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Effect of solution pH on complex formation between epi-type catechin and β-cyclodextrin

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Abstract
The effect of solution pH on the formation of an inclusion complex between (−)-epigallocatechin gallate (EGCg: p*K*a = 7.5) and β-cyclodextrin (β-CD) was investigated by isothermal titration calorimetry and ^{1}H-NMR spectroscopy. The formation of an inclusion complex (EGCg-β-CD) depended on the solution pH; two different types of inclusion complexes were formed at 1:1 molar ratio in acid/neutral solutions, and only one type of complex was formed in the basic solution. The first type of EGCg-β-CD with larger association constant was formed independently of pH, with the AC-ring of EGCg being deeply inserted into the cavity of β-CD and the B-ring existing near the secondary hydroxyl group of β-CD. On the other hand, the formation of the second type depended on the solution pH. The B′-ring of EGCg was included in the case of acid and neutral solutions, but the formation of an inclusion complex in the basic solution was difficult due to the ionization of the 4″-OH on the B′-ring. ^{1}H-NMR spectroscopy supported these results. These results suggested that when determining the structures of EGCg-β-CD in an aqueous solution, it is necessary to consider unionized and ionized forms of EGCg.

Keywords (−)-Epigallocatechin gallate · β-Cyclodextrin · Isothermal titration calorimetry · ROESY spectrum

Introduction

Catechins, numerous polyphenols contained in tea leaves, are important ingredients for flavors in some teas, and the main component is (−)-epigallocatechin gallate (EGCg). EGCg is made up by AC-, B-, and B′-rings as shown in Fig. 1. The B- and B′-rings of EGCg cross each other at right angles to the AC-ring, and hydroxyl groups are placed at each of the rings of EGCg [1]. The value of p*K*a is 7.5, since 4″-OH on the B'-ring is ionized [2, 3]. It was reported that unionized EGCg formed an insoluble complex with some other molecules and stabilized them by an intermolecular hydrogen bonding interaction [1, 4, 5]. EGCg has not only beneficial actions such as antioxidant activity, suppression of cholesterol absorption, antimicrobial activity and anticancer actions but also strong bitterness and astringency [6–11]. To suppress the unpleasant taste in a beverage containing EGCg at a high concentration, β-cyclodextrin (β-CD) is often used as an additive. Since β-CD consists of a hydrophobic cavity and hydrophilic rims containing primary and secondary hydroxyl groups (Fig. 1), it is capable of producing inclusion complexes with various molecules [12–17]. In the case of complexation between EGCg and β-CD, it was reported that only the AC-ring of EGCg was included [17]. However, EGCg has some insertable sites (AC-, B-, or B′-ring) in the cavity of β-CD. In this study, the effect of solution pH on the formation of an inclusion complex was investigated by isothermal titration calorimetry (ITC) and ^{1}H-NMR spectroscopy.

Experimental

Materials

EGCg was purchased from ChromaDex, Inc. (Irvine, CA, USA) and β-CD was purchased from Pfanstiehl Laboratories, Inc. (Waukegan, IL, USA) and they were used without further purification. Potassium dihydrogenphosphate, disodium hydrogen phosphate and tetramethylsilane

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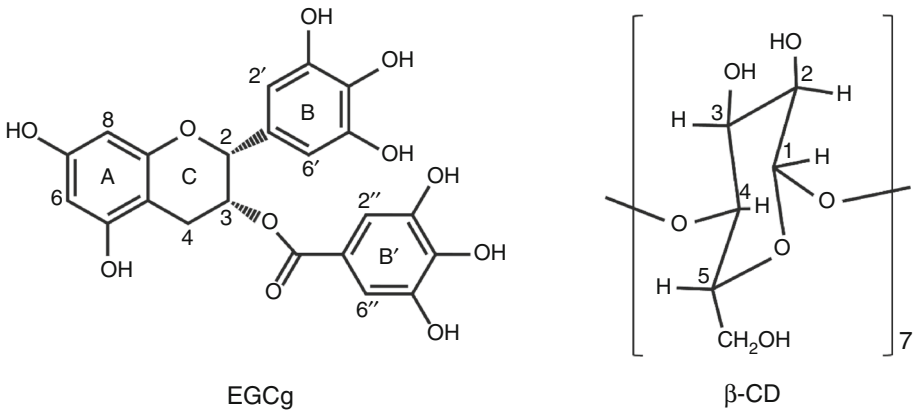

Fig. 1 Chemical structures of EGCg and β-CD. The numbers in these structures represent the hydrogen atom attached to the ring according to IUPAC. Symbols in the phenyl group of EGCg represent the names of the rings

were purchased from Nacalai Tesque, Inc. (Kyoto, Japan), and deuterium oxide was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other materials in this study were of analytical grade.

Isothermal titration calorimetry and data analysis

All experiments were performed at 298.15 K using Thermal Activity Monitor 2277 (Thermometric, Järfälla, Sweden). EGCg and β-CD were dissolved in various 1/15 M phosphate buffers with pH ranging from 4.5 to 8.5. The reaction cell put in the calorimeter was initially filled with 3 mL of 0.5 mM EGCg as a titrand. A solution of 15 μL of 10 mM β-CD as a titrant was automatically injected into the reaction cell 16 times every 15 min, and the heat of reaction was measured. Dilution heat of β-CD was not detected.

The calorimetric titration curves were analyzed using a two-type binding model assuming that two inclusion complexes were produced simultaneously [18].

$$\mathrm{EGCg} + n_1\beta\text{-CD} \rightleftarrows \mathrm{EGCg}-\beta\text{-CD}_{n1} \quad K_1, \Delta H_1 \tag{1}$$

$$\mathrm{EGCg} + n_2\beta\text{-CD} \rightleftarrows \mathrm{EGCg}-\beta\text{-CD}_{n2} \quad K_2, \Delta H_2, \tag{2}$$

where K_1 and K_2 ($K_1 > K_2$) represent association constants for the first and second complexes, respectively. The heat of reaction (ΔQ) was proportional to the quantity of the complexes and could be expressed as a function of the total concentration of β-CD:

$$\Delta Q = [\mathrm{EGCg}]_t V \left(\frac{n_1 \Delta H_1 K_1 [\mathrm{CD}]_f}{1 + K_1 [\mathrm{CD}]_f} + \frac{n_2 \Delta H_2 K_2 [\mathrm{CD}]_f}{1 + K_2 [\mathrm{CD}]_f} \right), \tag{3}$$

where $[\mathrm{EGCg}]_t$ represents the total concentration of EGCg, $[\mathrm{CD}]_f$ is the free concentration of β-CD and V is the volume of the EGCg solution. The best-fit values of n, K and ΔH were estimated using nonlinear regression analysis. The values of ΔG and ΔS were calculated by the general formulas $\Delta G = -RT\ln K$ and $\Delta S = (\Delta H - \Delta G)/T$.

^{1}H-NMR spectroscopy

^{1}H-NMR spectra were recorded at 308 K using a JEOL JNM-ECZ600R spectrometer at 600 MHz and were analyzed using Delta NMR Processing and Control Software v 5.0.5.1. The mixture of EGCg and β-CD (1:1 molar ratio) was dissolved in 1.0 mM HCl (pH 3.0) or 0.1 M $NaHCO_3$ (pH 8.5). ^{1}H-NMR chemical shifts (δ) were measured by the presaturation method, because H-6 and H-8 protons in the A-ring of EGCg were easily exchanged with deuterium in D_2O. The rotating frame nuclear overhauser effect spectroscopy (ROESY) spectra were acquired with 16 scans, sampling point was set to 256, the relaxation delay was 1.5 s and the spin-lock mixing time was 250 ms.

Results and discussion

Calorimetric study for complex formation between EGCg and β-CD

Figure 2 shows some calorimetric titration curves of EGCg with β-CD at various pH values. The heat of reaction increased as the concentration of β-CD increased. The titration curve of pH 4.5 was the highest in all pH values. The best-fit values for all parameters calculated using Eq. 3 are summarized in Table 1. In this case, the value of n_1 was fixed to 1.0, and the other parameters (K_1, K_2, ΔH_1, ΔH_2 and n_2) were estimated.

Assuming that the first type of inclusion complex (EGCg–β-CD) was formed at a molar ratio of 1:1 (n_1 = 1.0), then the values of n_2 decreased with the increase in pH to be 0.3 at pH 8.0 and pH 8.5. The values of $-\Delta G_1$ and $-\Delta H_1$ for the first type with higher affinity to β-CD ($K_1 > K_2$) were almost constant in all solutions at various pH values. On the other hand, the values of $-\Delta G_2$ and $-\Delta H_2$ for the second type

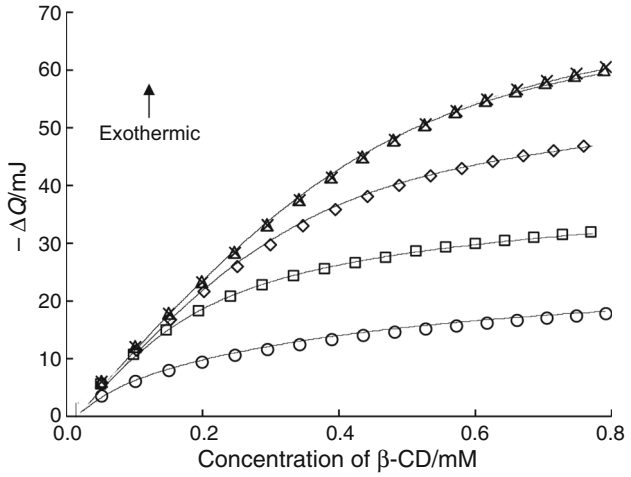

Fig. 2 Calorimetric titration curves of EGCg-β-CD systems in various pH solutions at 273.15 K. In all experiments, the initial concentrations of EGCg as a titrand and β-CD as a titrant were 0.5 and 10 mM, respectively. Each solid line represents a computer fitting curve by using a two-type binding model. Each point represents experimental value in various solutions, ×: pH 4.5, △: pH 6.0, ◇: pH 7.0, □: pH 7.5, ○: pH 8.5

decreased with the increase in pH, and they sharply decreased at pH 8.0 and pH 8.5, at which both n_2 and K_2 were very small. It was thought that the second type might be disregarded in basic solutions. When the titration curves of pH 8.0 and pH 8.5 were reanalyzed using a one-type binding model expressed by Eq. 1, the average values of the parameters were $n_1 = 0.8$, $\Delta G_1 = -14.4 \pm 0.3$ kJ mol^{-1} and $\Delta H_1 = -49.5 \pm 0.3$ kJ mol^{-1}. It was thought that EGCg could form only one type of inclusion complex in basic solutions. Thus, there should be two different types of EGCg–β-CD in acid and neutral solutions. The first complex with K_1 was formed independently of the solution pH with constant thermodynamic parameters, $\Delta G_1 = -21.8 \pm 0.6$ kJ mol^{-1}, $\Delta H_1 = -44.7 \pm 1.9$ kJ mol^{-1} and $\Delta S_1 = -76.6 \pm 6.4$ J mol^{-1}K^{-1}.

ROESY spectra of EGCg and β-CD mixture

Figure 3 shows two partial contour plots of the ROESY spectra of EGCg and β-CD mixtures. In the acid solution at pH 3.0, there were shown some strong intermolecular cross-peaks between H-6 (H-8) of EGCg and 5-H of β-CD and between H-3, H-6 (H-8), H-2′ (H-6′) and H-2″ (H-6″) of EGCg and 3-H of β-CD. It seemed that the AC-ring of EGCg was deeply included in the β-CD cavity with B- and B′-rings being inserted into the cavity near the broad rim of the secondary hydroxyl group of β-CD. In the basic solution at pH 8.5, two weak cross-peaks between H-6 (H-8) of EGCg and 5-H of β-CD and between H-2′ (H-6′) of EGCg and 3-H of β-CD were confirmed, suggesting that only the AC-ring was included in the cavity of β-CD with the B-ring being inserted near the rim of β-CD.

Formation of inclusion complexes between EGCg and β-CD

The EGCg molecule has multiple sites (AC-, B- and B′-rings) to be included by β-CD in an aqueous solution. Since the 4″-OH group on the B′-ring of EGCg was ionized (pKa = 7.5), the ionized ratio increased with the increase in pH: 0.10% at pH 4.5, 2.9% at pH 6.0, 50% at pH 7.5, 76% at pH 8.0 and 91% at pH 8.5. The results indicated that the formation of an EGCg-β-CD inclusion complex depended on the solution pH: two different types in acid/neutral solutions (pH ≤ pKa) and only one type in basic solution (pH > pKa).

Two types of inclusion complexes were simulated using the data obtained by ^{1}H-NMR measurement, and the structures are shown in Fig. 4. The first type with larger K_1 was formed independently of pH, with β-CD including the AC-ring of EGCg deeply in its cavity and the B-ring being inserted near the secondary hydroxyl group of β-CD (left in Fig. 4). Some clear cross-peaks between H-6 (H-8) on the

Table 1 Best-fit values of stoichiometries (n), association constants (K) and thermodynamic parameters for complexation between EGCg and β-CD at 298.15 K and 101.32 kPa

pH	n_1	K_1/M^{-1}	$-\Delta G_1$/ kJ mol^{-1}	$-\Delta H_1$/ kJ mol^{-1}	ΔS_1/ J mol^{-1} K^{-1}	n_2	K_2/M^{-1}	$-\Delta G_2$/ kJ mol^{-1}	$-\Delta H_2$/ kJ mol^{-1}	ΔS_2/J mol^{-1} K^{-1}
4.5	1.0	5040	21.1	44.3	− 77.8	1.0	3250	20.0	59.8	− 133.6
5.3	1.0	6470	21.7	48.4	− 89.5	0.9	1640	18.3	42.3	− 80.6
6.0	1.0	7120	22.0	45.1	− 77.7	0.8	1220	17.6	39.5	− 73.4
6.5	1.0	5020	21.1	42.3	− 71.2	0.7	599	15.8	28.1	− 41.2
7.0	1.0	9450	22.7	44.2	− 72.1	0.6	292	14.1	24.1	− 33.8
7.5	1.0	8210	22.3	43.7	− 71.5	0.5	180	12.9	19.1	− 20.7
8.0	1.0	8800	22.5	55.2	− 109.6	0.3	43	9.3	5.8	11.8
8.5	1.0	9320	22.6	54.6	− 107.2	0.3	50	9.7	7.7	6.5

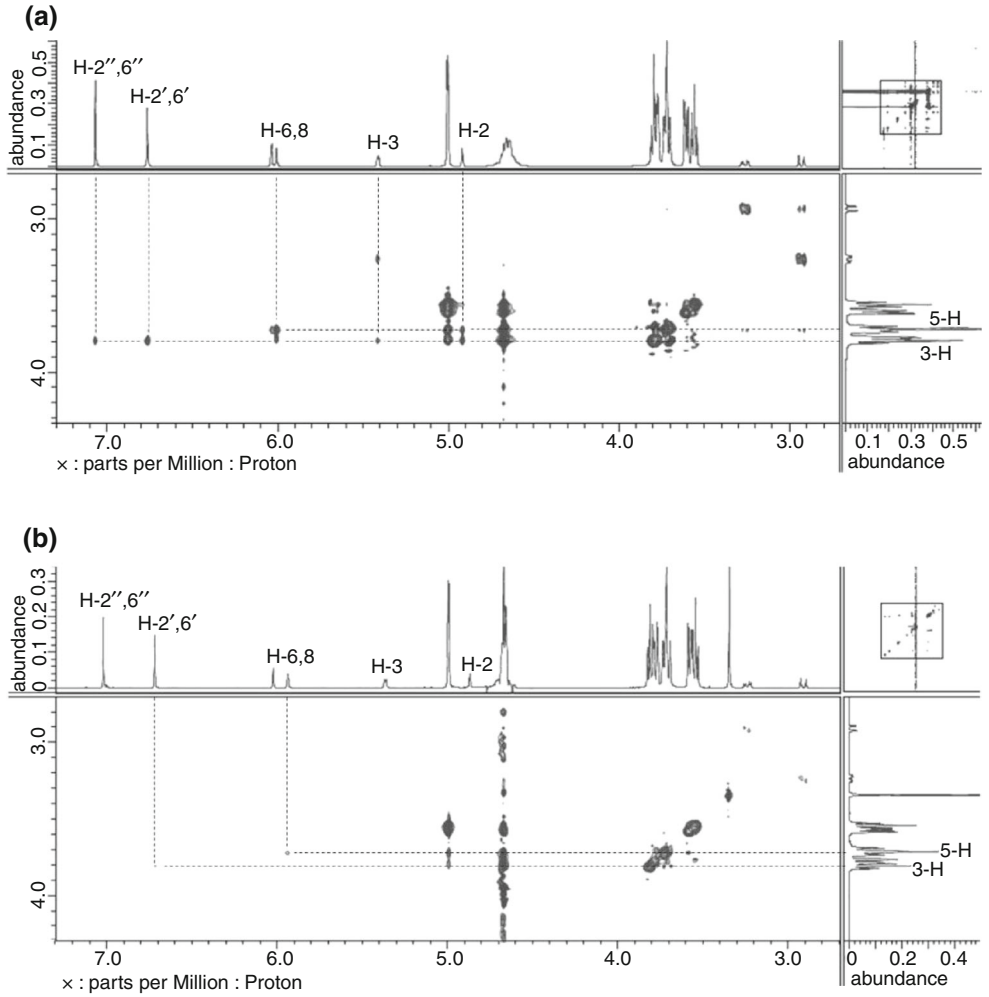

Fig. 3 ROESY spectra of the EGCg and β-CD mixtures in pH 3.0 HCl solution (**a**) and pH 8.5 $NaHCO_3$ solution (**b**) at 308 K. In ROESY spectra, the symbols shown on the X and Y axes represent the protons of EGCg and β-CD molecules, respectively

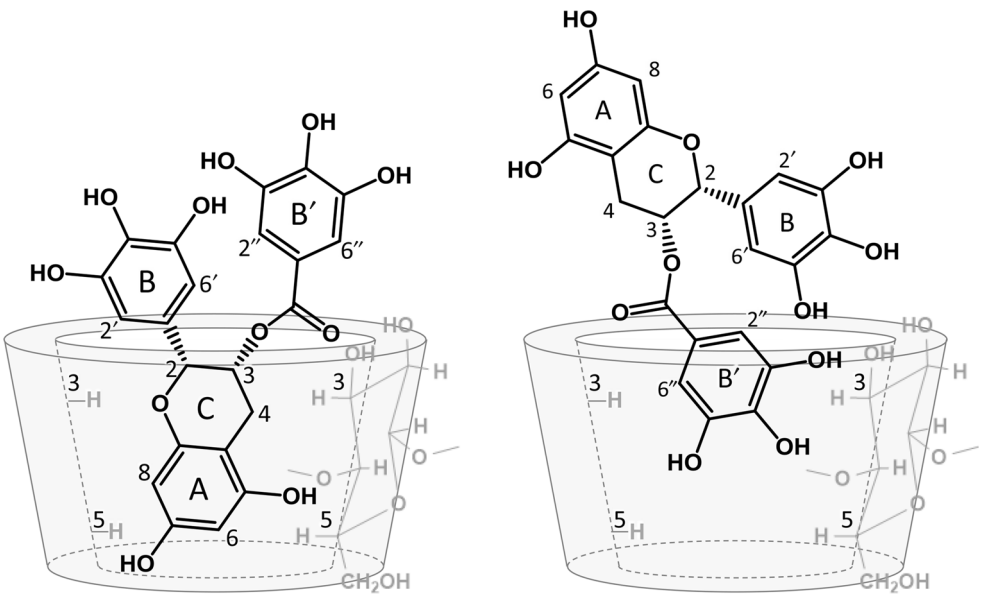

Fig. 4 Two expected types of inclusion complexes for EGCg-β-CD. Illustration of the donut shape represents β-CD

A-ring of EGCg and 5-H situated in a deep position of the β-CD cavity and between H-2′ (H-6′) on the B-ring and 3-H in a shallow position of the cavity were observed in both of the ROESY spectra at pH 3.0 and pH 8.5 (Fig. 3). The large negative values of ΔH_1 and ΔS_1 would reflect hydrogen bonding between an OH group on the A-ring of EGCg and an internal O atom of β-CD. On the other hand, the formation of the second type depended on the solution pH, because the B′-ring of EGCg was shallowly inserted in the β-CD cavity (right in Fig. 4). The cross-peak between H-2″ (H-6″) on the B′-ring of EGCg and 3-H of β-CD was confirmed in the ROESY spectrum only at pH 3.0. Decreases in n_2 and K_2 with the increase in pH indicated that complexation at a 1:1 molar ratio became difficult due to the ionization of the 4″-OH group on the B′-ring. Decreases in the negative values of ΔH_2 and ΔS_2 would indicate that the hydrogen bonding between an OH group on the B′-ring of EGCg and an internal O atom of β-CD disappeared as pH increased.

In this study, it was found that EGCg with some insertable sites formed two types of inclusion complexes with β-CD. The stoichiometry (n), association constant (K) and thermodynamic parameters (ΔG, ΔH and ΔS) could be calculated from the calorimetric titration curves. ITC might be indispensable to discuss the inclusion mechanism between cyclodextrin and compounds such as EGCg.

Conclusions

In the complexation of EGCg-β-CD, the AC-ring was easily inserted into the cavity of β-CD independently of pH to form the first type of the complex. The B′-ring of EGCg would be included by β-CD in the acid and neutral solutions to form the second type, but the formation of the complex in the basic solutions (≥ pH 8.0) would be difficult due to the ionization of the B′-ring.

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