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A highly sensitive method for quantifying gallocatechin gallate and its epimer using a catechin-specific peptide

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ABSTRACT

We have developed a method for quantifying gallocatechin gallate (GCg) and epigallocatechin gallate (EGCg) using a catechin-binding peptide (part of the 67-kDa laminin receptor). Using micro titer plates, we investigated various conditions, including the quantifiable range of EGCg concentrations, the optimal concentration of the catechin-binding peptide, and the optimal reaction conditions. In this microplate assay, after each well was coated with bovine serum albumin, sample containing GCg and EGCg was added at pH 8.0, and allowed to stand at 37 °C for 2 h. After washing, biotinylated-peptide solution was added at 1 μg mL^{-1} and allowed to react for 1 h at 37 °C. Each well was added with streptavidin–horseradish peroxidase conjugate, followed by chromogenic reaction for 25 min at room temperature. After the reaction, absorbance was measured at 405 nm. Our method is capable of quantifying EGCg in the range of approximately 0.1–2.0 mg L^{-1} with a high degree of sensitivity and a high correlation ($R^2 = 0.98$) between EGCg concentration and absorbance. The method was specific to GCg and EGCg and seems capable of estimating GCg and EGCg contents in the presence of other catechin compounds. The method is simple and highly sensitive for quantitative GCg and EGCg measurement that requires no special equipment or operation and can measure multiple samples simultaneously.

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1. Introduction

Catechins are polyphenol compounds that are found in abundance in green tea. Catechins have attracted attention in recent years for their various physiological effects, i.e., antibacterial (Hamilton-Miller, 1995; Lee et al., 2009; Shiota et al., 1999), anticancer (Yang, Maliakal, & Meng, 2002), and anti-inflammatory (Sano, Suzuki, Miyase, Yoshino, & Maeda-Yamamoto, 1999) action, and they are expected to have practical applications in a range of different fields.

Some of these reported antibacterial effects of catechins include antitoxin effects (Hamilton-Miller, 1995), antipathogen effects (Lee et al., 2009), and reduction of the minimum inhibitory concentration (MIC) of β-lactam antibiotics for methicillin-resistant *Staphylococcus aureus* (Shiota et al., 1999). Catechins are known to be more effective against Gram-positive than Gram-negative bacteria (Yoda, Hu, Zhao, & Shimamura, 2004), and the catechins found in green tea, epigallocatechin gallate (EGCg) and gallocatechin gallate (GCg), have the highest antibacterial effects (Hamilton-Miller, 1995). Mechanisms for catechin antibacterial action that have been postulated involve damage to bacterial cells by binding to their surfaces (Ikigai, Nakae, Hara, & Shimamura, 1993) and the bactericidal action of hydrogen peroxide given off as a result of catechin autooxidation (Akagawa, Shigemitsu, & Suyama, 2003; Arakawa, Maeda, Okubo, & Shimamura, 2004). Our investigations of antibacterial effects of catechins against various bacteria (Miyamoto et al., 2009; Nakayama, Shigemune, Tokuda, Furuta, Matsushita, Mekada et al., 2008; Nakayama, Shigemune, Tokuda, Furuta, Matsushita, Yoshizawa et al., 2008) indicated that catechins tend to have strong antibacterial effects against Gram-positive bacteria, such as *S. aureus* and *Bacillus cereus*, and weak effects against Gram-negative bacteria, such as *Escherichia coli* and *Salmonella*. It seems that the differences in the catechin sensitivity of bacteria appear to be the result of differences in the amount of catechins binding to the surface of the bacteria due to variations in the surface structure. Catechins are frequently determined by Liquid chromatography. In practice, the liquid chromatographic techniques resulted in low sensitivity suggesting that the concentration of the catechin should

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be several tens of milligrams per milliliter or more (Dalluge & Nelson, 2000). For rapid and simple determination, we have developed a turbidimetric method to determine catechins based on the fact that many polyphenols produce hydrogen peroxide in an alkaline environment and that hydrogen peroxide oxidizes cerium to generate cerium oxide precipitates. Catechins are quantitatively determined by the method in a range from 10 to 1000 mg L^{-1}. However, much more sensitive determination method is required for determination of catechins to measure the amounts of catechins attached on the surface of bacterial cells. Here, we report a highly sensitive method of quantifying EGCg using a specific EGCg-binding peptide derived from part of the 67-kDa laminin receptor protein (Tachibana, Koga, Fujimura, & Yamada, 2004) that binds to catechins.

2. Materials and methods

2.1. Catechin-binding peptide

The catechin-binding peptide was derived from terminal biotinylation as described by Tachibana et al. (2007) and synthesized by Thermo Fisher Scientific. The amino acid sequence of the peptide is biotinyl—NH—YV DIA IPC NNK GAH S—COOH (molecular weight 1828), and it was used in phosphate-buffered saline (PBS) containing 137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, and 1.47 mM KH_2PO_4 (pH 7.4).

2.2. Quantification of GCg and EGCg by microplate assay using catechin-specific peptide

After investigating various reaction conditions, we constructed the following measurement method. First, 360 μL of 0.01 M Na Phosphate buffer (pH 7.0) containing 10 g L^{-1} bovine serum albumin (BSA) was added to each well of a 96-well microplate (high binding type, 400 ng cm^{-2} IgG, Nalge Nunc International), and allowed to stand overnight at 4 °C. Each plate was then washed three times with 360 μL PBS containing 0.5 g L^{-1} Tween 20 (TPBS). To each well, 100 μL gallocatechin gallate (GCg) or epigallocatechin gallate (EGCg) dissolved in PBS (pH 8.0) was added and allowed to stand for 2 h at 37 °C. The plate was then washed three times with TPBS, and 360 μL of 1.0 μg mL^{-1} biotinylated-catechin-specific peptide solution was added to each well and allowed to stand for 1 h at 37 °C. The plate was washed three times with TPBS; after which, 360 μL streptavidin—horseradish peroxidase (HRP) conjugate (GE Healthcare) diluted 1000-fold with PBS (pH7.4) containing 1 g L^{-1} Tween 20 was added and allowed to stand for 1 h at 37 °C. The plate was then washed three times with TPBS; after which, 180 μL chromogenic substrate solution was added and reacted at room temperature. The chromogenic substrate solution was prepared by mixing 0.2 M phosphate-citrate buffer (0.2 M Na_2HPO_4 and 0.2 M $C_6H_8O_7 \cdot H_2O$, pH 4.0) containing 0.3 g L^{-1} hydrogen peroxide with an equal volume of 0.6 mg mL^{-1} 2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS, Sigma Aldrich). After 25 min, the reaction was stopped by adding 180 μL of 15 g L^{-1} oxalic acid, and absorbance was measured at 405 nm using a microplate reader (Model 680, Bio-Rad).

To determine the conditions for GCg and EGCg detection, reactions were carried out under the following conditions.

1. To investigate the effect of catechin-specific peptide concentration, 360 μL of green tea extracts (GTE) (Polyphenon 70A, Mitsui Norin Co., Ltd., Japan) was added in place of the EGCg solution, and allowed to stand for 1 h at 37 °C. After washing, peptide solution dissolved in PBS at a range from 0.1 to 5.0 μg mL^{-1} was added and allowed to react. The content by percentage of catechins in the GTE preparation was previously reported as: 54.7% EGCg; 15.0% epicatechin gallate (ECg); 4.0% gallocatechin gallate (GCg); 2.1% epigallocatechin (EGC); 1.8% epicatechin; 0.9% catechin gallate; 0.5% gallocatechin; and 0.3% catechin (Nakayama, Shigemune, Tokuda, Furuta, Matsushita, Yoshizawa et al., 2008).
2. To investigate the effect of different EGCg immobilization conditions, 100 μL EGCg was added to each well and allowed to stand for 1, 2, and 4 h at 37 °C and overnight at 4 °C.
3. To investigate the effect of pH of the EGCg solution being immobilized, 100 μL EGCg solutions at pH levels of 5.0, 6.0, 7.0, 7.4, 8.0, and 9.0 were added to each well and allowed to stand for 2 h at 37 °C.
4. To investigate the effect of chromogenic reaction time, the chromogenic substrate solution was added and reacted at 37 °C for 15, 25, and 60 min. The reaction was stopped by adding 100 μL of 15 g L^{-1} oxalic acid and absorbance was measured at 405 nm.
5. To investigate specificity, GCg, EGC and ECg were used in addition to EGCg and GTE.

To determine relationship between GCg or EGCg concentration and absorbance, experiments were conducted with three or four replications under the reaction conditions determined above.

3. Results and discussion

3.1. Optimal conditions for EGCg quantification using catechin-binding peptide

First, we investigated the optimal concentration of catechin-binding peptide for quantifying GTE in the peptide solutions at a range from 0.1 to 5.0 μg mL^{-1} (Fig. 1). Absorbance was highest at 1.0 μg mL^{-1} and lower at 0.1 and 0.5 μg mL^{-1}; at peptide concentration of 5 μg mL^{-1}, absorbance remained below 0.14. The catechin-specific peptide was therefore used at a concentration of 1.0 μg mL^{-1} in all subsequent experiments.

We next investigated the temperature and time for EGCg immobilization on the microplate. EGCg immobilization was measured after 1, 2, and 4 h at 37 °C and overnight at 4 °C (Fig. 2). Up to 2.0 μg mL^{-1}, absorbance for EGCg was the highest for the overnight reaction at 4 °C, followed by the 2-h reaction at 37 °C,

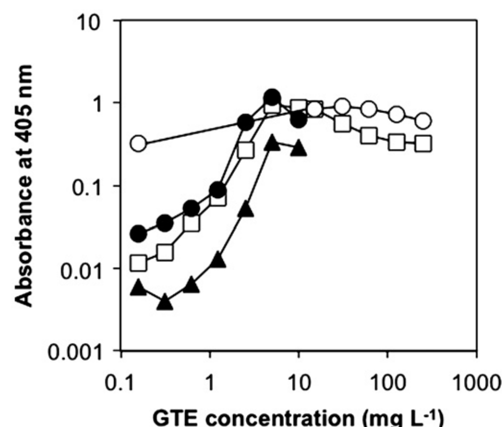

Fig. 1. Effect of concentration of catechin-binding peptide on detection of GTE. To each well coated with BSA, GTE (pH 8) was added at various concentrations and allowed to stand for 2 h at 37 °C. After washing, biotinylated-peptide solution was added at 0.1 μg mL^{-1} (▲), 0.5 μg mL^{-1} (□), 1 μg mL^{-1} (●), 5 μg mL^{-1} (○) and allowed to react for 1 h at 37 °C. Each well was added with streptavidin—HRP, followed by chromogenic reaction at room temperature and measurement of absorbance at 405 nm.

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