Note

Influence of the Galloyl Moiety in Tea Catechins on Binding Affinity for Human Serum Albumin

Kanako MINODA¹, Tatsuya ICHIKAWA¹, Tomoharu KATSUMATA¹, Ken-ichi ONOBORI¹, Taiki MORI¹, Yukiko SUZUKI², Takeshi ISHII^{1,*} and Tsutomu NAKAYAMA¹

¹Department of Food and Nutritional Sciences, and Global COE Program, University of Shizuoka, 52–1 Yada, Suruga-ku, Shizuoka 422–8526, Japan

²ULVAC, Inc., 2500 Hagazono, Chigasaki, Kanagawa 253–8543, Japan

(Received April 27, 2010)

Summary The major catechins of green tea extract are (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECg), and (–)-epigallocatechin gallate (EGCg). Recent research has indicated that catechins form complexes with human serum albumin (HSA) in blood, and differences in their binding affinity toward HSA are believed to modulate their bioavailability. In this study, we kinetically investigated the interaction between the catechins and HSA immobilized on a quartz-crystal microbalance (QCM). The association constants obtained from the frequency changes of QCM revealed interactions of ECg and EGCg with HSA that are 100 times stronger than those of EC and EGC. Furthermore, comparisons of these catechins by native-gel electrophoresis/blotting with redox-cycling staining revealed that, in a phosphate buffer, ECg and EGCg have a higher binding affinity toward HSA than EC and EGC. These observations indicate that catechins with a galloyl moiety have higher binding affinities toward HSA than catechins lacking a galloyl moiety.

Key Words catechin, human serum albumin, interaction, binding affinity, galloyl moiety

Tea is the most widely consumed beverage in the world. Its diverse biological activities have been attributed to a group of polyphenolic compounds, namely catechins, present in green tea leaves (1, 2). The major catechins of green tea extract are (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECg), and (–)-epigallocatechin gallate (EGCg) (Fig. 1). The number of the hydroxyl groups on the B-ring and the presence or absence of a galloyl moiety result in differences in the structures of these catechins. The hydroxyl group on the B-ring is the most significant factor in the scavenging of reactive oxygen species. Catechins with a pyrogallol-type structure on the B-ring, such as EGC and EGCg, have strong antioxidant activities (3). On the other hand, many experiments have shown that catechins with a galloyl moiety, such as ECg and EGCg, are more biologically active than their homologues lacking the galloyl moiety (4–6).

Recently, much attention has focused on the identification of a promising target protein that can interact

with food chemicals. Predicting target molecules has resulted in the identification of a number of proteins that can directly bind with tea catechins (7–11). Previous research has indicated that catechins form complexes with human serum albumin (HSA), and differences in binding affinity toward HSA are believed to determine their bioavailability. Several reports using spectrofluorometry, circular dichroism, and capillary electrophoresis have indicated that green tea catechins bind to HSA (12–14). Recently, we revealed that EGCg non-covalently binds to HSA after incubation of human serum with EGCg (15). In a separate study, we demonstrated, using high performance liquid chromatography (HPLC) with an immobilized HSA column, that the hydroxyl groups on the galloyl moiety and the number of hydroxyl groups on the B-ring of each catechin influence binding affinity toward HSA under acidic conditions (16).

In the present study, we investigated the interaction kinetics between the catechins and HSA immobilized on a quartz-crystal microbalance (QCM), and compared the binding affinity of catechins in phosphate buffer.

Materials and Methods

Materials. EC, EGC, ECg, and EGCg were kindly provided by Mitsui Norin Co. Ltd. (Shizuoka, Japan). HSA, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and 3,3'-dithiodipropionic acid (DTDP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enhanced chemiluminescence (ECL) western blotting detection reagents were obtained from GE

*To whom correspondence should be addressed.

E-mail: ishii_t@u-shizuoka-ken.ac.jp

Abbreviations: DTDP, 3,3'-dithiodipropionic acid; EC, (–)-epicatechin; ECg, (–)-epicatechin gallate; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; EGC, (–)-epigallocatechin; EGCg, (–)-epigallocatechin gallate; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; HSA, human serum albumin; NHS, N-hydroxysuccinimide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; QCM, quartz crystal microbalance; SDS, sodium dodecyl sulfate.

Fig. 1. Chemical structures of tea catechins.

Healthcare UK, Ltd. (Buckinghamshire, UK). All other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Kinetic analysis using QCM. A 27-MHz QCM (Affinix Q4, Initium, Inc., Tokyo, Japan) was employed to analyze the interaction between catechins (EC, EGC, ECg or EGCg) and HSA. HSA was immobilized on a ceramic sensor chip using an amine coupling reaction (17). Both sides of the AT-cut quartz plate (Initium, Inc.) bore gold electrodes with 2.5-mm diameters (4.9-mm² area). When an AC voltage at a specific frequency is applied, a resonant oscillation is generated due to the piezoelectric effect of the crystal, and mass changes associated with adsorbed or dissociated material on the crystal oscillator surface cause a frequency change in the resonant oscillator. Sauerbrey's Eq. (1) was applied to calculate the mass of the adsorbed molecules:

$$\Delta F = -(2F_0^2/A\sqrt{\mu_q\rho_q})\Delta m \quad (1)$$

where ΔF is the measured change in frequency (Hz), F_0 the fundamental frequency of the QCM (27 MHz), Δm the mass change (g), A the electrode area (cm²), μ_q shear modulus (2.95×10^{11} dyn/cm²), and ρ_q the density of quartz (2.65 g/cm³). The result shows that a frequency decrease of 1-Hz corresponds to 30 pg binding to the electrode. A 1.0 mM of DTDP in ethanol was applied to a sensor chip to immobilize the DTDP directly on the gold electrode surface by an Au-thiol interaction. The sensor chip was then activated by addition of a coupling solution consisting of 520 μ M EDC and 870 μ M N-hydroxysuccinimide (NHS). Subsequently, the sensor chip was immersed in phosphate-buffered saline (PBS), 1.0 mg/mL HSA (5 μ L) was added to the PBS solution, and HSA immobilization was followed by monitoring the frequency change resulting from changes in the mass at the electrode surface. The immobilized HSA generated a signal of about 350 Hz, indicating that about 10.5 ng of HSA was bound. Individual catechins at four different concentrations (0.1, 1.0, 5.0, and 10 μ M) were then

Table 1. Association constants (K_a) of catechins toward HSA immobilized on QCM.

Catechins	K_{on} [M ⁻¹ s ⁻¹]	K_{off} [s ⁻¹]	K_a [M ⁻¹]
EC	5.88	2.75×10^{-3}	2.14×10^3
EGC	8.50	2.99×10^{-3}	2.84×10^3
ECg	4.97×10^2	3.59×10^{-3}	1.38×10^5
EGCg	2.24×10^2	8.85×10^{-4}	2.53×10^5

added to 100 mM phosphate buffer (pH 7.4) at 25°C. AQUA ver 1.5 software (Initium, Inc.) was used to monitor the frequency change.

Native-polyacrylamide gel electrophoresis (PAGE)/blotting and redox-cycling staining. EC, EGC, ECg, or EGCg, at a concentration of 500 μ M, was incubated in 500 μ L of 200 mM sodium phosphate buffer (pH 6.2) containing ascorbic acid (1.0 mM) with 60.0 mg/mL HSA at 37°C for 10 min. The samples were subjected to centrifugal filtration using Microcon-30 (molecular weight cut-off of 30 kDa; Millipore, Bedford, MA, USA) to obtain a filtrate. The filtrates were separated by native-PAGE using 10% gels (NativePAGE-NOVEX-Bis-Tris Gel System, Invitrogen Co., Carlsbad, CA, USA). The proteins were stained with Simply Blue SafeStain (Invitrogen). For redox-cycling staining, gels were transblotted onto PVDF membranes. The catechins-HSA complexes were detected by staining with nitro blue tetrazolium (240 μ M) in potassium glycinate (2.0 M, pH 10.0). Fresh human serum was prepared from whole blood.

Results

Determination of catechin binding constants toward immobilized HSA by QCM analysis

We first investigated the interaction kinetics between tea catechins (EC, ECg, EGC, and EGCg) and HSA immobilized on a QCM. The interaction of bioactive compounds with protein-coated QCM has been investigated previously (17). The binding process of [B] (HSA) to [A] (catechin) is described by Eq. (2). The amount of the [C] (HSA-catechin) complex formed at time t after the injection is given by Eqs. (3) and (4).

$$[C]_t = [C]_{\max} \{1 - \exp(-t/\tau)\} \quad (3)$$

$$\Delta m_t = \Delta m_{\max} \{1 - \exp(-t/\tau)\} \quad (4)$$

$$1/\tau = K_{on}[B]_0 + K_{off} \quad (5)$$

The relaxation time (τ) of catechin binding was calculated from the decreases in curve fitting QCM frequency at various catechin concentrations. The catechin binding and dissociation rate constants (K_{on} [M⁻¹s⁻¹] and K_{off} [s⁻¹]) were obtained from the slope and intercept of the plot of τ^{-1} against catechin concentration using Eq. (5). The binding constants (K_a [M⁻¹]) were calculated from K_{on}/K_{off} . The K_a values for the four catechins are summarized in Table 1. The results revealed that the affinity of EGCg to HSA was similar to that of ECg. ECg and EGCg showed affinity toward HSA that is ca. 100 times stronger than that of EC or EGC, which lack the galloyl moiety, suggesting that the galloyl moiety plays

Fig. 2. Interaction of tea catechins with HSA in phosphate buffer. HSA (60.0 mg/mL) was incubated with EC, EGC, ECg or EGCg at a concentration of 200 μ M in 200 mM phosphate buffer (pH 6.2) at 37°C for 10 min. The reaction mixtures were subjected to centrifugal filtration to obtain filtrates. The filtrates were analyzed by native-PAGE followed by redox-cycling staining (upper) and CBB staining (lower).

an important role in this interaction.

Interaction of catechins with HSA in phosphate buffer

To evaluate the binding affinity of the catechins with HSA in phosphate buffer, reaction mixtures of 500 μ M of individual catechins with 60.0 mg/mL HSA, followed by centrifugal filtration, were analyzed by native-PAGE/blotting with redox-cycling staining. As shown in Fig. 2, the catechin-treated HSA generated positive bands around 66 kDa when assayed by redox-cycling staining, suggesting that the catechins having a galloyl moiety bound to HSA. The order of relative staining strength is as follows: EGCg>ECg>EGC>EC. This correlates well with our previous study using HPLC with an immobilized HSA column (16). These observations suggest that the HSA binding affinities of the catechins with a galloyl moiety were higher than those of the corresponding catechins lacking a galloyl moiety.

Discussion

Serum albumin, the most abundant protein in blood, is a 66-kDa protein that plays an important role in the reversible binding of many compounds, such as free fatty acids, steroids, and some metals and drug metabolites. The binding ability of HSA is an important factor in the transport and release of various endogenous and exogenous compounds (18). Protein complexes of these compounds may replenish free compounds removed by metabolic processes. Therefore, interaction with HSA directly influences the transport and metabolism of such compounds, and consequently contributes to the magnitude of their biological activities in vivo. From our evaluation of the binding affinities of tea catechins with HSA, we found that the galloyl moiety of the catechins influences the affinity for HSA.

Catechins have flavan-3-ol structures with A, B, and C rings, or a galloyl group. The major green tea catechins used in this study included EC, ECg, EGC, and EGCg (Fig. 1). Comparing these four catechins by QCM analysis (Table 1), we showed that catechins having a galloyl moiety (ECg and EGCg) have higher binding affinities toward HSA immobilized on QCM than the catechins lacking a galloyl moiety (EC and EGC). The

data are consistent with our previous report using HPLC with immobilized HSA column (16). In addition, we also revealed that catechins having a galloyl moiety have higher binding affinities with non-immobilized HSA than catechins lacking a galloyl moiety. These results and previous findings indicate that the galloyl moiety is responsible for the binding affinity of catechins for HSA. A previous report has shown that the hydrophobicity of the catechins obtained for a 1-octanol/PBS binary liquid was increased drastically by the presence of the galloyl moiety (19). On the other hand, studies of serum albumin-polyphenol interactions suggest that binding of polyphenols with serum albumin are stabilized by hydrophobic interactions, hydrogen-bonding forces, and electrostatic forces (20, 21). HSA is a globular protein composed of three structurally similar domains (I, II, and III), each containing a number of hydrophobic cavities. In particular, there are two hydrophobic sites located in subdomains IIA and IIIA. These two subdomains have well-determined cavity sites I and II, respectively. It has been believed that flavonoids bind to the hydrophobic pockets on site I of HSA. Nozaki et al. reported that EGCg was located on sites I and II of HSA (22). Taken together, this suggests that the affinity of tea catechins for HSA is influenced by their hydrophobicity, derived from the galloyl moiety.

Many experiments have shown that catechins with a galloyl moiety, such as ECg and EGCg, have more effective biological activities than their homologues lacking the galloyl group, including growth inhibition of a human lung cancer cell line: ECg>EGCg>EGC>EC (23); antibacterial activity against *Helicobacter pylori*, EGCg>ECg>EGC>EC (5); inactivation effects on human type-A influenza virus, ECg>EGCg>EGC (6); and inhibitory effects on the oxidative modification of LDL, EGCg>ECg>EGC>EC or EGCg>ECg>EC>EGC (24, 25). These results indicate that the activities of ECg and EGCg were always higher than those of EC and EGC. This is in good agreement with the order of the K_a values in the present study, although the biological activities did not always show the 100-fold difference between ECg/EGCg and EC/EGC demonstrated here with HSA immobilized on QCM.

In conclusion, this study indicates that the galloyl moiety of individual catechins influences its binding affinity toward HSA. Notably, it should be emphasized that, for the first time, the affinity of catechins toward HSA was demonstrated by K_a values measured with QCM. The results provide fundamental information on the relationship between the chemical structure of catechins and binding affinity toward HSA.

Acknowledgments

This research was supported in part by Shizuoka Prefecture and Shizuoka City Collaboration of Regional Entities for the Advancement of Technological Excellence, Japan Science and Technology Agency (JST). Support was also provided by two Grants-in-Aid (No. 22780122 to T.I. and No. 21580148 to T.N.) for scien-

tific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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