and substrate inhibition in green tea extract

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Tannases are important enzymes in the antioxidant potential of tea leaves. In this study, we evaluated the effect of two tannases (T1 and T2) on biotransformation of tea polyphenols and antioxidative activities from catechins in green tea extract (GTE). The T1 tannase-catalyzed reaction was inhibited by the addition of >2.0% GTE substrate, whereas the T2-catalyzed reaction was not inhibited, even by addition of 5.0% GTE. Furthermore, the T1 tannase-catalyzed reaction was inhibited by addition of 10 mg mL^{-1} EGCG, whereas the T2 tannase-catalyzed reaction did not display any inhibitory effect. These results indicate that T2 tannase was more tolerant than T1 tannase to substrate inhibition in degallation reactions. Specifically, the substrate EGCG (90,687.1 $\mu g\ mL^{-1}$) was transformed into gallic acid (50,242.9 $\mu g\ mL^{-1}$) and EGC (92,598.3 $\mu g\ mL^{-1}$) after 1-h treatment with T2 tannase (500 U g^{-1}). The tannase-mediated product displayed higher *in vitro* radical-scavenging activity than the control. IC_{50} value of GTE on ABTS and DPPH radicals (46.1 $\mu g\ mL^{-1}$ and 18.4 $\mu g\ mL^{-1}$, respectively) decreased markedly after T2 tannase treatment (to 35.8 $\mu g\ mL^{-1}$ and 15.1 $\mu g\ mL^{-1}$, respectively). These results indicate that T2 tannase treatment of GTE enhanced its radical-scavenging activity, an increase that was also observed in the reaction using EGCG substrate. Taken together, our results revealed that T2 tannase is more suitable for biotransformation of catechins in GTE than T1 tannase, and T2 treatment provides an enhanced radical-scavenging effect.

[**Key words:** Tannase; Biotransformation; Tea catechins; Antioxidative activity; Substrate inhibition]

Tea polyphenols constitute one of several essential components of tea that affect physiological functions. Catechins are the primary polyphenols found in tea, accounting for 75–80% of its soluble ingredients (1), including epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC), gallocatechin (GC) and catechin (C). EGCG constitutes over 50% of catechin content in tea (2).

Tannase or tannin acyl hydrolase (EC, 3.1.1.20) catalyzes the hydrolysis of ester and depside bonds present in hydrolyzable tannins or gallic acid esters, such as EGCG or ECG, thereby releasing glucose or gallic acid as products (3). The release of gallic acid is physiologically beneficial due to its significant antioxidant potency (4). We previously reported that increase of gallic acid levels by tannase treatment was associated with an increase in antioxidant activity (5). Gallic acid is used in the synthesis of propyl gallate, which is an antioxidant primarily found in fats and oils, as well as in beverages. Tannase is also extensively used in the preparation of instant tea, acorn wine, and coffee-flavored soft drinks; the clarification of beer and fruit juices; and the detannification of foods (3,6,7).

Many studies of tannase-mediated biotransformation of tea catechins have been performed (5,8,9). Our previous study (10) reported that tea catechins (such as EGCG or ECG) were hydrolyzed by tannase action (to generate EGC or EC, respectively, as well as gallic acid) by using >1% tea extract as a substrate. However, biotransformation of catechins by tannase was observed to be affected by substrate-mediated dose-dependent inhibition (5). Albertse (11) also reported the inhibition of tannase at higher concentrations of EGCG. Tea polyphenols, such as EGCG, are known to be involved in enzyme inhibition as well as the formation of precipitants in tea drinks (12,13). Substrate-mediated inhibition has been an important limitation on the widespread use of tannases. Therefore, the discovery of a tannase that is not susceptible to substrate inhibition is an important goal for the use of tannases in the biotransformation of tea catechins.

At the present, many companies manufacture tannases of varying catalytic properties (7) using multiple methods. Tannases from Kikkoman (Japan) and Biocon (India) are manufactured by solid-state fermentation (SSF). The reactivity of tannase on substrates can depend on the enzyme source, culture methods, degree of purification, etc. (7). In general, tannases have been isolated from microbial sources, such as fungi, using SSF or submerged fermentation (SmF).

Several differences in tannase production from microbes have been observed between SSF and SmF methods (14,15). For example, substrate concentrations are recognized to affect the regulatory mechanisms of tannase production (14,15).

In this study, we examined the effect of two fungi-derived tannases from different manufacturers on biotransformation of tea

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catechins and substrate inhibition. The antioxidative effects of tannase hydrolysates between the two types of tannases were also compared. This study shows that T2 tannase treatment leads to vastly improved biotransformation of tea catechins over T1 tannase treatment, without concomitant substrate inhibition compared.

MATERIALS AND METHODS

Materials and chemicals Green tea extract (GTE) was purchased from Zhejiang Huang Minghuang Natural Products Development Co., Ltd. (Beijing, China). The extract was determined by high-performance liquid chromatography (HPLC) to be composed of 71.9% catechins, including 40.9% EGCG. Acetic acid and acetonitrile (HPLC analytical grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). HPLC-grade standard gallic acid (GA), EGC, EC, EGCG, ECG, and standards were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). The remaining reagents used were of analytical grade. Tannases were purchased from Kikkoman Corporation (Tokyo, Japan) and Bision Biochem. Co. (Sungnam, Republic of Korea).

Tannase treatment and determination of catechins by HPLC Thirty mg of tannase (500 U g^{-1}) was added to 50 mL of 1% GTE solution, and the reactions were performed at 35°C. The tannase reactant of green tea was obtained at regular intervals until the completion of hydrolysis (10). The inactivation of hydrolysis was performed by heating the sample at 100°C for 10 min after reaction.

The content of each catechin was measured using an HPLC system equipped with a hypersil C18 column (5 μm, 25 × 0.46 cm ID) and a UV–VIS detector. The mobile phase contained 1% acetic acid (solvent A) and acetonitrile (solvent B) with a linear gradient starting at an A/B ratio of 92/8 and completing at 73/27 over a 40-min period at a flow rate of 1 mL min^{-1}. Identification of individual catechins and GA was based on a comparison of the retention times of the sample peaks with those of authentic reference standards. The quantity of each constituent in green tea was estimated from the integrated data (16).

Inhibitory activity of EGCG and gallic acid The effect of EGCG and gallic acid addition was tested with GTE. Gallic acid (5 and 10 mg mL^{-1}) and EGCG (1, 2, 5, and 10 mg mL^{-1}) were added to the enzymatic reaction mixture (50 mL) of tannase. Thirty mg of tannase (500 U g^{-1}) was added to 50 mL of 1% GTE solution, and the reactions were performed at 35°C for 2 h. The inactivation of hydrolysis was performed by heating at 100°C for 10 min after reaction. Samples were removed at regular intervals until the completion of hydrolysis and assayed to determine changes in the level of catechins.

Scaleup for the biotransformation of GTE Six gram of tannase was added to 10 L of 10% GTE solution, with the reactions performed at 35°C. The tannase reactant of green tea was obtained at regular intervals until the completion of hydrolysis. The inactivation of hydrolysis was performed by heating at 100°C for 10 min after the reaction. The reactant was lyophilized and used for the assay of catechin and radical-scavenging activity.

Antioxidant activities of green tea extract The scavenging activity of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was measured, according to the method of Cheung et al. (17) The scavenging activity of the 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical was measured according to the method of Re et al. (18) The DPPH and ABTS radical-scavenging activities were calculated as follows:

$$\text{Radical scavenging activity}(\%) = \left(1 - A_{\text{sample}} / A_{\text{control}}\right) \times 100 \quad (1)$$

where A_{sample} is the absorbance in the presence of sample, and A_{control} is the absorbance in the absence of sample. The half-maximal inhibitory concentration (IC_{50}) value is the effective concentration at which DPPH and ABTS radicals were scavenged by 50%.

Statistical analysis All expressed values are mean values of triplicate determinations. All statistical analyses were performed using the Statistical Package for Social Sciences version 12.0 (SPSS, Chicago, IL, USA). Differences among the samples were evaluated statistically by one-way analysis of variance (ANOVA) and Tukey's New Multiple Test. All data were two-sided at a 5% significance level, and have been reported as mean ± deviation (SD).

RESULTS

Effect of GTE substrate concentration on tannase T1 and T2 are *Aspergillus niger*-derived tannases produced using different methods. T1 tannase obtained from Kikkoman was produced by SSF, while T2 tannase from Bision was produced by SmF. To measure the effect of substrate concentration in tannase T1- and T2-mediated reactions, several concentrations of green tea extract (0.5–5.0%) were added to the reaction mixture, followed by HPLC analysis of their products (Fig. 1). The addition of 0.5% GTE resulted in an increase of the quantity of gallic acid as a product of the T1

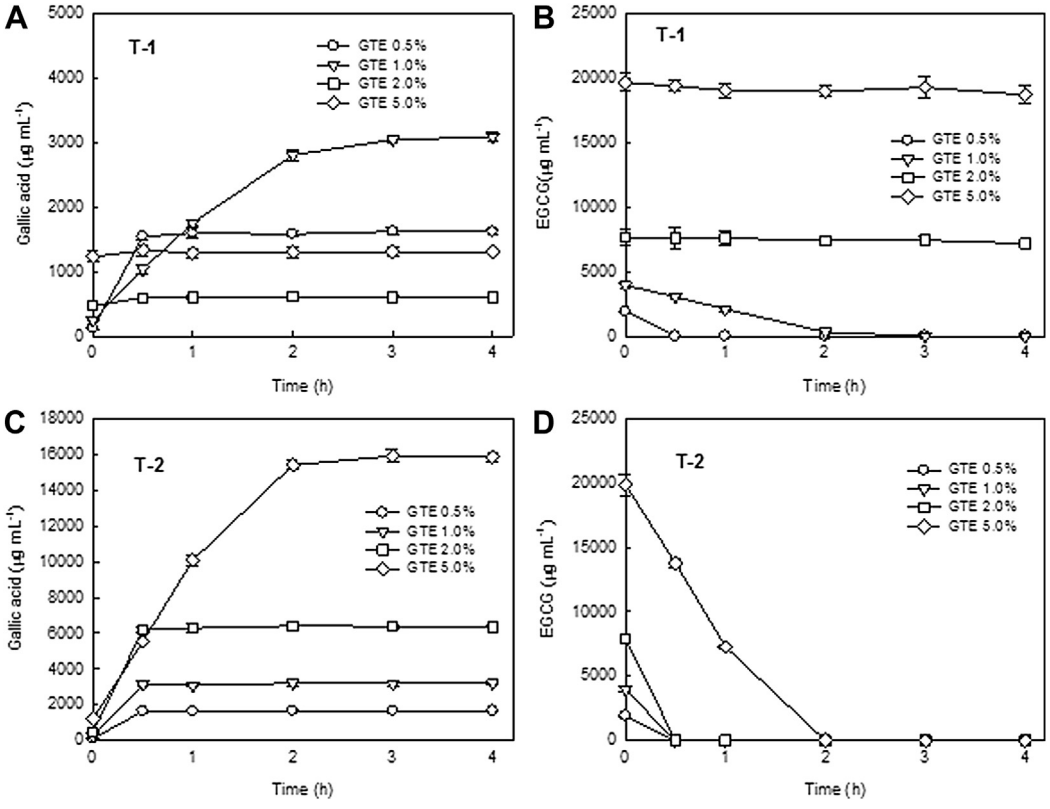

FIG. 1. Effect of GTE concentration on tannase T1 (A, B) and T2 (C, D) activity. The reactions were performed at 35°C and pH 5.0 at various GTE concentrations.