Properties of Precipitate of Creaming Down by (−)-Epigallocatechin-3-O-gallate and Caffeine

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An aqueous solution of equimolecular amounts of gallated catechin (−)-epigallocatechin-3-O-gallate (EGCg) and caffeine afforded a crude precipitate by creaming, which crystallized slowly for about three months at 10°C to give a colorless block crystal. The crystal was determined to be a 2:2 complex of EGCg and caffeine by X-ray crystallographic analysis. The 2:2 complex was formed with the cooperative effect of three intermolecular interactions, π–π and CH–π interactions, and intermolecular hydrogen bonds. Upon formation of the 2:2 complex, a caffeine molecule was captured by a hydrophobic space formed by the aromatic rings A, B, and B’ rings of two EGCg molecules. Judging from the entropy value, the shift value in the chemical shift of proton signals in 1H-NMR spectra, the \( NT_1 \) value, and nuclear Overhauser effects (NOEs), it was thought that the structure of complexes of EGCg and caffeine in aqueous solution were the same as their crystal structure.

Key words creaming down; (−)-epigallocatechin-3-O-gallate; caffeine; π–π interaction; entropy; \( NT_1 \) value

Tea is drunk for its taste and, especially in recent years, as a healthy beverage all over the world. Tea is commonly prepared by pouring hot or boiling water over leaves of the tea plant, *Camellia sinensis*, Theaceae, which includes caffeine, tannins, vitamins, and theanine.1) When a hot tea beverage cools down, it becomes turbid and brown-white particles settle out. This phenomenon is called a “creaming” or “creaming-down reaction.” Since creaming is a trigger deforming the original appearance and color of tea, it is one of the most serious problems in making a tea beverage.

Previously, Ina and colleagues reported that all the main signals were assigned to gallated catechins such as (−)-epigallocatechin-3-O-gallate (EGCg) and (−)-epicatechin-3-O-gallate (ECg) and caffeine in the 13C-NMR spectrum of a hot water solution of a crude precipitate formed by the creaming of a tea infusion 2) (Fig. 1). We reported that quantitative analysis of a crude precipitate of creaming from a catechin mixture and caffeine suggested that gallated catechins were predominantly responsible for creaming rather than non-gallated catechins.3) Furthermore, in complex formation with caffeine, non-gallated catechin (−)-epicatechin (EC) formed a 1:1 complex with caffeine, and gallated catechin ECg formed a 2:4 complex with caffeine. The π–π complexation site of EC with caffeine was only the A ring, whereas that of ECg was all aromatic rings, A, B, and B’. It was thought that the hydrophobicity of the 2:4 complex of ECg and caffeine was stronger than that of the 1:1 complex of EC and caffeine, with the result that the 2:4 complex of ECg and caffeine precipitated by creaming more predominantly than the 1:1 complex of EC and caffeine in aqueous solution.

However, the green tea catechin most contained in tea is EGCg; therefore, it cannot be said only by studying ECg and EC complex with caffeine that creaming down has been elucidated sufficiently. Ina and colleagues reported that when an aqueous caffeine solution is poured into an aqueous EGCg solution.2) Also, Hayashi et al. reported that EGCg and caffeine formed a 1:1 complex in aqueous solution, and all aromatic rings participated in complex formation.4) Thus, we investigated the crystal structure of the precipitate formed by creaming made from an aqueous solution of EGCg and caffeine, and detail properties of the complex of EGCg and caffeine in aqueous solution on the basis of the crystal structure of EGCg complex with caffeine.

![Fig. 1. (−)-Epigallocatechin-3-O-gallate, (−)-Epicatechin-3-O-gallate, (−)-Epicatechin and Caffeine](image-url)

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Results and Discussion

Preparation of Creaming Precipitate Made from EGCg and Caffeine

EGCg and caffeine were dissolved in water by heating. When the aqueous solution cooled down, it divided into the supernatant liquid and sticky precipitate, which is a crude precipitate formed by creaming. The sticky precipitate crystallized slowly to afford a colorless block crystal, which contained EGCg and caffeine at a molar ratio of 1:1, based on measurement of the integral volume of $^1$H-NMR signals (Fig. 2).

Determination of the Stereochemical Structure of the Complex of EGCg and Caffeine

A single crystal of the colorless block crystal, which was prepared from the aqueous solution of EGCg and caffeine, was determined to be a 2:2 complex of EGCg and caffeine by X-ray crystallographic analysis. An ORTEP drawing of the 2:2 complex of EGCg, caffeine is shown in Fig. 3a. The 2:2 complex was formed from two crystallographically different EGCgs (EGCg A, B) and two caffeines (caffeine A, B), and the B' rings of each EGCg faced the caffeines. One unit cell contained four units of the 2:2 complex of EGCg and caffeine, and sixty water molecules as crystal solvent (Fig. 3b).

The torsion angles of EGCg moieties (EGCg A, B) of the 2:2 complex of EGCg and caffeine indicated that B' rings of EGCgs A and B were both in equatorial positions and B' rings of EGCgs A and B were both in axial positions with respect to C rings of EGCg molecules (Table 1). The torsion angles of caffeine moieties indicated that caffeine moieties (caffeine A, B) were both almost in a plane form, as shown in Table 2.

The layer structure of the 2:2 complex of EGCg and caffeine was formed from two layers (Fig. 4a). Namely, caffeine A was stacked between B' rings of EGCg A and caffeine B was stacked between B' rings of EGCg B, and caffeine A or

| Torsion Angle of EGCg Moieties of the 2:2 Complex of EGCg and Caffeine |
|---------------------------------------------------------------|
| Torsion angle of | Torsion angle of |
| EGCg A           | EGCg B           |
| $\angle C_1-C_2-C_3-O$ | 69.2(8)$^\circ$ | 67.1(8)$^\circ$ |
| $\angle H_2-C_2-C_3-H_3$ | $-72.2^\circ$ | $-68.4^\circ$ |
| $\angle H_2-C_2-C_3-O$ | 166.9$^\circ$ | 171.1$^\circ$ |

| Torsion Angle of Caffeine Moieties of the 2:2 Complex of EGCg and Caffeine |
|---------------------------------------------------------------|
| Torsion angle of | Torsion angle of |
| caffeine A       | caffeine B       |
| $\angle N_1-C_7-C_8-O_{13}$ | 0.5(13)$^\circ$ | 3.4(13)$^\circ$ |
| $\angle O_{13}-C_8-N_2-N_10$ | $-1.8(10)^\circ$ | 2.3(10)$^\circ$ |
| $\angle O_{13}-C_8-N_2-C_{12}$ | $-5.1(10)^\circ$ | 1.0(11)$^\circ$ |
| $\angle N_1-C_8-N_2-N_9$ | $-179.6(7)^\circ$ | 178.1(7)$^\circ$ |
| $\angle N_1-C_8-N_2-O_{11}$ | $-0.4(10)^\circ$ | $-4.3(10)^\circ$ |
| $\angle N_1-C_8-N_2-N_9$ | $6.2(11)^\circ$ | $-0.1(12)^\circ$ |

B was located almost in the middle of two B' rings of EGCg A or B. These were in parallel in the same direction as the $b$-axis.

In the 2:2 complex of EGCg and caffeine, intermolecular interactions forming between EGCg and caffeine moieties were elucidated (Fig. 4b). $\pi-\pi$ Interactions formed between the B' ring of EGCg and a six-membered ring of caffeine, and A rings of EGCg A, B. Also, CH–π interactions formed between amino group N$_1$-CH$_2$, N$_2$-CH$_2$ of caffeine and B rings of EGCg A and B. Two O–H...N and two O–H...O intermolecular hydrogen bonds were also observed between EGCg and caffeine (Table 3).
As shown in Fig. 5a, the caffeine moieties of the 2:2 complex were located in the space surrounding the top and lower walls of \( \text{B}'/\text{uni} \) rings of EGCg moieties and right and left walls of A and B rings of EGCg moieties. As a result, caffeine molecules were captured by the hydrophobic space formed by three aromatic A, B, \( \text{B}'/\text{uni} \) rings of EGCg in the 2:2 complex. In Fig. 5b, the 2:2 complex of EGCg and caffeine, water molecules as a crystal solvent are displayed and caffeine molecules are not displayed. The water molecules existed outside the space formed by three aromatic A, B, \( \text{B}'/\text{uni} \) rings of EGCg and caffeine moieties as not displayed. The water molecules existed outside the space formed by three aromatic A, B, \( \text{B}'/\text{uni} \) rings of EGCg and caffeine A and were not observed in the space, suggesting that the space had high hydrophobicity. It was therefore thought that the sticky precipitate formed by creaming precipitated from the aqueous solution of EGCg and caffeine due to its high hydrophobicity. Also this phenomenon is thought to be a kind of tannin activity.

The only structural difference between the gallated catechins EGCg and ECg is the presence of a hydroxyl group in 5' position (Fig. 1); however, a large difference occurred in complex formation with caffeine, as shown in Fig. 5a and Fig. 5c. While EGCg formed a 2:2 complex with caffeine, ECg formed a 2:4 complex with caffeine. Furthermore, EGCg formed \( \pi-\pi \) interactions between \( \text{B}'/\text{uni} \) rings of EGCg and caffeine, and A rings of EGCg A, whereas ECg formed \( \pi-\pi \) interactions between all aromatic rings (A, B, \( \text{B}'/\text{uni} \) rings) of ECg and caffeine. 3)

**Properties of the Complex of EGCg, EC and Caffeine in Aqueous Solution State**  The stability constants for the for-
mation of complexes of EGCg, EC and caffeine $K_c$ at 40–80°C were estimated by Eq. 1, which assumed the order of the reaction $n$.\(^6\) Here, CF and CX mean caffeine and the complexes of EGCg, EC and caffeine, respectively, and $\Delta \delta_{\text{obs}}$ and $\Delta \delta_{\text{CX}}$ represent $\left(\delta_{\text{EGCg or EC}} - \delta_{\text{obs}}\right)$ and $\left(\delta_{\text{EGCg or EC}} - \delta_{\text{CX}}\right)$, respectively. $\delta_{\text{EGCg or EC}}, \delta_{\text{CX}},$ and $\delta_{\text{obs}}$ represent the chemical shift (ppm) of the H$_2$ proton of EGCg and the H$_5$ proton of EC in a free state, complexes of EGCg, EC and caffeine, and the mixture of EGCg, EC and caffeine in 1H-NMR spectra, respectively. The stability constants $K_c$ of the complexes of EGCg, EC and caffeine at 40°C were 940.4 and 577.2 M$^{-1}$, respectively (Table 4).

From the dependency of $K_c$ on temperature, the change of free energy $\Delta G$, enthalpy $\Delta H$, and entropy $\Delta S$, of the complex formation were estimated as shown in Table 4. The entropy ($\Delta S$) for the formation of the complex of EGCg and caffeine takes a large negative value ($-34.6$ kJ mol$^{-1}$ K$^{-1}$), suggesting that caffeine was fixed tightly in the complex of EGCg and caffeine. On the other hand, the entropy ($\Delta S$) for the formation of the complex of EC and caffeine takes a small negative value ($-5.3$ kJ mol$^{-1}$ K$^{-1}$), suggesting that caffeine fitted loosely

| $T$ (°C) | $K_c$ (M$^{-1}$) | $\Delta G$ (kJ mol$^{-1}$) | $\Delta H$ (kJ mol$^{-1}$) | $\Delta S$ (kJ mol$^{-1}$ K$^{-1}$) | $n$ |
|----------|-----------------|--------------------------|--------------------------|--------------------------|-----|
| 40       | 940.7           | -17.8                   | 0.49                     |                           |     |
| 45       | 762.4           | -17.5                   | 0.51                     |                           |     |
| 50       | 710.9           | -17.6                   | 0.53                     |                           |     |
| 55       | 568.6           | -17.3                   | -28.7                    | -34.6                    | 0.61|
| 60       | 482.4           | -17.1                   | 0.62                     |                           |     |
| 70       | 348.3           | -16.7                   | 0.67                     |                           |     |
| 80       | 274.8           | -16.5                   | 1.01                     |                           |     |

| $T$ (°C) | $K_c$ (M$^{-1}$) | $\Delta G$ (kJ mol$^{-1}$) | $\Delta H$ (kJ mol$^{-1}$) | $\Delta S$ (kJ mol$^{-1}$ K$^{-1}$) | $n$ |
|----------|-----------------|--------------------------|--------------------------|--------------------------|-----|
| 40       | 577.2           | -16.5                   | 0.58                     |                           |     |
| 45       | 495.9           | -16.4                   | 0.61                     |                           |     |
| 50       | 439.8           | -16.3                   | 0.65                     |                           |     |
| 55       | 413.6           | -16.4                   | -18.1                    | -5.3                     | 0.94|
| 60       | 351.9           | -16.2                   | 0.71                     |                           |     |
| 70       | 311.2           | -16.3                   | 0.61                     |                           |     |
| 80       | 255.9           | -16.3                   | 0.65                     |                           |     |
in the complex of EC and caffeine.

Judging from the crystal structure of the 2:2 complex of EGCg and caffeine (Fig. 5a), caffeine was thought to be fixed tightly in the space formed by three aromatic A, B, B' rings of EGCg. On the other hand, judging from the crystal structure of the 1:1 complex of EC and caffeine (Fig. 5d), caffeine was thought to fit loosely between the A rings of EC.

Nextly, changes in chemical shifts of proton signals of EGCg and EC in 1H-NMR spectra by adding regular amounts of caffeine were observed. As shown in Fig. 6a, the upfield shift of the proton signal of H$_2$ and H$_6$ was most prominent in the EGCg proton signals. The upfield shift of the signal of H$_2$ and H$_6$ may result from magnetic anisotropic shielding by the ring current from the xanthine skeleton of caffeine, suggesting that caffeine moieties in the complex of EGCg and caffeine were positioned in the vicinity of the B' ring of EGCg (Fig. 7a).

Figure 6b shows that the proton signals of H$_6$ and H$_8$ of the A ring of EC shifted most predominantly upfield in the proton signals of EC. The upfield shifts of proton signals of H$_6$ and H$_8$ were considered to result mainly from the magnetic anisotropy of the aromatic ring of the xanthine skeleton of caffeine, suggesting that caffeine moieties in the complex of EC and caffeine were positioned in the vicinity of the A ring of EC (Fig. 7b).

Information regarding the structural flexibility of caffeine moieties upon complex formation with EGCg and EC in D$_2$O can be experimentally obtained from the relaxation times of

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![Fig. 6. The Shifts of 1H-NMR Signals of the Protons of EGCg and EC upon Addition of Caffeine to the Solution of EGCg and EC in D$_2$O](image)
carbon resonances ($T_1$). The $NT_1$ values ($N$=number of attached protons, $T_1$=longitudinal relaxation time) are corrected directly with molecular mobility. The $NT_1$ values of carbon of caffeine moieties in complexes of EGCg, EC and caffeine are shown in Table 5. The $NT_1$ values of N1-CH3, N7-CH3, and C8 of caffeine in the complex of EGCg and caffeine were smaller than those in the complex of EC and caffeine, indicating that the mobility of the caffeine moieties in the complex of EGCg and caffeine was restricted more strongly than those in the complex of EC and caffeine. Judging from the crystal structure of the 2:2 complex of EGCg and caffeine (Fig. 5a) and 1:1 complex of EC and caffeine (Fig. 5d), upon complex formation with EGCg, the xanthine skeleton of caffeine was fixed strongly in the hydrophobic space formed by three aromatic A, B, B′ rings of EGCg. Furthermore, the mobility of N1-CH3, N7-CH3 of methyl groups of the caffeine moieties in the complex of EGCg and caffeine were thought to be restricted by the force of CH–π interaction with the B rings of EGCg.

The nuclear Overhauser effect (NOE) difference analysis of aqueous solution containing equimolar amounts of EGCg and caffeine was conducted. Intermolecular NOEs between H2,6 of EGCg and N1-CH3, N7-CH3 and C8 of caffeine in the complex of EGCg and caffeine were observed (Fig. 7a).

It was therefore considered that the structure of complexes of EGCg, EC and caffeine in aqueous solution were the same as their crystal structure. Upon a formation of the 2:2 complex of EGCg and caffeine, EGCg captured caffeine in the hydrophobic space formed by three aromatic A, B, B′ rings of EGCg. We are going to investigate a molecular capture of various compounds by EGCg in aqueous solution using this findings.

**Experimental**

**Materials** EGCg (≥95%, from green tea), EC (≥90% (HPLC)) and caffeine (≥100%) were purchased from Sigma-Aldrich Co. EGCg, EC and caffeine were used without further purification.

**NMR Experiments** $^1$H-NMR spectra were recorded at room temperature on a JEOL JMN-LA500 (Tokyo, Japan) operating at 500 MHz for $^1$H and 125.65 MHz for $^{13}$C, using a 5 mmϕ sample tube. D2O was used as a solvent (99.9 atom% D; Wako Pure Chemical Industries, Ltd.). Chemical shift values are expressed in ppm downfield using sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard. $T_1$ values of solutions containing equimolar amounts of EGCg and caffeine, EC and caffeine in D2O (40 mM) were estimated using the standard inversion-recovery sequence to determine the null in signal intensity. The NOE difference experiments of a solution containing equimolar amounts of EGCg and caffeine in D2O (10 mM) were typically conducted with 32 K data points covering a spectral width of 10000 Hz and with ca. 5 s presaturation time.

**Preparation of Creaming Precipitate Made from EGCg and Caffeine** EGCg (10.00 mg, 2.18×10⁻² mmol) and caffeine (4.24 mg, 2.18×10⁻² mmol) were dissolved in distilled water (130 µL) by heating at 90°C for 5 min. The aqueous solution was left at room temperature for 12 h, and then divided into the supernatant liquid and sticky precipitate, which is a crude precipitate formed by creaming. They were left at 10°C for about 3 months, and the sticky precipitate crystallized slowly to afford a colorless block crystal (Fig. 2).

**X-Ray Crystal Structure Analysis of the 2:2 Complex of EGCg and Caffeine** A single crystal of a 1:1 complex of EGCg and caffeine was determined by X-ray crystallographic analysis at 213K. X-Ray intensity data of 18735 reflections (of which 8514 were unique) were collected on a Rigaku RAXIS RAPID imaging plate area detector with graphite
monochromated CuKα radiation (λ=1.54187 Å). The structure was solved by direct methods using SIR2004⁹ and expanded using Fourier techniques.⁹ The non-hydrogen atoms were refined anisotropically. Hydrogen atoms were refined using the riding model. The final cycle of full-matrix least-squares refinement on F² was based on 8514 observed reflections and 979 variable parameters and converged with unweighted and weighted agreement factors of: \(R = \frac{\sum |F_o| - |F_c|}{\sum |F_o|} = 0.0583\) (I>2.0σ(I)), \(R_w = \frac{\sum w(F_o^2 - F_c^2)^2}{\sum w(F_o^2)^2} = 0.1894\). The standard deviation of the unit weight observation was 1.10. The maximum and minimum peaks on the final difference Fourier map corresponded to 1.01. Unit weights were used. The maximum and minimum peaks on the final difference Fourier map corresponded to 1.01. Unit weights were used. The maximum and minimum peaks on the final difference Fourier map corresponded to 1.01.

X-Ray Crystal Structure Analysis of the 1:1 Complex of EC and Caffeine. A crystal of a 1:1 complex of EC and caffeine was determined by X-ray crystallographic analysis at 213 K. X-Ray intensity data of 8547 reflections (of which 3326 were unique) were collected on a Rigaku RAXIS RAPID II imaging plate area detector with graphite monochromated CuKα radiation (λ=1.54187 Å). The data were corrected for Lorentz and polarization effects. The structure was solved by direct methods using SIR2004⁹ and expanded using Fourier techniques.⁹ The final cycle of full-matrix least-squares refinement on F² was based on 3326 observed reflections and 335 variable parameters and converged with unweighted and weighted agreement factors of: \(R = \frac{\sum |F_o| - |F_c|}{\sum |F_o|} = 0.0699\) (I>2.0σ(I)), \(R_w = \frac{\sum w(F_o^2 - F_c^2)^2}{\sum w(F_o^2)^2} = 0.1710\). The standard deviation of the unit weight observation was 1.01. Unit weights were used. The maximum and minimum peaks on the final difference Fourier map corresponded to 0.32 and −0.24 e/Å³, respectively. All calculations were performed using the CrystalStructure¹² crystallographic software package except for refinement, which was performed using SHELXL-97.¹¹ Crystallographic data reported in this manuscript have been deposited with Cambridge Crystallographic Data Center as supplementary publication No. 765712 for a 1:1 complex of EC and caffeine.

Stoichiometry and Thermodynamic Parameters of EGCg and EC Complexes with Caffeine in Aqueous Solution. The stoichiometry and stability constants \(K_c\) of the EGCg and EC complexes with caffeine were determined by monitoring the chemical shifts of the \(H_2\alpha:\) proton signals of EGCg and the \(H_\beta\) proton signals of EC in the \(^1H\)-NMR measurements when the concentration of caffeine continuously was increased from 0 to 54 mm in a constant concentration of EGCg and EC (10 mm) and using the equation of Eq. 1 in the range 40–80°C. Also the changes of free energy \(\Delta G\), enthalpy \(\Delta H\), entropy \(\Delta S\) for formation of the EGCg and EC complexes with caffeine on temperature were estimated by using the results of the stability constants.

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