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Effect of antioxidant activity of caffeic acid with cyclodextrins using ground mixture method

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ABSTRACT

In the current study, we prepared a ground mixture (GM) of caffeic acid (CA) with α -cyclodextrin (α CD) and with β -cyclodextrin (β CD), and then comparatively assessed the physicochemical properties and antioxidant capacities of these GMs. Phase solubility diagrams indicated that both CA/ α CD and CA/ β CD formed a complex at a molar ratio of 1/1. In addition, stability constants suggested that CA was more stable inside the cavity of α CD than inside the cavity of β CD. Results of powder X-ray diffraction (PXRD) indicated that the characteristic diffraction peaks of CA and CD disappeared and a halo pattern was produced by the GMs of CA/ α CD and CA/ β CD (molar ratios = 1/1). Dissolution testing revealed that both GMs had a higher rate of dissolution than CA alone did. Based on the ¹H-¹H NOESY NMR spectra for the GM of CA/ α CD, the vinylene group of the CA molecule appeared to be included from the wider to the narrower rim of the α CD ring. Based on spectra for the GM of CA/ β CD, the aromatic ring of the CA molecule appeared to be included from the wider to the narrower rim of the β CD ring. This suggests that the structures of the CA inclusion complexes differed between those involving α CD rings and those involving β CD rings. Results of a DPPH radical-scavenging activity test indicated that the GM of CA/ α CD had a higher antioxidant capacity than that of the GM of CA/ β CD. The differences in the antioxidant capacities of the GMs of CA/ α CD and CA/ β CD are presumably due to differences in stability constants and structures of the inclusion complexes.

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1. Introduction

In recent years, cardiovascular diseases have become the second leading cause of death in Japan, after cancer [1]. Therefore,

several health foods are being investigated to treat the risk factors of cardiovascular diseases, which include dyslipidemia and diabetes-induced atherosclerosis. Chlorogenic acid, a coffee polyphenol, is one such health food that has been reported to protect against lifestyle diseases, such as dyslipidemia and

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diabetes [2-4]. Caffeic acid (CA) is a decomposition product of chlorogenic acid that has the same physiological activity as chlorogenic acid does. Although CA has been reported to protect against dyslipidemia and blood glucose elevation owing to its antioxidant activity, its application is limited by poor water solubility and oral absorption [5,6]. Cyclodextrin (CD) is a ring-shaped molecule consisting of 6, 7, or 8 glucose units for α , β , or γ CD, respectively, which are linked by α -(1 \rightarrow 4)-glucosidic bonds. In the safety assessment of the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) Joint Expert Committee on Food Additives (JECFA), the acceptable daily intake (ADI) was set as "not specified" for α - and γ CD and "up to 5 mg/kg/day" for β CD. CD molecules have a hydrophobic cavity in their center that can incorporate various guest molecules to form inclusion complexes [7]. Inclusion within CD is known to stabilize pharmaceuticals [8], and improve their bioavailability by improving their solubility [9]. Various methods, such as coprecipitation [10], kneading [11], and lyophilization [12], have been reported for the preparation of inclusion complexes. Co-grinding, a solvent-free, mechanochemical method of forming inclusion complexes, causes changes in physicochemical properties because of the application of mechanical energy (i.e., by the grinding of the solid material), and has been used for the complexation of piperine, which is poorly water soluble [13,14]. The preparation of (R)- α -lipoic acid and CD inclusion complexes, which is affected by the cavity size of CD, has also been reported [15]. As the steric properties and antioxidant capacity of drugs are reported to be affected by the type of CD [16], it is useful to evaluate the physicochemical properties of inclusion complexes prepared using CDs of different cavity sizes. In addition, CDs have been reported to increase antioxidant capacity by their inclusion of polyphenols, such as chlorogenic acid [17]. However, there is no report of increased antioxidant capacity of CA using CDs. Fundamental research to evaluate the antioxidant capacity of inclusion complexes prepared using CDs of different cavity sizes to improve the antioxidant capacity of CA may have clinical applications. We have already reported the preparation of CA and γ CD inclusion complexes by co-grinding and demonstrated that the antioxidant capacity is retained owing to improvement in CA inclusion and elution [18]. Since α CD and β CD have a narrower cavity than γ CD does, they may form different inclusion complexes and thus affect the antioxidant capacity of drugs differently. Therefore, in this study, we prepared CA/CD inclusion complexes using α CD and β CD, and evaluated their physicochemical properties, dissolution properties, and antioxidant capacities.

2. Materials and methods

2.1. Materials

CA (Fig. 1A) was purchased from Wako Pure Chemical Industries Co. Ltd (Japan). α CD and β CD (Fig. 1B) were a generous gift from CycloChem Co. Ltd. (Tokyo, Japan) and were stored at 40 °C and 82% relative humidity for 7 days, prior to use. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich Co. Ltd. All other chemicals and solvents were of

analytical grade and were purchased from Wako Pure Chemical Industries Co. Ltd.

2.2. Preparation of the physical mixture (PM) and ground mixture (GM)

The PM was prepared by mixing CA and CD in different molar ratios (2/1, 1/1, and 1/2) using a vortex mixer for 1 min. The GM was prepared by placing the PM (1 g) in an alumina cell and grinding for 60 min using a vibration rod mill (TI-500ET, CMT Co. Ltd., Japan).

2.3. Methods

2.3.1. Phase solubility studies

Phase solubility studies were performed as previously described by Higuchi and Connors [19]. A supersaturated amount of CA (100 mg) was added to an aqueous solution (10 ml) of α CD (0-40 mM) or β CD (0-15 mM), and the mixture was placed in a medium-sized, shaking instrument for 24 h at 200 rpm and 25 ± 0.5 °C. After the suspension reached equilibrium, the solution was immediately filtered through a 0.45 μ m membrane filter. The solubility was quantified by high-performance liquid chromatography using a Waters® e2795 separations module (Nippon Waters Co., Ltd., Japan) and Cosmosil 5C₁₈-AR-II packed column (4.6 mm \times 150 mm) at a detection wavelength of 285 nm. The sample injection volume was 30 μ l, and the column temperature was 40 °C. The mobile phase consisted of distilled water/acetonitrile/methanol/acetic acid (862/113/20/5), and the CA retention time was 10 min. The apparent stability constant (Ks) of the CA/CD inclusion complexes was calculated from the slope of the phase solubility diagram and solubility of CA in the absence of CD (S₀) using Equation (1).

$$Ks = \frac{\text{slope}}{S_0(1 - \text{slope})} \quad (1)$$

2.3.2. Differential scanning calorimetry (DSC)

The thermal behavior of samples was recorded using a differential scanning calorimeter (Thermo plus EVO, Rigaku Co., Japan). All samples were weighed (2 mg) and heated at a scanning rate of 5.0 °C/min under nitrogen flow (60 ml/min). Aluminum pans and lids were used for all samples.

2.3.3. Powder X-ray diffraction (PXRD)

PXRD studies were performed using a powder X-ray diffractometer (MiniFlex II, Rigaku Co., Japan) with Cu K α radiation (30 kV, 15 mA). The diffraction intensity was measured using a NaI scintillation counter. The samples were scanned from $2\theta = 3-35^\circ$ at a scan rate of 4°/min. The powder sample was spread on a glass plate to maintain a flat sample plane, and measurements were performed.

2.3.4. Fourier transform infrared (FTIR) spectroscopy

The FTIR spectra of samples were recorded using an FTIR spectrometer (FT/IR-410, JASCO Inc., Japan) by the potassium bromide (KBr) disk method. The samples were scanned 32 times over a range of 650-4000 cm⁻¹ with a resolution of 4 cm⁻¹. The tablets were prepared by adding KBr to the sample

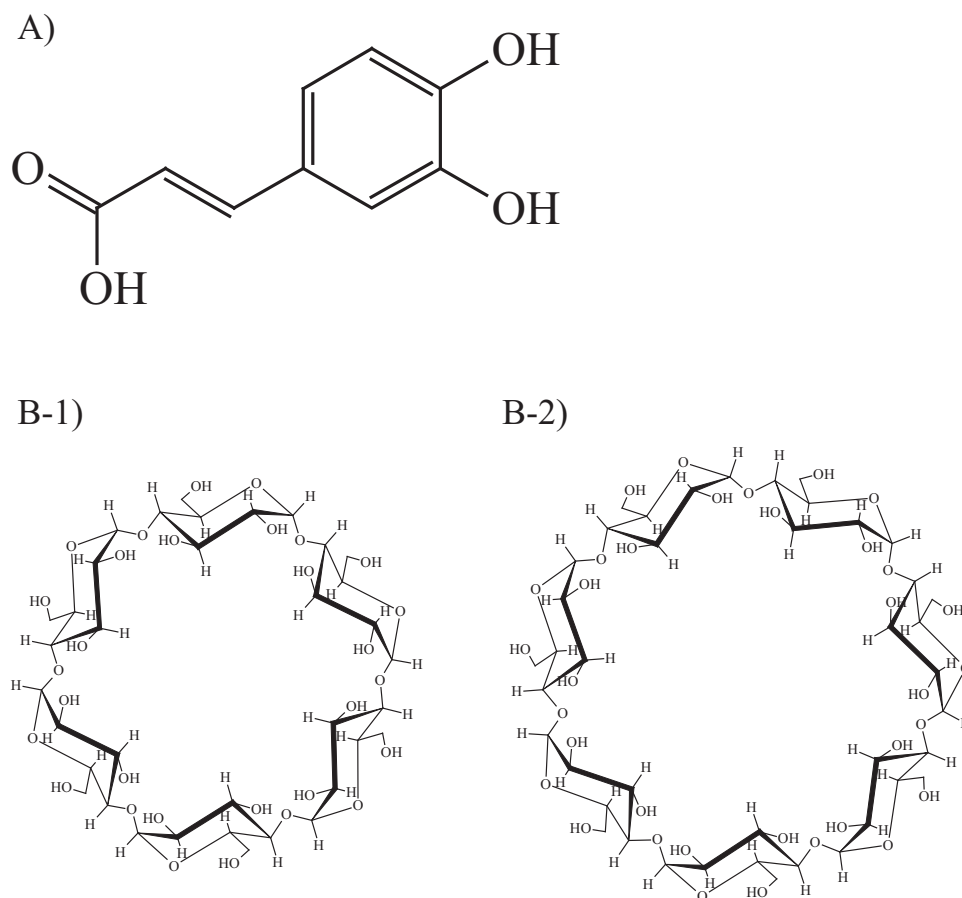


Fig. 1 – Chemical structure. (A) Caffeic acid (CA), (B-1) α Cyclodextrin (α CD), (B-2) β Cyclodextrin (β CD)

(sample/KBr = 1/10 (w/w)) and manually compressing the mixture. Background correction was performed using a blank KBr tablet.

2.3.5. Measurement of 1H-1H nuclear overhauser effect spectroscopy (NOESY) NMR spectra

The NOESY spectra were recorded on a 700 MHz Varian NMR spectrometer (Agilent Technologies) under the following conditions: solvent: D_2O ; resonant frequency: 699.6 MHz; pulse width: 90°; relaxation delay: 0.500 s; scan time: 0.500 s; temperature: 25 °C; 256 increments.

2.3.6. Dissolution profile

Dissolution testing of samples was performed using a dissolution apparatus (NTR-593, Toyama Sangyo Co. Ltd., Japan) with 900 ml of distilled water at 37 ± 0.5 °C and 50 rpm, according to the paddle method (Japanese Pharmacopoeia 17th Edition). CA (100 mg) was weighed accurately and loaded into the paddle apparatus. Dissolved samples (10 ml) were collected at 5, 10, 15, 30, 60, 90, and 120 min through 0.45 μ m membrane filters, with replacement of an equal volume of fresh dissolution medium at the same temperature, to maintain a constant volume of dissolution medium. The concentration of CA in the samples was determined by the same method as described for the phase solubility studies (Section 2.3.1).

2.3.7. DPPH radical scavenging activity

The DPPH radical scavenging activity of the samples was measured using a microplate reader (SpectraMax 190, Molecular Devices, Japan). Briefly, each sample and DPPH methanolic solution (100 μ M) were mixed in a microplate at a ratio of 1/1 (v/v) and incubated at 25 °C for 5 min. The absorbance of DPPH in samples (A_s) was measured at 517 nm. The absorbance of a mixture of methanol/water (1/1 (v/v)) (Br), which has a radical removal rate of 100%, and a mixture of DPPH/water (1/1 (v/v)) (A_0), which has a radical removal rate of 0%, was also measured. The radical scavenging rate was calculated using Equation (2) [17].

$$\text{Radical scavenging rate} = [1 - (A_s - Br)/(A_0 - Br)] \times 100$$

3. Results and discussion

3.1. Phase solubility studies

Phase solubility studies were conducted to assess the molar ratio of the CA/CD inclusion complexes and their stability constants in an aqueous solution. Results of the solubility testing indicated that the solubility of CA increased linearly for α CD and β CD, producing an A_L type of phase solubility diagram

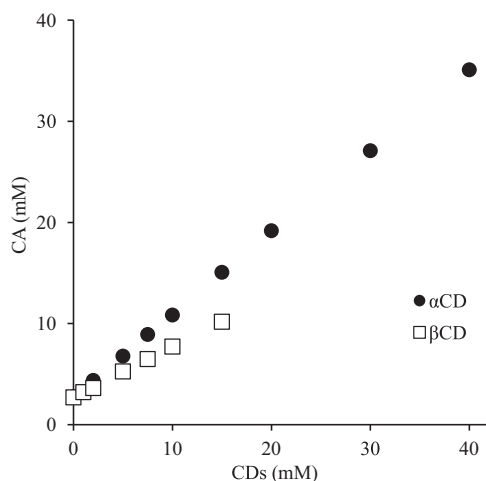


Fig. 2 – Phase solubility diagram of CA/CDs. Results are expressed as means \pm SD ($n = 3$).

Table 1 – Apparent stability constant (K_s) of CA inclusion complexes.

CA complex	$K_s \pm SD (M^{-1})$
α CD	1547.5 ± 30.9
β CD	371.4 ± 6.22
γ CD	391.2 ± 25.8

according to the classification system of Higuchi and Connors [19], (Fig. 2). Typically, an A_L diagram indicates that a complex is formed at a molar ratio of 1/1 [20], so both CA/ α CD and CA/ β CD formed a complex at a molar ratio of 1/1 in solution. The stability constant (K_s) was determined next. K_s was $1547.5 M^{-1}$ for α CD and $371.4 M^{-1}$ for β CD (Table 1). A study by Iacovino et al. reported that, when a complex has a stability constant exceeding $1000 M^{-1}$, the guest molecule and CD are unlikely to dissociate [21]. CA included in α CD had a stability constant of $1547.5 M^{-1}$, so CA was more stable inside the cavity of α CD than it was inside the cavity of β CD.

3.2. Differential scanning calorimetry (DSC)

Phase solubility diagrams suggested that both CA/ α CD and CA/ β CD formed a complex at a molar ratio of 1/1 in an aqueous solution. When CA/ α CD and CA/ β CD are ground together, they presumably still form an inclusion complex at a molar ratio of 1/1. It has been reported that when a guest molecule does not melt or its peak shifts as a result of inclusion complex formation caused by co-grinding, changes in its thermal behavior become evident [22]. Thus, DSC was performed in the current study to examine the thermal behavior of the GMs. Results of DSC revealed an endothermic peak due to melting at $223^\circ C$ for intact CA (Fig. 3A). The PM of CA/ α CD (molar ratio = 1/1) had an endothermic peak due to the melting of CA at $210^\circ C$, and the PM of CA/ β CD (molar ratio = 1/1) had an endothermic peak due to the melting of CA at $216^\circ C$, so CA crystals were presumably present (Fig. 3B and F). The GM of CA/ α CD (molar ratio = 2/1) had an endothermic peak due to CA at $207^\circ C$ (Fig. 3C). The endothermic peak due to CA disappeared for the

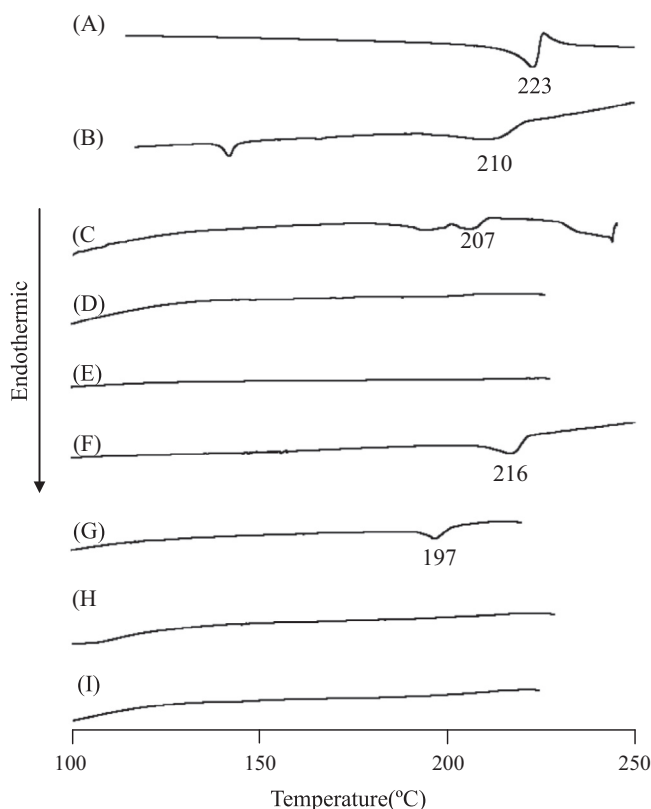


Fig. 3 – DSC curves of CA/CD systems. (A) CA intact, (B) PM (CA/ α CD = 1/1), (C) GM (CA/ α CD = 2/1), (D) GM (CA/ α CD = 1/1), (E) GM (CA/ α CD = 1/2), (F) PM (CA/ β CD = 1/1), (G) GM (CA/ β CD = 2/1), (H) GM (CA/ β CD = 1/1), (I) GM (CA/ β CD = 1/2)

GM of CA/ α CD at molar ratios of 1/1 and 1/2 (Fig. 3D and E). The GM of CA/ β CD (molar ratio = 2/1) had an endothermic peak due to CA at $197^\circ C$ (Fig. 3G). The endothermic peak due to CA disappeared for the GM of CA/ α CD at molar ratios of 1/1 and 1/2 (Fig. 3H and I). In our previous study, the disappearance of the CA melting peak was confirmed by DSC measurement for the GM of the CA/ γ CD complex [18]. Therefore, the GMs of CA/ α CD and CA/ β CD are also considered inclusion complexes. The endothermic peak due to CA disappeared because the CA and α CD molecules and the CA and β CD molecules formed inclusion complexes. The GM of CA/ α CD (molar ratio = 2/1) and the GM of CA/ β CD (molar ratio = 2/1) showed an endothermic peak due to CA. The reason may be that there was excess CA that did not complex with the CDs. This suggests that both CA/ α CD and CA/ β CD were present in the GMs at a molar ratio of CA/CD of 1/1.

3.3. Powder X-ray diffraction (PXRD)

The results of DSC suggested that CA could form an inclusion complex with either α CD or β CD at a stoichiometric ratio of 1/1. Thus, PXRD was performed to examine the crystalline state of the CA/CDs in the GMs. Intact CA and ground CA had a characteristic diffraction peak at $2\theta = 26.9^\circ$, α CD had a characteristic diffraction peak at $2\theta = 11.8^\circ$, and β CD had a characteristic diffraction peak at $2\theta = 12.3^\circ$ (Fig. 4A, C and I). The PM of CA/ α CD (molar ratio = 1/1) and PM of CA/ β CD (molar

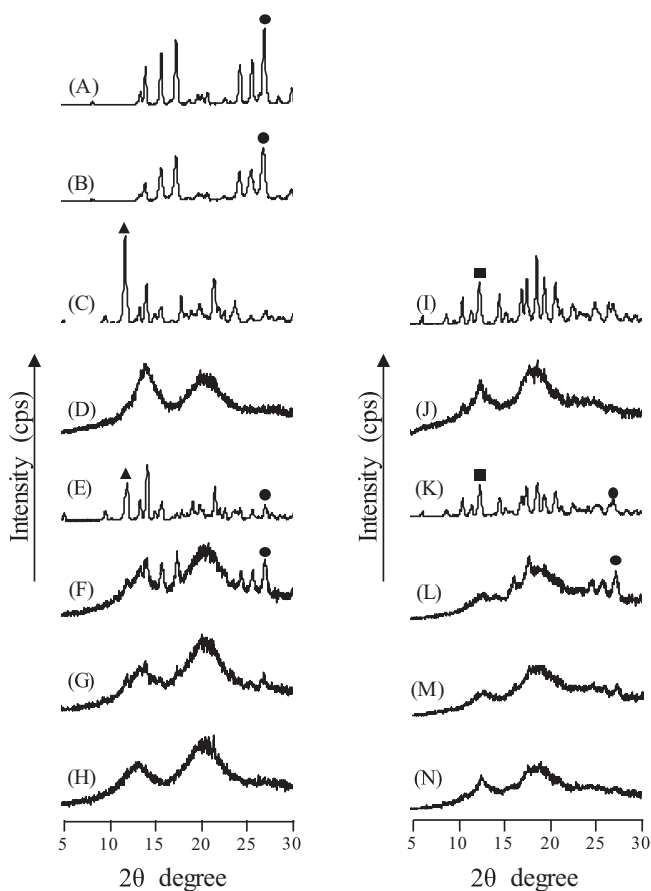


Fig. 4 – PXRD patterns of CA/CDs. (A) CA intact, (B) CA ground, (C) α CD, (D) α CD ground, (E) PM (CA/ α CD = 1/1), (F) GM (CA/ α CD = 2/1), (G) GM (CA/ α CD = 1/1), (H) GM (CA/ α CD = 1/2), (I) β CD, (J) β CD ground, (K) PM (CA/ β CD = 1/1), (L) GM (CA/ β CD = 2/1), (M) GM (CA/ β CD = 1/1), (N) GM (CA/ β CD = 1/2)

ratio = 1/1) had diffraction peaks due to CA at $2\theta = 26.9^\circ$, α CD at $2\theta = 12.1^\circ$, and β CD at $2\theta = 12.3^\circ$ (Fig. 4E and K). The GM of CA/ α CD (molar ratio = 2/1) had a diffraction peak due to CA at $2\theta = 26.9^\circ$, but the peak disappeared and a halo pattern was produced with the GM of CA/ α CD at molar ratios of both 1/1 and 1/2 (Fig. 4F, G and H). The GM of CA/ β CD (molar ratio = 2/1) had a diffraction peak due to CA at $2\theta = 26.9^\circ$, but the peak disappeared and a halo pattern was produced by the GM of CA/ β CD at molar ratios of both 1/1 and 1/2 (Fig. 4L, M and N). A halo pattern has been confirmed in the GM of the CA/ γ CD complex [18]. The GM of CA/ α CD and CA/ β CD also has similar patterns, which suggests that they form inclusion complexes. Co-grinding disrupts the structures of crystals, and these amorphous solids can produce a halo pattern [23]. A previous study has reported that supplying mechanical energy by grinding and friction can result in the formation of amorphous inclusion complexes [14]. The GMs of CA/ α CD and CA/ β CD (both molar ratios of 2/1) had an endothermic peak due to CA. The reason may be that excess CA exists outside the CD complexes. This suggests that both CA/ α CD and CA/ β CD in a GM (molar ratio = 1/1) form an inclusion complex.

3.4. Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy was performed in order to examine the molecular state of the complexes in a solid state. The intact CA had a peak due to the OH groups of its aromatic ring at 3433 cm^{-1} . A peak due to the carbonyl group (C = O) was produced at 1644 cm^{-1} , and a peak due to the aromatic ring (C = C) was produced at 1622 cm^{-1} (Fig. 5A). In the PM of CA/ α CD (molar ratio = 1/1) and the PM of CA/ β CD (molar ratio = 1/1), the OH groups from the aromatic ring in the CA molecule produced a peak at 3433 cm^{-1} , the carbonyl group (C = O) produced a peak at 1644 cm^{-1} , and the aromatic ring (C = C) produced a peak at 1622 cm^{-1} (Fig. 5D and E). In the GM of CA/ α CD (molar ratio = 1/1) and the GM of CA/ β CD (molar ratio = 1/1), the O-H stretching band attributed to the hydroxyl groups of the aromatic ring in the CA molecules were still observed at the same position as with the CA crystals. However, the peaks assigned to aromatic ring (C-C) and the carbonyl group of CA were significantly shifted to 1622 and 1685 cm^{-1} , respectively. The results suggest a molecular interaction between the CA and CD molecules (Fig. 5F and G). Shifts in the CA aromatic rings and carbonyl group peaks have already been confirmed in the GM of CA/ γ CD complexes [18]. The GMs of CA/ α CD and CA/ β CD have similar peak shifts, which suggest that they also form inclusion complexes. CA is known to form a dimeric structure as a result of intermolecular hydrogen bonding between the

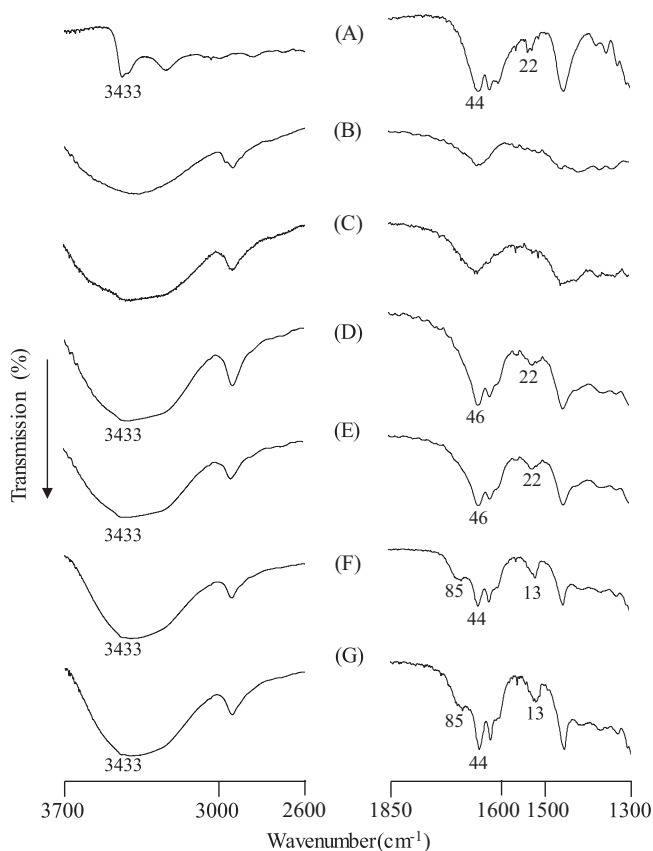


Fig. 5 – FTIR spectra of CA/CD systems. (A) CA intact, (B) α CD, (C) β CD, (D) PM (CA/ α CD = 1/1), (E) PM (CA/ β CD = 1/1), (F) GM (CA/ α CD = 1/1), (G) GM (CA/ β CD = 1/1)

carbonyl group (C = O) of one carboxylic acid and the hydroxyl group (-OH) of another [24]. Higashi et al. reported that complex formation by molecules with this dimeric structure causes hydrogen bonds between molecules to dissociate, resulting in an upward shift in the peak due to carbonyl groups (C = O) [25]. Thus, molecular interaction is presumably occurring between CA and α CD and between CA and β CD in the GMs (both molar ratios of 1/1) in the solid state. Since the peak corresponding to the hydroxyl groups (-OH) of the aromatic ring of CA at 3433 cm^{-1} did not change, the CD cavity and OH groups of CA presumably do not interact in the GM of CA/ α CD or in the GM of CA/ β CD (both molar ratios of 1/1).

3.5. Dissolution profile

The results of the DSC, PXRD, and FTIR spectra suggest that CA and α CD as well as CA and β CD in the GMs (both at molar ratios of 1/1) form an inclusion complex in the solid state. Thus, dissolution testing was performed next to ascertain changes in the dissolution behavior of CA as a result of the formation of the inclusion complexes. Test samples were intact CA, ground CA, PM of CA/ α CD, PM of CA/ β CD, GM of CA/ α CD, and GM of CA/ β CD (all mixtures at molar ratios of 1/1). The dissolution rate of CA after 5 min was 24% for intact CA and 23% for ground CA, so both had a slow rate of dissolution (Fig. 6). The dissolution rate of CA after 5 min was 37% for the PM of CA/ α CD and 59% for the PM of CA/ β CD, so the PMs showed a higher rate of dissolution than that of the intact CA. The dissolution rate of CA after 5 min was 100% for both the GM of CA/ α CD and the GM of CA/ β CD, so the GMs demonstrated improved dissolution in comparison to that of intact CA and the PMs. It has been reported that disrupting the molecular arrangement of a drug by rendering it amorphous and inclusion complex formation were possible mechanisms of enhanced dissolution

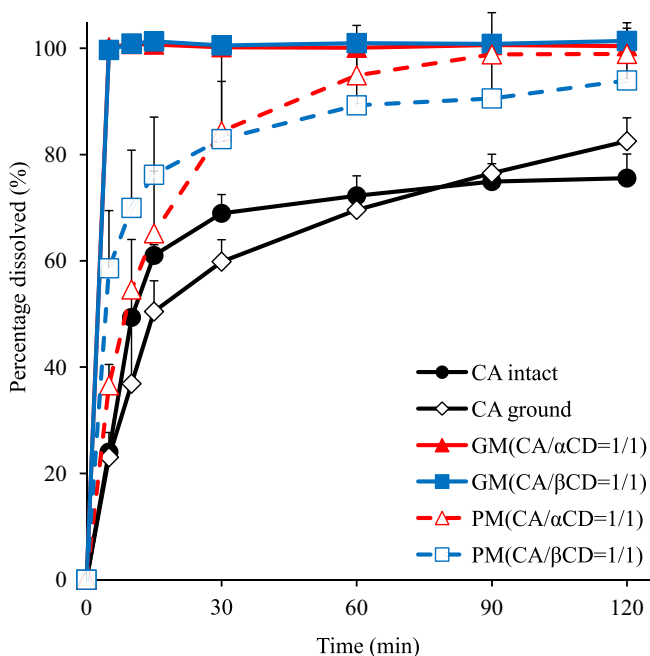


Fig. 6 – Dissolution profiles of CA/CD systems. Results are expressed as means \pm SD ($n = 3$).

[26,27]. The results of the PXRD revealed that CA was amorphous, and they indicated inclusion complex formation (Fig. 2). The FTIR spectra revealed the interaction between the aromatic ring of CA (which is a hydrophobic group) and the CDs. Thus, multiple factors, such as CA in an amorphous form and inclusion complex formation, help improve the elution of CA in CA/CDs. The improved dissolution in the PM of CA/ α CD and in the PM of CA/ β CD (both molar ratios of 1/1) was presumably due to the formation of inclusion complexes in an aqueous solution. In addition, improvement of dissolution was confirmed in the GM of the CA/ γ CD complex [18]. Similarly, the GM of CA/ α CD and the GM of CA/ β CD have improved solubility. It is likely that the formation of inclusion complexes contributes to the dissolution property.

3.6. Measurement of ^1H - ^1H nuclear Overhauser effect spectroscopy (NOESY) NMR spectra

The results of the dissolution tests revealed that the GMs had an improved rate of dissolution compared to that of intact CA. The molecular interaction between CA and α CD and between CA and β CD (both molar ratios of 1/1) in the GMs in solution may affect their solubility. ^1H - ^1H NOESY NMR spectroscopy was performed to examine these molecular interactions in detail. ^1H - ^1H NOESY NMR spectroscopy can reveal spatial interactions between a guest molecule and the CD cavity, so it is used to predict the positioning of the guest molecule within the inclusion complex [28]. In the GM of CA/ α CD (molar ratio = 1/1), the H-3 proton (3.66 ppm) in the CD cavity, the H-a proton (6.08, 6.06 ppm) and H-b proton (7.38, 7.40 ppm) of the vinylene group of CA, and the H-c proton (6.98, 6.99 ppm) and H-e proton (6.96 ppm) of the aromatic ring produced cross peaks (Fig. 7A). The H-a and H-b protons of the vinylene group of CA and the H-3 proton in the cavity of α CD in particular produced intense cross peaks. Typically, the H-3 proton is found at the wider rim of the ring of CD and the H-6 proton is found at the narrower rim of the ring of CD [28]. The appearance of cross peaks indicates that the distance between protons is less than 4 \AA , and a more intense peak indicates a smaller distance [29]. Thus, the vinylene group of the CA molecule appears to be oriented from the wider to the narrower rim of the α CD ring (Fig. 8A). In the GM of CA/ β CD (molar ratio = 1/1), cross peaks were produced by H-3 (3.67 ppm), H-5 (3.53, 3.54 ppm), and H-6 (3.60 ppm) in the cavity, by H-a (6.02, 6.05 ppm) and H-b (7.31, 7.33 ppm) in the vinylene group of CA, and by H-c (6.80, 6.81 ppm), H-d (6.68, 6.69 ppm), and H-e (6.88 ppm) in the aromatic ring (Fig. 7B). In the GM of CA/ β CD, intense cross peaks were produced by H-b in the vinylene group of CA, by H-e in the aromatic ring, and by H-5 in the cavity of β CD. No cross peaks were produced by H-c or H-d in the benzene ring or by H-3 in the cavity of β CD. Thus, the aromatic ring of the CA molecule appears to be oriented from the wider to the narrower rim of the ring of β CD (Fig. 8B). The results of the FTIR revealing an interaction between the aromatic ring and vinylene group of the CA and the CDs has been confirmed. This result suggests that the inclusion complex of the GM CA/CDs also involves interactions of the aromatic ring and vinylene group of CA similar to that observed in aqueous solution. In the GM of CA/ α CD (molar ratio = 1/1), the aromatic ring was partially included, while the aromatic ring was completely included in the GM

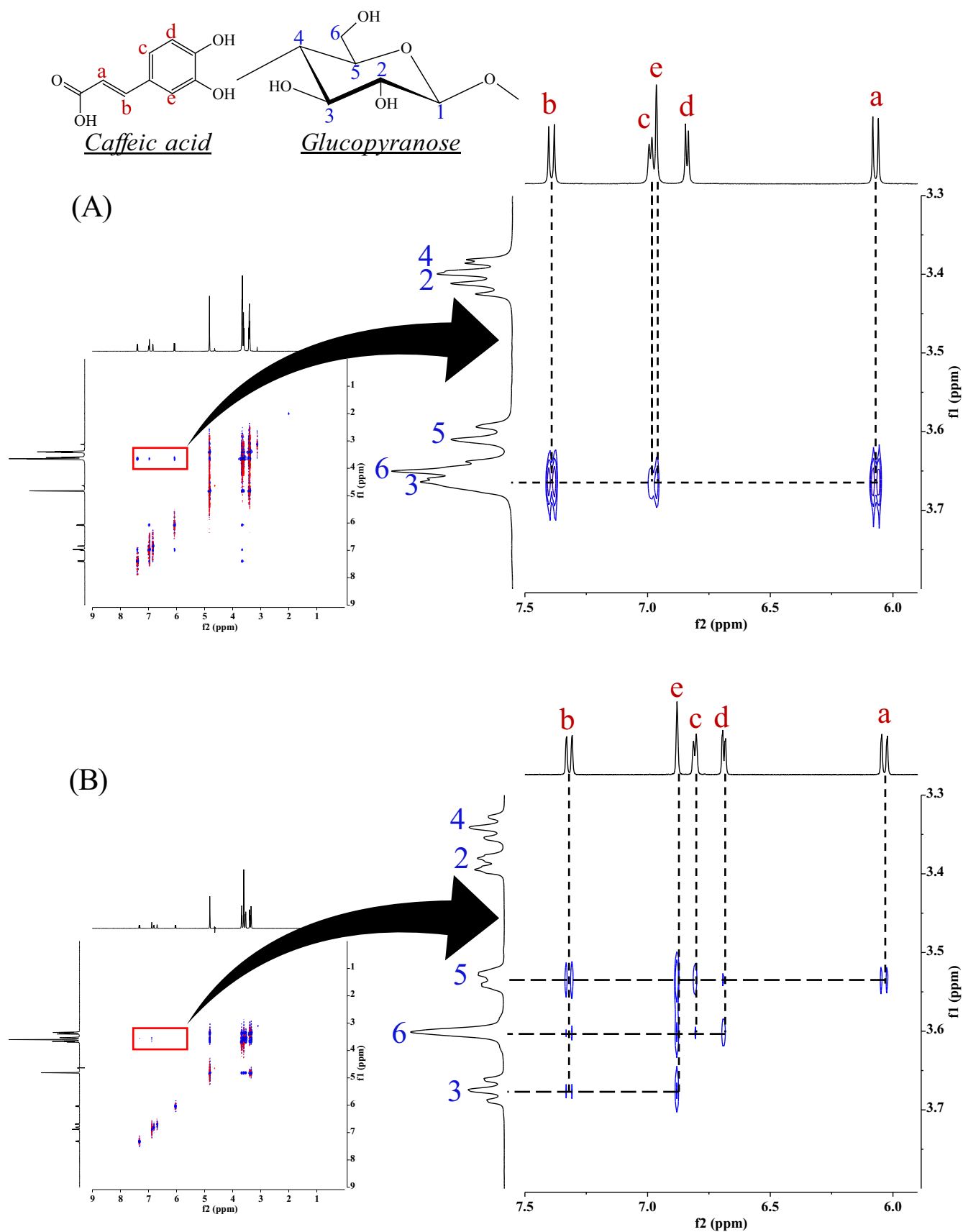


Fig. 7 – ^1H - ^1H NOESY NMR spectra of CA/CD systems. (A) GM (CA/ α CD = 1/1), (B) GM (CA/ β CD = 1/1)

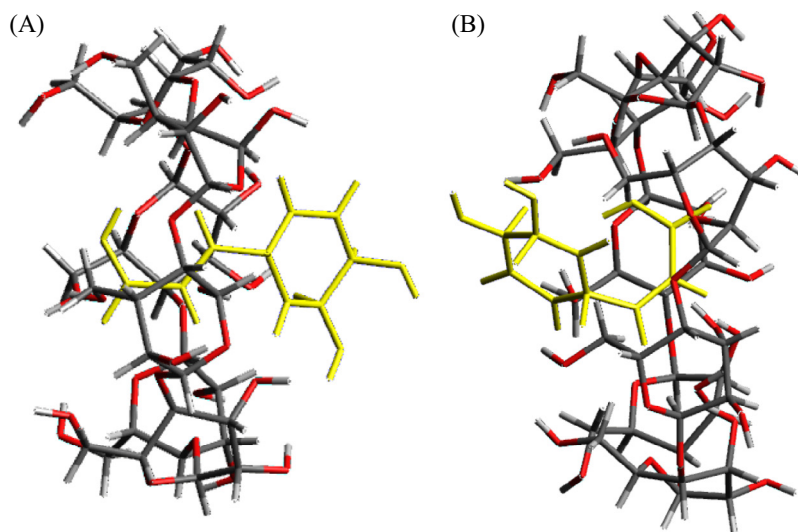


Fig. 8 – Proposed structural images of CA/CDs complex. (A) side view of GM (CA/αCD), (B) side view of GM (CA/βCD)

of CA/βCD (molar ratio = 1/1). In the CA/γCD complex, there was a strong cross peak between the proton derived from the aromatic ring of the CA molecule and the narrow edge of γCD [18]. Thus, the aromatic ring of the CA molecule appears to be oriented from the wider to the narrower rim of the ring of γCD. This result suggests that the GM of CA/αCD has a different inclusion form compared to that of CA/βCD and CA/γCD.

3.7. DPPH radical scavenging test

It has been reported that inclusion complex formation increases antioxidant capacity [17]. The presence of a guest molecule in a CD molecule results in increased electron density, resulting in a greater likelihood that the guest molecule will release protons as radicals to quench the DPPH radical. Thus, a DPPH radical-scavenging activity test was conducted to assess antioxidant capacity in the GM of CA/αCD (molar ratio = 1/1) and the GM of CA/βCD (molar ratio = 1/1). The results of this test indicate that the 50% inhibitory concentration (IC_{50}) of DPPH radical scavenging was 2.57 μg/ml for intact CA, 1.42 μg/ml for the GM of CA/αCD, and 1.77 μg/ml for the GM of CA/βCD (Fig. 9). The IC_{50} was significantly lower for the GMs of CA/αCD and CA/βCD (both molar ratios of 1/1) compared to that of intact CA, and increased antioxidant capacity was noted. The GM of CA/αCD (molar ratio = 1/1) tended to have higher antioxidant capacity. This might be explained by the stability constant in aqueous solution affecting the antioxidant capacity; the results of solubility testing indicated that CA/αCD had a higher stability constant than CA/βCD did [30]. 1H - 1H NOESY NMR spectra revealed that the form of the inclusion complex in the GM of CA/αCD (molar ratio = 1/1) was different from that of the GM of CA/βCD (molar ratio = 1/1) (Fig. 7). Additionally, βCD has a more hydrophobic cavity than αCD does [31]. Thus, the form of the CA inclusion in the GM of CA/βCD involves capture of the aromatic ring. In the GM of CA/αCD, the inclusion primarily involves the vinylene group and not the aromatic ring. The antioxidant capacity of CA is due to the hydroxyl groups attached to the aromatic ring, suggesting that the differences in

the orientation of the CA within the CD affected the antioxidant capacity [32]. The GM of the CA/γCD complex did not improve antioxidant capacity [18]. The GM of the CA/γCD complex has a lower stability constant than the GM of the CA/αCD complex has, and the CA of the latter is complexed in a manner that captures the aromatic rings. We believe that these two reasons explain the improved antioxidant capacity of the GM of CA/αCD.

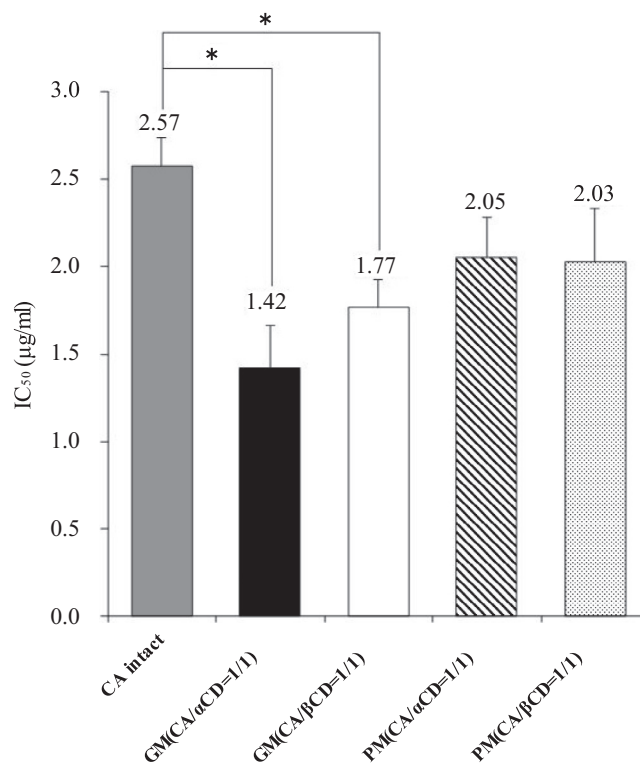


Fig. 9 – IC_{50} of DPPH radical scavenging test of CA/CD systems. Results are expressed as means \pm SD (n = 3) * P < 0.05 vs CA (Tukey's test).

4. Conclusion

In the current study, phase solubility diagrams indicated that both CA/ α CD and CA/ β CD form a complex at a molar ratio of 1/1. In addition, the results revealed that CA was accommodated more stably inside the cavity of α CD than inside the cavity of β CD.

The results of the DSC, PXRD, FTIR, and dissolution tests revealed that both CA/ α CD and CA/ β CD could form a 1/1 inclusion complex with an enhanced dissolution rate through the co-grinding method. ^1H - ^1H NOESY NMR spectra revealed that the orientation of the guest molecule inclusion differed depending on the form of CD. The results of a test of DPPH radical-scavenging activity revealed that differences in stability constants and forms of inclusion affected the antioxidant capacity of CA. These findings indicate that the complexes of CA/ α CD and CA/ β CD have improved solubility and improved antioxidant capacity, suggesting that CA may warrant increased use in functional foods.

Conflict of interests

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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