Interaction of Polyphenols with Proteins: Binding of (−)-Epigallocatechin Gallate to Serum Albumin, Estimated by Induced Circular Dichroism

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The binding of (−)-epigallocatechin gallate (EGCG), a representative natural polyphenol, to human serum albumin (HSA) and bovine serum albumin (BSA) was investigated using induced circular dichroism (CD). The site of the binding EGCG-HSA was analyzed based on the competition with drugs with known binding sites on HSA, such as phenylbutazone (PB) and diazepam (DP). Double-reciprocal plot analyses showed the competitive relations with the site-I- (PB and tolbutamide, TB) and site-II-binding drugs (DP and ibuprofen, IP) indicating the binding of EGCG to sites I and II on HSA, while digitoxin (DG), a site-III-binding drug, did not affect the binding of EGCG. In an analogous way, the competitive relations were observed between EGCG and the site-I- (PB and TB) and site-II-binding (ethacrynic acid, EA) drugs for the binding of EGCG and BSA. The site-III drug DG also showed competitive binding with EGCG to BSA. The binding of EGCG to the albumins indicated its affinity to sites I and II on HSA, while competitive binding for all three sites was observed on BSA.

Key words (−)-epigallocatechin gallate; serum albumin; interaction; polyphenol; binding; circular dichroism

Polyphenols are regarded as a group of natural compounds that have various pharmacological properties such as antioxidant, anti-tumor, antibacterial and antiviral effects.[1—3] These properties are, at least in part, attributed to the interaction between the polyphenols and biomolecules such as proteins and lipids. Polyphenol interactions with proteins and peptides have been studied using NMR,[4,5] and MS has also been used to visualize the polyphenol complex with bovine serum albumin (BSA).[6] However, the complete structure of the binding of polyphenols with proteins, which is needed to understand polyphenolic properties in interactions with biomolecules, has not yet been clarified.

Previously, we have reported the formation of large water-soluble complexes between BSA and some polyphenols such as (−)-epigallocatechin gallate (EGCG) and pentagalloylglucose (PGG).[7,8] Size exclusion chromatography (SEC) and polyacrylamide gel electrophoresis (PAGE) are valuable tools for observing the formation of such large complexes. However, although these methods are adequate for observing large differences in the molecular sizes of the complexes, the formation of these complexes requires several hours. For example, PGG and BSA form a complex with a molecular weight as large as several hundred thousand after 24 h.

On the other hand, circular dichroism (CD) has been used to analyze the binding of drugs to human serum albumin (HSA). Although HSA has its own Cotton effects on the CD spectrum, the CD measurements of mixtures of HSA and drugs show induced Cotton effects in some cases, even if the drugs are optically inactive. This phenomenon has been utilized for the quantitative estimation of the binding of drugs to HSA.[9] In contrast to equilibrium dialysis and ultrafiltration, CD can demonstrate binding directly without the adsorption of the compounds to the membrane. CD requires only a small sample, and reflects changes in protein conformation or ligand sensitively. Since the binding sites of several drugs have been established, the binding site or binding mode of a certain compound to HSA can be determined by using these drugs.[10] When a compound suppresses an induced CD for a combination of HSA and one of those drugs competitively, the binding site of the compound on HSA can be assigned to be the same as that of the drug.[11—13]

We examined competitive binding between site-specific binding drugs and EGCG on HSA, since EGCG is a representative polyphenol with known biological/pharmacological properties,[14,15] and its absorption and fate after oral administration have also been reported.[16] The present study investigated the binding of EGCG to HSA, and also the differences between binding to BSA and HSA, based on the quantitative estimation of changes in the induced CD.

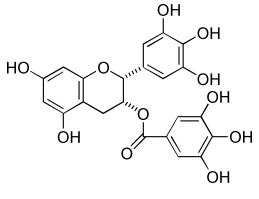

Fig. 1. Structure of EGCG

Experimental

Reagents and Polyphenol HSA (cat. No. A3782) and BSA (cat. No. A0281) used in this study were of the fatty acid free grade (≥99%). These two proteins, phenylbutazone (PB), tolbutamide (TB), ibuprofen (IP), and digitoxin (DG) were purchased from Sigma (St. Louis, MO, U.S.A.). Diazepam (DP) and ethacrynic acid (EA) were purchased from Wako (Osaka, Japan) and Tokyo Kasei (Tokyo, Japan), respectively. EGCG was isolated from leaves of *Thea sinensis* L. (=*Camellia sinensis* O. KUNTZE), as reported previously.[17]

CD Spectral Measurements CD spectra between 355 and 245 nm were recorded on a JASCO J-720W spectropolarimeter (Tokyo, Japan), using a 0.1-cm path-length cell at 298—301 K for the experiments using HSA, or at 295—297 K for the BSA experiments. Time constant, scan speed, resolution, and sensitivity were set at 1 s, 100 nm/min, 1.0 nm and 5 mdeg, respectively. Twelve scanned spectra were accumulated for the spectral measurements.

The changes in the amplitudes of the induced Cotton effects were measured when the resolution was set at 0.5 nm, and the data from 32 scans were given as the means of the three points around the indicated wavelength.

All experiments were performed in the solution of 0.1 M K_2HPO_4–KH_2PO_4 buffer (pH 7.0), and the reagent (EGCG and drugs) were soluble in the buffer at the concentrations used. The final concentration of HSA was set

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at 6.02×10^{-5} M and that of EGCG was varied in the range of 0—1.75×10^{-4} M. The wavelength was set considering the minimum overlap of the induced CD, when the induced CD for HSA-EGCG and a HSA-drug that showed site-specific binding overlapped. In the case of the competitive experiments with DP, the concentration of DP was varied, because the induced Cotton effect due to the interaction between DP and HSA was larger than that observed for the combination EGCG-HSA, as shown later.

The induced CD for HSA-EGCG was expressed by the following equation:

$$\text{induced CD}_{\text{HSA}-\text{EGCG}}=\text{CD}_{\text{HSA}+\text{EGCG}}-(\text{CD}_{\text{HSA}}+\text{CD}_{\text{EGCG}})$$

The competition of EGCG and other drugs was analyzed by double-reciprocal plots, and the mode of inhibition on the binding sites on HSA or BSA was assigned.

The analogous examinations were also performed for the binding of EGCG to BSA, where the final concentration of BSA was set at 2.90×10^{-5} M.

Results and Discussion

Interaction between HSA and EGCG The induced CD curves for HSA and EGCG interaction are shown in Fig. 2, in which the negative and positive Cotton effects appear at around 325 and 280 nm, respectively. The amplitude of the induced CD was dependent on the concentration of EGCG (Figs. 2, 3A). The induced CD was attributable to the conformational change of HSA. However, the change in the conformation of EGCG may also be attributed to the induced CD.

Effects Observed for the Site I Binding Drug To investigate the binding of EGCG to site I on HSA, the effects adding PB, a drug that binds to site I, on the induced CD for HSA-EGCG were observed. As shown in Fig. 3A, the induced CD at 325 nm was inhibited by the addition of PB, in a dose-dependent manner.

The double-reciprocal plots, indicating relations between $1/D_f$ and $1/r$ for EGCG-HSA in the absence and presence of PB, are shown in Fig. 4A. D_f represents the molar concentration of EGCG unbound, estimated by subtraction of the concentration of EGCG bound to HSA, D_b, from the concentration of EGCG added. The calculation of D_b was based on the method indicated by Rosen.[9] The number of EGCG molecules bound to a molecule of HSA, r, corresponds to D_b/P_t, where P_t represents the molar concentration of HSA added

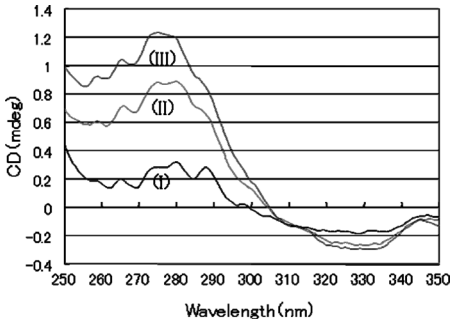

Fig. 2. Induced CD for HSA-EGCG

The presence of (I) 4.36×10^{-5} M, (II) 1.31×10^{-4} M, and (III) 2.18×10^{-4} M of EGCG in the solution of HSA (6.02×10^{-5} M) caused the induced CD, which were obtained by subtraction of the sum of the CDs for HSA and EGCG from those of the combination of HSA and EGCG.

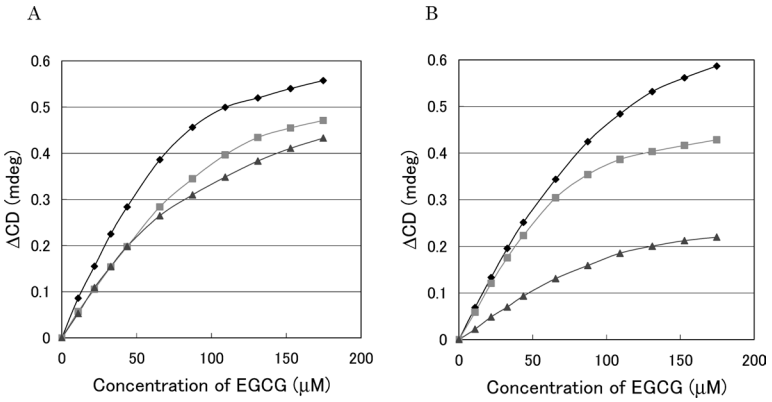

Fig. 3. Effects of Addition of PB (A) and TB (B) on the HSA-EGCG-Induced CD

(A) Amplitudes of the induced CD at 325 nm for HSA (6.02×10^{-5} M) and various concentrations of EGCG in the absence (◆) and presence (■, 4.86×10^{-4} M; ▲, 9.73×10^{-4} M) of PB were plotted. (B) Those at 330 nm for HSA (6.02×10^{-5} M) and various concentrations of EGCG in the absence (◆) and presence (■, 2.77×10^{-4} M; ▲, 5.55×10^{-4} M) of TB were plotted.

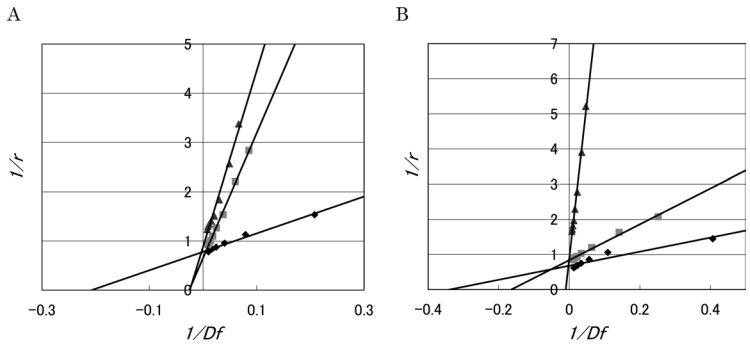

Fig. 4. Competitive Relationship of EGCG and PB (A) or TB (B) on Site I of HSA

Double-reciprocal plots were based on the induced CD at 325 nm (A) or 330 nm (B). (A) Effects in the presence of 4.86×10^{-4} M (■), 9.73×10^{-4} M (▲), or absence (◆) of PB on the binding of EGCG-HSA were shown. (B) Effects in the presence of 2.77×10^{-4} M (■), 5.55×10^{-4} M (▲), or absence (◆) of TB on the binding of EGCG-HSA were shown.

(bound and unbound, in total).

The all three lines for the presence/absence of PB cross at around 1 of the *y*-axis, indicating that PB, which is bound to HSA at site I, inhibited binding of EGCG to HSA competitively, and therefore binding of EGCG was assigned to site I on HSA.

TB, which also binds to site I on HSA, was then used to verify binding to site I during the binding of EGCG to HSA. The decrease of the induced CD of EGCG-HSA was shown in Fig. 3B, and the double-reciprocal plots are shown in Fig. 4B, in which the three lines in the presence/absence of TB cross at around 1 of the *y*-axis. This result substantiates the binding of EGCG to site I on HSA.

Effects Observed for the Site II Binding Drug The combination of HSA and DP, a representative site-II-binding drug, induced CD with noticeable amplitude as shown in Fig. 5. This spectrum appeared in the region overlapped with that observed for the combination HSA-EGCG, and the amplitude for HSA-DP was larger than that observed for HSA-EGCG. Therefore, the effects of adding EGCG on the induced CD of HSA-DP were examined for the quantitative analysis of the relationship between DP and EGCG. As shown in Fig. 6A, the addition of EGCG caused a decrease in the induced CD caused by HSA-DP, in a dose-dependent manner.

The effects of adding EGCG were analyzed using double-reciprocal plots. The plots for the absence and presence of EGCG shown in Fig. 7A indicated that the inhibition of the binding of DP to HSA by EGCG was competitive. Therefore, EGCG also bound to site II on HSA.

To confirm the competitive inhibition of the binding of the site-II drugs by EGCG, IP was used, which is a representative site-II-binding drug. The effects of adding IP to the HSA-EGCG-induced CD are shown in Figs. 6B and 7B. The inhibition by IP was competitive, and the binding of EGCG to site II on HSA is also shown here.

Effects Observed for the Site III Binding Drug DG was used as a representative drug that binds to site III on HSA. The addition of DG did not affect the HSA-EGCG-induced CD (Fig. 8), which indicates that site III does not par-

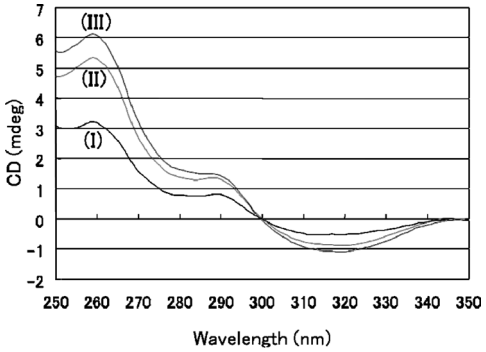

Fig. 5. Induced CD for HSA-DP

(I) 7.02×10^{-5} M, (II) 2.11×10^{-4} M, and (III) 3.51×10^{-4} M DP combination with HSA (6.02×10^{-5} M) induced Cotton effects, which were obtained by subtraction of CD for HSA from those of the combination of HSA and DP.

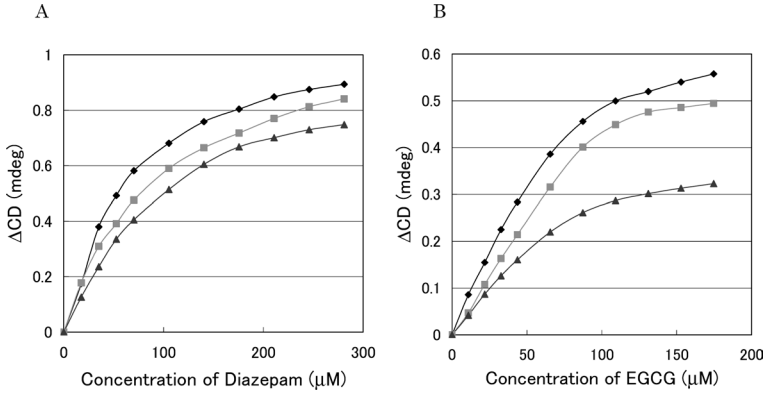

Fig. 6. Effects of Addition of EGCG on the HSA-DP-Induced CD (A), and That of IP on the HSA-EGCG-Induced CD (B)

Amplitudes of the induced CD at 310 nm for HSA (6.02×10^{-5} M) and various concentrations of DP in the absence (◆) and presence (■, 5.27×10^{-4} M; ▲, 1.05×10^{-3} M) of EGCG were plotted (A). In an analogous way, the amplitude of the induced CD at 325 nm for the induced CD for HSA-EGCG in the absence (◆) and presence (■, 3.64×10^{-4} M; ▲, 7.27×10^{-4} M) of IP were plotted (B).

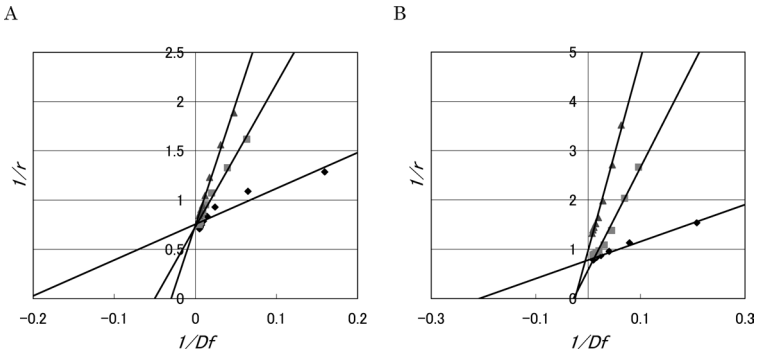

Fig. 7. Competitive Relationship of EGCG and DP (A) or IP (B) on Site II of HSA

Double-reciprocal plots were based on the induced CD at 310 nm (A) or 325 nm (B). (A) Effects in the presence of 5.27×10^{-4} M (■), 1.05×10^{-3} M (▲), or absence (◆) of EGCG on the binding of DP-HSA were shown. (B) Effects in the presence of 3.64×10^{-4} M (■), 7.27×10^{-4} M (▲), or absence (◆) of IP on the binding of EGCG-HSA were shown.

ticipate in the binding of EGCG to HSA.

Based on these findings, it appears that EGCG binds to sites I and II, but not site III. A docking study[18] assumed that EGCG could bind to both of sites I and II. The present results substantiate this assumption experimentally, and the analyses using CD spectra may be useful when the induced CD is available for a combination of polyphenols and HSA.

These analyses are based on the assumption that the direct interactions between EGCG and the drugs are negligible. However, if the interactions are stronger enough to affect the induced CD, their participation should be considered further.

Interaction between BSA and EGCG The binding of EGCG to BSA was then analyzed to investigate the effect of slight changes in the amino-acid sequence of the albumins. The BSA-EGCG-induced CD was also observed as shown in Fig. 9.

The negative and positive Cotton effects appeared at around 315 and 270 nm, respectively. The change in amplitude was dependent on the concentration of EGCG. However, the relative amplitudes for the longer and shorter wavelength differed somewhat from those observed for HSA-EGCG, which suggests the conformational difference between the two albumins.

The effects of PB and TB (bound to site I of HSA), EA (site II), and DG (site III) on the BSA-EGCG-induced CD were examined, to investigate whether the structural differences in the albumins affect the binding of EGCG. Since the induced CD for BSA-DP overlapped with that for BSA-EGCG, EA was used as a drug that shows binding to site II. The relationships between EGCG and these drugs in the binding sites were analyzed by double-reciprocal plots shown in Fig. 10.

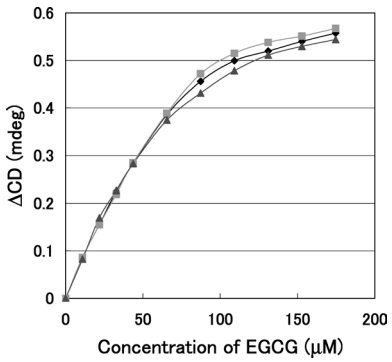

Fig. 8. The Effect of Addition of DG on the HSA-EGCG-Induced CD

Amplitudes of the induced CD at 325 nm for HSA (6.02×10^{-5} M) and various concentrations of EGCG in the absence (◆) and presence (■, 1.96×10^{-4} M; ▲, 3.92×10^{-4} M) of DG were plotted.

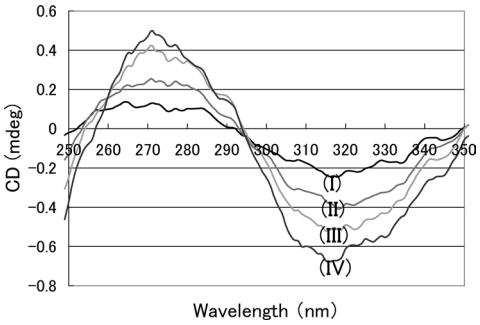

Fig. 9. Induced CD for BSA-EGCG

(I) 1.09×10^{-5} M, (II) 2.18×10^{-5} M, (III) 4.36×10^{-5} M, and (IV) 6.54×10^{-5} M EGCG in combination with BSA (2.90×10^{-5} M) induced CD, which were obtained by subtraction of the sum of the CDs for BSA and EGCG from those of the BSA combined with EGCG.

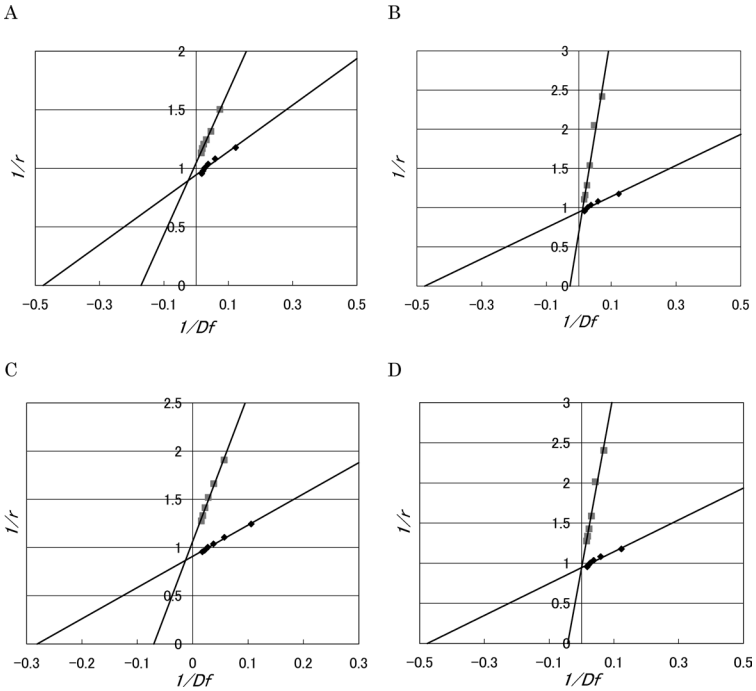

Fig. 10. Competitive Relationship of EGCG and Drugs of Specific Binding on BSA

Double-reciprocal plots were based on the induced CD at 320 nm (A, B, D), or 310 nm (C). (A) Effects in the presence of 1.95×10^{-4} M (■), or absence (◆) of PB on the binding of EGCG-BSA. (B) Effects in the presence of 2.22×10^{-4} M (■), or absence (◆) of TB on the binding of EGCG-BSA. (C) Effects in the presence of 1.98×10^{-4} M (■), or absence (◆) of EA on the binding of EGCG-BSA. (D) Effects in the presence of 7.84×10^{-5} M (■), or absence (◆) of DG on the binding of EGCG-BSA.

The lines for the presence and absence of PB cross at around 1 on the *y*-axis (Fig. 10A), and the competitive relationship between EGCG and PB is indicated. Analogously, TB (Fig. 10B) and EA (C) also showed competitive relationships with EGCG.

Although DG did not affect the HSA-EGCG interaction, the BSA-EGCG-induced CD was suppressed by adding DG, and double-reciprocal plot analysis showed competitive relationship between DG and EGCG (Fig. 10D).

Slight differences in the amino acid sequences of HSA and BSA affect their binding, which indicates a difference in the tertiary structure or conformation of the two albumins. The CD method was found to be a sensitive tool for the analysis of conformational differences in proteins.

Conclusions

The double-reciprocal plots based on the induced CD were utilized for the analyses of the binding of EGCG to HSA and BSA. Binding sites I and II on HSA were assigned based on the competitive inhibition of the site-I- and site-II-binding drugs. On the other hand, the competitive relationship was also observed between EGCG and the drug binds to site III in the case of the interaction of EGCG to BSA.

The results of the present study indicate that the binding of the polyphenol EGCG to protein is susceptible to the small differences in the structure or conformation of the protein, and does not show non-specific binding. Recent studies have revealed that EGCG interacts with various biomolecules,[19,20] and therefore application of the polyphenol as a tool for investigating the structure of such biomolecules is expected. Further studies on the effects of structural differences of polyphenols on their interaction with biomolecules, especially proteins, are awaited.

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