Original paper

NMR Spectroscopic Characterization of Inclusion Complexes of Theaflavin Digallate and Cyclodextrins

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Cyclodextrins (CDs) are a family of cyclic oligosaccharides which possess the unique ability to include a number of compounds inside their cavities. CDs have been used in tea to mask the bitter taste caused by catechins. In this study we investigated the complexes formed between theaflavin-3,3'-di-O-gallate (TFDg), a major constituent in black tea, and CDs (α -, β -, and γ -CD) using several NMR techniques and a computational simulation. ¹H NMR spectral titration results and DOSY analyses suggested that TFDg interact with each CD. NMR experiments suggested that β -CD binds TFDg more tightly than α - or γ -CD. Additionally, a model for the complex formed between TFDg and β -CD was estimated by NOE measurements. The analysis showed that the galloyl moiety of TFDg was present in the β -CD cavity; this result was supported by computational simulation. The present study characterized the inclusion complex of TFDg and β -CD.

Keywords: theaflavin-3,3'-di-O-gallate (TFDg), cyclodextrin (CD), NMR, DOSY, NOESY, computational simulation

Introduction

Theaflavins (TFs), a family of red pigments in black tea, are produced by the oxidation and dimerization of catechins during the preparation of black tea or oolong tea from tea leaves. TFs are the major polyphenolic components in black tea. The most abundant TFs in black tea are theaflavin (TF), theaflavin-3-O-gallate (TF-3-g), theaflavin-3'-O-gallate (TF-3'-g) and theaflavin-3,3'-di-O-gallate (TFDg) (Figure 1). The dry weight of TF, TF-3-g, TF-3'-g and TFDg in black tea are 0.08, 0.3, 0.2 and 0.4%, respectively (Davies *et al.*, 1999; Hara *et al.*, 1987; Shiraki *et al.*, 1994; Tanaka *et al.*, 2001).

It has been reported that TFs show health benefits such as antioxidant effects (Shiraki *et al.*, 1994), antimutagenic effects (Shiraki *et al.*, 1994), anti-inflammation effects (Lin *et al.*, 1999), cancer prevention (Leone *et al.*, 2003), reduction of blood cholesterol levels (Ikeda *et al.*, 2010) and anti-hyperglycemic effects (Matsui *et al.*, 2007). However, the distinctive bitter taste,

insolubility in water, and easy oxidation of tea polyphenols such as catechins and/or TFs are problematic for their use as food and/or beverage additives. In the case of green tea catechins, these problems have been resolved by the addition of a cyclodextrin (Gaudette and Pickering, 2012).

Cyclodextrins (CDs) are cyclic oligosaccharides. Typical CDs are composed of six, seven, or eight D-(+)-glucopyranose units connected by α -1,4-glycosidic linkages and are known as α -, β -, and γ -CDs, respectively (Eastburn and Tao, 1994). CDs are synthesized from starch in a reaction catalyzed by cyclomaltodextrin glucanotransferase (Kobayashi *et al.*, 1979). In general, CDs can interact with guest molecules bound in their hydrophobic cavities depending on cavity size. The formation of a CD-guest complex can alter the physical, chemical, and biological properties of the guest molecule, resulting in a guest compound with new properties (Wong and Yueu, 2003; Dell, 2004).

Inclusion complexes of catechins and CDs have been studied by

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	R ₁	R ₂
TF	H	H
TF-3-g	galloyl	H
TF-3'-g	H	galloyl
TFDg	galloyl	galloyl

Fig. 1. Structures of the four major theaflavins (TFs) found in black tea leaves, and of β -cyclodextrin

of EGCg, which has been reported to interact well with β -CD. Furthermore, TFDg is a major polyphenol and bioactive constituent of black tea (Li *et al.*, 2013).

In the present study, NMR techniques and computational simulation were used to study and find the optimum ratio between TFDg and CDs, and to reveal the binding mechanism.

Materials and Methods

NMR Experiments ^1H NMR (400 MHz) and 2D NMR spectra were recorded at 300 K on a Bruker BioSpin AVANCE III (Rheinstetten, Germany). The chemical shift values are reported in ppm (δ). Deuterium oxide (D_2O) was used as a solvent (99.9 atom% D, Acros Organics, Geel, Belgium). Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DDS, Wako Pure Chemical Industries Ltd., Osaka, Japan) was used as an internal standard. Diffusion ordered spectroscopy (DOSY) spectra were acquired using the following conditions: pulse delay time, 4.0 s; diffusion time, 100 ms; gradient pulse, 1.5 ms; 8 scans; 32×16384 data points. The data were processed with a sine-bell and an exponential multiplication window function. Nuclear Overhauser effect spectroscopy (NOESY) spectra were acquired using the following conditions: pulse delay time, 2.0 s; mixing time, 500 ms; 8 scans; 1024×1024 data points. The data were processed with a sine-bell squared window function.

Chemicals Cyclodextrins (α -, β -, and γ -CDs) were supplied by Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Theaflavin-3,3'-di-*O*-gallate (TFDg) was purified from black tea leaves by repeated column chromatography. The purity of the compound was >99% as determined from its ^1H NMR spectrum.

Stoichiometric ratio between TFDg and CDs A stock solution of TFDg (2.0 mM) and each CD (2.0 mM) in D_2O were prepared separately. Mixtures were prepared in an NMR tube using 600 μL of TFDg and CDs in the following ratios: $[\text{TFDg}] / ([\text{TFDg}] + [\text{CD}]) = 0.0, 0.25, 0.4, 0.5, 0.55, 0.65, 0.75, 0.9, 1.0$, where $[\text{TFDg}]$ and $[\text{CD}]$ represent the initial concentration of the TFDg and the CD, respectively.

Binding constant between TFDg and CDs A stock solution of each CD (10.0 mM) in D_2O was prepared separately. For each TFDg / CDs system, NMR tubes were filled with a different concentration of each CD (0.1, 0.3, 0.5, 0.7, 1.0, 1.5, 2.0, 3.0, 5.0, 7.0, 10.0 mM) and a constant concentration of TFDg (1.0 mM) in a final volume of 600 μL .

DOSY experiment A stock solution of TFDg (2.0 mM) and each CD (2.0 mM) in D_2O were separately prepared. For the TFDg / CD system, an NMR tube was filled with a constant concentration of TFDg (1 mM) and each CD (1 mM) in a final volume of 600 μL .

NOESY experiment A sample solution in D_2O (600 μL) containing TFDg (10 mM) and β -CD (10 mM) was prepared. For the TFDg / CD system, an NMR tube was filled with a constant concentration of TFDg (10 mM) and each CD (10 mM) in a final

NMR techniques and theoretical approaches (Ishizu *et al.*, 2006, 2008, 2009, and 2011). NMR experiments showed that (–)-epigallocatechin gallate (EGCg), a green tea catechin, forms a 1:1 complex with β -CD. The inclusion sites of EGCg and β -CD were determined to be the aromatic A-ring and a part of the heterocyclic C-ring. In addition, the complexes and the structure formed between β -CD and other catechin-gallates have been investigated (Ishizu *et al.*, 2008). Therefore, our focus has been directed at the interaction between CDs and the theaflavins of black tea, because theaflavins are dimeric catechins. In this study, we specifically focused on TFDg in black tea because it is a dimeric catechin

Fig. 2. Job plots for the inclusion complex of TFDg and each CD. The values on the x-axis correspond to the mole ratio $X = [\text{TFDg}] / ([\text{TFDg}] + [\text{CD}])$ at a total concentration $[\text{TFDg}] + [\text{CD}] = 2.0 \text{ mM}$, and those on the y-axis correspond to $\Delta\delta$ of the H-G2'/G6' proton signal of TFDg in the ^1H NMR spectrum in D_2O at 300 K multiplied by the mole ratio X . $\Delta\delta$ is the difference between the observed ^1H NMR chemical shift of TFDg with and without CD.

○(A): TFDg and α -CD, ●(B): TFDg and β -CD, △(C): TFDg and γ -CD

from the chemical shift at H-G2'/G6' (δ_{H} 6.44 in free) and estimated by the maximum variation range near the minimum value using the Job plot method (Job, 1928). Figures 2A-2C show the Job plot curves of α -, β -, and γ -CD, respectively. These results indicate that the stoichiometry of TFDg complexed with α - and γ -CD is expected to be 1:1, because each maximum variation range is around 0.5 (Figs 2A and 2C). In contrast, the maximum variation range with β -CD was between 0.5 – 0.65 (Fig. 2B), indicating that the stoichiometry of TFDg complexed with β -CD is 1:1 and/or 1:2.

Calculation of binding constants between TFDg and CDs ^1H NMR titration was used to estimate the binding constant between TFDg and each CD. Figure 3 shows the titration curve for each CD. The binding constant of TFDg for each CD was calculated according to eq. 1; the constants are shown in Table 1. The binding constant of TFDg to β -CD ($K_a = 2520 \text{ M}^{-1}$) is larger than that to α -CD ($K_a = 172 \text{ M}^{-1}$) and γ -CD ($K_a = 30.3 \text{ M}^{-1}$), indicating that the interaction between TFDg and β -CD is the strongest amongst the three CDs. In addition, the Gibbs energy change (ΔG) for the CDs was calculated from eq. 2; the results are shown in Table 1. The ΔG of each CD decreases upon interaction with TFDg. The results show that TFDg interacts with each CD, and that TFDg tends to interact most strongly with β -CD.

$$\Delta\delta_{\text{obs}} = \frac{\Delta\delta_{1:1}}{2} \left\{ 1 + K_a [H] + K_a [G] - \sqrt{(1 + K_a [H] + K_a [G])^2 - 4K_a^2 [H][G]} \right\} \quad \text{.....Eq. 1}$$

$$K_a = \exp \left(\frac{-\Delta G}{RT} \right) \quad \text{.....Eq. 2}$$

volume of 600 μL .

Computational simulation Initial coordinates of β -CD were extracted from the X-ray structure of AMP-activated protein kinase subunit β with bound β -CD (Protein Data Bank entry code: 1Z0N). Assigning bond orders, adding hydrogen atoms, and constraint energy minimization of the structure of β -CD were performed using the OPLS2005 force field in MacroModel (Schrödinger LLC., Portland, OR). A three dimensional model of TFDg was constructed and minimized using the Molecular Builder module and MacroModel in Maestro (Schrödinger LLC.). Docking of TFDg into the β -CD was carried out using Glide SP mode, with the grid center defined as the geometrical center of the β -CD molecule (Schrödinger LLC.). The 100 predicted best-binding poses of TFDg were written to output files.

Results

Determination of stoichiometric ratios between TFDg and CDs Stoichiometric ratios between TFDg and each CD were determined

Diffusion constants between TFDg and CDs by DOSY spectrum
Diffusion-ordered NMR spectroscopy (DOSY) is a method where the NMR signals of different species separate according to their diffusion coefficient. DOSY experiments have been used to monitor the interaction between proteins and low molecular weight

reveal the interaction between different compounds when the intrinsic diffusion constant of the compound is altered due to interactions in solution. If two compounds interact closely with each other, the diffusion constant obtained by a DOSY spectrum should approximate the diffusion constant of one of the compounds (Sughir *et al.*, 2010).

Figure 4 shows the DOSY spectrum of a mixture of TFDg and β -CD in D_2O , and Table 2 shows the diffusion coefficients of TFDg and each CD as calculated from the DOSY experiments. The diffusion constants of TFDg mixed with each CD shifted towards the diffusion constant of each CD. Among the three CDs, the diffusion constant of TFDg complexed with β -CD shifted the most towards that of the CD (β -CD), implying that the complex between TFDg and β -CD acts as one molecule due to their interaction with each other.

Estimation of interaction sites of TFDg and CDs by NOESY experiments
Nuclear Overhauser effect spectroscopy (NOESY) spectra can provide information about spatial protons. A NOESY experiment was conducted to determine the specific sites of the interaction between TFDg and β -CD. β -CD was used in this experiment because the DOSY results showed that TFDg interacts more with β -CD than with α - or γ -CD.

Fig. 3. 1H NMR titration curves for TFDg and the CDs
The shift in the 1H NMR signals in D_2O of H-G2'/G6' of TFDg upon addition of CD. [CD] is the total concentration of CD. Initial condition: a solution of TFDg (1.0 mM) in D_2O at 300 K.
○: TFDg (1.0 mM) and α -CD, ●: TFDg (1.0 mM) and β -CD, △: TFDg (1.0 mM) and γ -CD

Table 1. Binding constants K_a (M^{-1}) and changes in free energy ΔG (kJ) of the inclusion complex of TFDg and each CD

	K_a (M^{-1})	ΔG (kJ)
TFDg- α -CD	172	-12.8
TFDg- β -CD	2520	-19.5
TFDg- γ -CD	30.3	-8.5

Table 2. Diffusion constants ($\times 10^{-10} m^2/s$) determined from DOSY spectra of the inclusion complex of TFDg and each CD. TFDg in free solution (D_{free}) and in a complex (D_{obs}), CDs in free solution (D_{sugar}) and in a complex (D_{bound}).

	TFDg		CD	
	D_{free}	D_{obs}	D_{sugar}	D_{bound}
TFDg- α -CD	2.31	2.47	2.95	3.00
TFDg- β -CD	2.31	2.67	3.11	3.15
TFDg- γ -CD	2.31	2.32	2.60	2.55

compounds (Breckner and Husa, 2013). DOSY experiments can

Figure 5 shows the NOESY spectrum for a mixture of TFDg

Fig. 4. DOSY spectrum of a solution of TFDg (1.0 mM) and β -CD (1.0 mM) in D_2O at 300 K

Fig. 5. NOESY spectrum of a solution of TFDg (10.0 mM) and β -CD (10.0 mM) in D_2O at 300 K. Intermolecular NOEs are indicated in the square.

Fig. 6. Proposed model of the ground-state structure of the inclusion complex of TFDg and β -CD (1:1) determined by computational simulation

TFDg and β -CD are shown in stick and semitransparent space-filling presentation, respectively. Docking score of TFDg was -4.96 kcal/mol.

and β -CD in D_2O . Intermolecular NOE correlations between TFDg protons [H-g, H-G2/G6, H-G2'/G6', and H-6'/8'] and a β -CD proton [H-5] were observed. It is known that the proton at H-5 (δ_H 3.75) of β -CD is located on the inside of the structure of cyclodextrin (Veiga *et al.*, 2001). Therefore, these results imply that a galloyl moiety of TFDg is inside the cavity of β -CD.

Computational simulation between TFDg and β -CD To confirm the formation of an inclusion complex between TFDg and β -CD, we performed a computational binding analysis using 3D models and docking simulation. The results obtained from this

simulation indicated 83 possible complex candidates. One obtained docking score had a value of 5 kcal/mol; this, coupled with the results of the NOESY experiment, provided a candidate with a preferred 1:1 (TFDg : β -CD) binding interaction. A model showing this interaction is provided in Figure 6. This model suggests that the H-G2'/G6' galloyl group of bound TFDg is enclosed within the β -CD.

Discussion

In this study, we analyzed the mode of interaction between TFDg and three CDs (α -, β -, and γ -CDs) using several NMR techniques. These analyses included estimation of the binding constant and diffusion constant by DOSY. All the results suggested that TFDg interacts more with β -CD than with α - or γ -CD. The interaction site was observed by NOESY experiments, and data supporting the inclusion complex were obtained by computational simulation.

It has been reported that EGCg, one of the major tea catechins, interacts with β -CD, thereby suppressing the bitterness, improving the solubility, and increasing the stability of EGCg (Ishizu *et al.*, 2006, 2008, 2009, and 2011). In general, the conditions under which CDs incorporate a guest molecule are dependent on the inner diameter of the CD cavity and the compatibility of the hydrophobic nature of the guest molecule and the CD. The intermolecular interactions stabilizing the complex are relatively weak molecular attractions such as van der Waals interactions, hydrogen bonds, and electrostatic force. These molecular attractions are only effective at extremely short distances (within a few Å). Therefore, it is important that the inner diameter of the cavity should approximate the size of the guest molecule (Dell, 2004).

A number of mechanistic studies to elucidate the structure of

the complex formed between EGCg and β -CD have been performed. EGCg and β -CD form a tight interaction because the inner cavity diameter of β -CD is very close to the size of the interaction site of EGCg (Ishizu *et al.*, 2006). Ishizu *et al.* (2008) also reported that the A-ring moiety of EGCg is bound to β -CD, as observed by NMR techniques. Therefore, EGCg can form a complex with β -CD because of the very good match in size between the A-ring of EGCg and the β -CD cavity. In contrast, the inner cavity diameters of α - and γ -CD are smaller and bigger, respectively, than the interaction site of EGCg, making it difficult to form an inclusion complex between EGCg and these CDs.

In the case of TFDg, our NOESY and computational simulation results suggested that a galloyl moiety of TFDg is bound to β -CD. The NOESY spectrum showed the interaction between H-G2'/G-6' of TFDg (a galloyl moiety) and H-5 of β -CD (located in the inner cavity). The possible structure of the β -CD/TFDg complex at the same ratio in solution would be 1:1 because more cross peaks between H-G2'/G6' of TFDg and H-5 of β -CD were observed compared to H-G2/G-6 in the NOESY experiment (Figure 5). The three CDs used in this study could all interact with TFDg, as shown by binding constant and diffusion constant experiments. Our results showed that TFDg prefers to form an inclusion complex with β -, γ -, and α -CD, in that order. Based on these data, the investigation of the functional improvement of TFDg, such as solubility, stability, and enhanced activity resulting from complexation between TFDg and β -CD is in progress.

In conclusion, we characterized the inclusion complexes formed by TFDg and each of three CDs (α -, β -, and γ -CD). Even though the binding constant of β -CD with TFDg was smaller than that with EGCg, TFDg could still be encapsulated inside the CDs, especially β -CD. We also elucidated that the inclusion mechanism for TFDg and β -CD is different from that of EGCg and β -CD, since binding occurs through the galloyl moiety and not through the A-ring.

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References

- Brecker, L. and Husa, M. (2013). Protein-ligand interactions. *Nachr. Chem.*, **61**, 153-155.
- Davies, A.P., Goodsall, C., Cai, Y., Davis, A.L., Lewis, J.R., Wilkins, J., Wan, X., Clifford, M.N., Powell, C., Parry, A., Thiru, A., Safford, R., and Nursten, H.E. (1999). Black tea dimeric and oligomeric pigments - structures and formation. In "Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology," ed. by G.G. Gross, R.W. Hemingway, T. Yoshida, Kluwer Academic/Plenum Publishers, New York, NY, pp 697-724.
- Dell Valle, and E.M.M. (2004). Cyclodextrins and their uses: a review. *Process Biochem.*, **39**, 1033-1046.
- Eastburn, S.D. and Tao, B.Y. (1994). Applications of modified cyclodextrins. *Biotechnol. Adv.*, **12**, 325-339.
- Gaudette, N.J. and Pickering, G.J. (2012). The efficacy of bitter blockers on health-relevant bitterants. *J. Funct. Foods*, **4**, 177-184.
- Hara, Y., Matsuzaki, T., and Suzuki, T. (1987). Angiotensin I converting enzyme inhibiting activity of tea components. *Nippon Nogeikagaku Kaishi*, **61**, 803-808 (in Japanese).
- Ikedo, I., Yamahira, T., Kato, M., and Ishikawa, A. (2010). Black-tea polyphenols decrease micellar solubility of cholesterol *in vitro* and intestinal absorption of cholesterol in rats. *J. Agric. Food Chem.*, **58**, 8591-8595.
- Ishizu, T., Hirata, C., Yamamoto, H., and Harano, K. (2006). Structure and intramolecular flexibility of beta-cyclodextrin complex with (-)-epigallocatechin gallate in aqueous solvent. *Magn. Reson. Chem.*, **44**, 776-783.
- Ishizu, T., Kajitani, S., Tsutsumi, H., Yamamoto, H., and Harano, K. (2008). Diastereomeric difference of inclusion modes between (-)-epicatechin gallate, (-)-epigallocatechin gallate and (+)-gallocatechin gallate, with β -cyclodextrin in aqueous solvent. *Magn. Reson. Chem.*, **46**, 448-456.
- Ishizu, T., Tsutsumi, H., Yamamoto, H., and Harano, K. (2009). NMR spectroscopic characterization of inclusion complexes comprising cyclodextrins and gallated catechins in aqueous solution: cavity size dependency. *Magn. Reson. Chem.*, **47**, 283-287.
- Ishizu, T., Kajitani, S., Tsutsumi, H., Sato, T., Yamamoto, H., and Hirata, C. (2011). Configurational studies of complexes of tea catechins with caffeine and various cyclodextrins. *Planta Med.*, **77**, 1099-1109.
- Job, P. (1928). Formation and stability of inorganic complexes in solution. *Ann. Chim.*, **9**, 113-203.
- Kobayashi, S., Kainuma, K., and Suzuki, S. (1978). Purification and some properties of *Bacillus macerans* cycloamylose (cyclodextrin) glucanotransferase. *Carbohydr. Res.*, **61**, 229-238.
- Lavrado, J., Paulo, A., Bichenkova, E., Douglas, K. T., and Moreira, R. (2012). ¹H NMR spectroscopic identification of protonable sites in cryptolepines with C-11 substituents containing two amino functionalities. *Magn. Reson. Chem.*, **50**, 216-220.
- Leone, M., Zhai, D., Sareth, S., Kitada, S., Reed, J. C., and Pellicchia, M. (2003). Cancer prevention by tea polyphenols is linked to their direct inhibition of antiapoptotic Bcl-2-family proteins. *Cancer Res.*, **63**, 8118-8121.
- Li, S., Lo, C.Y., Pan, M.H., Lai, C.S., and Ho, C.T. (2013). Black tea: chemical analysis and stability. *Food Funct.*, **4**, 10-18.
- Lin, Y.L., Tsai, S.H., Lin-Shiau, S.Y., Ho, C.T., and Lin, J.K. (1999). Theaflavin-3,3'-digallate from black tea blocks the nitric oxide synthase by down-regulating the activation of NF- κ B in macrophages. *Eur. J. Pharmacol.*, **367**, 379-388.
- Matsui, T., Tanaka, T., Tamura, S., Toshima, A., Tamaya, K., Miyata, Y., Tanaka, K., and Matsumoto, K. (2007). α -Glucosidase inhibitory profile of catechins and theaflavins. *J. Agric. Food Chem.*, **55**, 99-105.
- Shiraki, M., Hara, Y., Osawa, T., Kumon, H., Nakayama, T., and Kawakishi, S. (1994). Antioxidant and antimutagenic effects of

- theaflavins from black tea. *Mutation Res.*, **323**, 29-34.
- Sughir, A., Skiba, M., Lameiras, P., Coadou, G., Lahiani-Skiba, M., and Oulyadi, H. (2010). Study of interaction between tiagabine HCl and 2-HP β CD: investigation of inclusion process. *J. Incl. Phenom. Macrocycl. Chem.*, **68**, 55-63.
- Tanaka, T., Inoue, K., Betsumiya, Y., Mine, C., and Kouno, I. (2001). Two types of oxidative dimerization of the black tea polyphenol theaflavin. *J. Agric. Food Chem.*, **49**, 5785-5789.
- Veiga, F.J.B., Fernandes, C.M., Carvalho, R.A., and Geraldes, C.F.G.C. (2001). Molecular modeling and ^1H -NMR: Ultimate tools for the investigation of tolbutamide: β -cyclodextrin and tolbutamide: hydroxypropyl- β -cyclodextrin complexes. *Chem. Pharm. Bull.*, **49**, 1251-1256.
- Wong, J.W. and Yuen, K.H. (2003). Inclusion complexation of artemisinin with α -, β -, and γ -cyclodextrins. *Drug Dev. Indian Pharm.*, **29**, 1035-1044.