Physicochemical and Structural Characterization of Quercetin-β-Cyclodextrin Complexes

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ABSTRACT: Quercetin is a bioactive flavonoid widely used as a health supplement. Being sparingly soluble and chemically unstable in aqueous intestinal fluids, quercetin is poorly absorbed orally. This study aimed to investigate the effects of three β-cyclodextrins, namely, unsubstituted β-cyclodextrin (β-CD), hydroxypropyl-β-cyclodextrin (HP-β-CD), and sulfobutyl ether β-cyclodextrin (SBE-β-CD) on the chemical stability and water solubility of quercetin, and to elucidate the complexation mechanisms of these β-CDs with quercetin. Quercetin-β-CD complexes in solution were characterized by stability assessment, phase solubility measurements, and 1H-nuclear magnetic resonance (NMR) spectroscopy. Molecular modeling was used to help establish the mode of interaction of the β-CDs with quercetin. Solubility enhancements of quercetin obtained with the three β-CDs followed the rank order: SBE-β-CD > HP-β-CD > β-CD. The stability of quercetin at alkaline pHs also showed substantial improvement. NMR spectroscopic analysis suggested that the B-ring, C-ring, and part of the A-ring of quercetin display favorable interaction with the hydrophobic cavity of the β-CDs, which was confirmed by molecular dynamics (MD) simulations using a solvated model of the quercetin-β-CD complex. An inclusion complex model has been established for explaining the observed augmentation of solubility and stability of quercetin in water by β-CDs.

INTRODUCTION

Quercetin (Figure 1A) belongs to the chemical class of flavonoid and is widely distributed in vegetables and plants. It has been demonstrated to possess a wide array of biological effects that are considered beneficial to health, including antioxidative, free radical scavenging, anticancer, and antiviral activities. However, quercetin is sparingly soluble in water, which has limited its absorption upon oral administration. In addition, it is chemically unstable, especially in aqueous alkaline medium, although acidic conditions can offer it some protection against degradation. The compound is also known to undergo extensive metabolism in the gut and the liver following absorption, and the resulting metabolites still retain some biological activity. All these problems lead to an extremely low oral bioavailability of quercetin (based on the unchanged quercetin) in human.

In pharmaceutical product development, β-cyclodextrins, a category of pharmaceutical excipients with unique solubilizing properties, have been widely used to tackle the aforementioned physicochemical problems associated with the formulation of particular drug candidates. β-cyclodextrins (β-CDs) are cyclic (α-1,4)-linked...
oligosaccharides composed of seven α-D-glucopyranose units, which together form a rigid cone-shaped structure (Figure 1B). This structure possesses a hydrophobic inner cavity and hydrophilic outer surface. It has been well established that the ability of β-CDs to enhance the stability and solubility of drugs is mediated through the formation of inclusion complexes. Unmodified or unsubstituted β-cyclodextrin (β-CD), that is, with no substituent on the glucopyranose unit, has poor water solubility and is parenterally unsafe due to its nephrotoxicity. Therefore, several synthetically modified and relatively safe β-CDs have been made and used in parenteral formulations, such as hydroxypropyl-β-cyclodextrin (HP-β-CD) and sulfobutyl ether β-cyclodextrin (SBE-β-CD).

It can be envisaged that if the absorption of a drug is limited by solubility rather than by permeability across the epithelial lipid membrane, the use of solubility enhancers such as β-CDs should, in principle, be able to increase the drug absorption. Our previous study using a Caco-2 cell monolayer model showed that permeability is not a limiting factor for the absorption of quercetin. In addition, it has been shown that co-administration of quercetin with HP-β-CD orally in rat afforded an oral bioavailability of quercetin (based on total quercetin measured) of as high as 59.1%, suggesting that factors other than permeability (notably solubility) may be more critical in limiting quercetin absorption.

The present study was designed to investigate the feasibility of utilizing three β-CDs (β-CD, HP-β-CD, and SBE-β-CD) to improve the chemical stability and aqueous solubility of quercetin, and to elucidate the mode of complexation between quercetin and these β-CDs. With this aim in mind, stability testing, phase solubility measurements, and proton nuclear magnetic resonance (NMR) spectroscopy have been employed together with molecular modeling as a complementary tool for characterizing the complexes in the present investigation.

MATERIALS AND METHODS

Chemicals

Quercetin dihydrate, fisetin, protocatechuic acid (PCA), and 1,3,5-trihydroxybenzene were purchased from Sigma Chemical Co., St. Louis, MO. β-CD was supplied by ICN Biomedicals, Inc., Aurora, OH. HP-β-CD (M.S. (average molar degree of substitution) = 0.8) and 2,4,6-trihydroxybenzoic acid was purchased from Sigma-Aldrich, Inc., St. Louis, MO. SBE-β-CD (T.D.S (total degree of substitution) = 7; Captisol®) was donated by CyDex, Overland Park, KS. All other chemicals and solvents were of analytical or high-performance liquid chromatography (HPLC) grade, and all water used was deionized and double-distilled.

Phase-Solubility Measurements

Phase-solubility measurements were carried out according to the method of Higuchi and Connors. Excess amount of quercetin was added to 10 mL of phosphate buffer (0.05 M, pH 3) containing various concentrations of β-CD, HP-β-CD, and SBE-β-CD. The resulting mixture was equilibrated in a Haake Swb20 thermostatic shaking waterbath (HAAKE Mess-Technik GmbHu. Co., Karlsruhe, Germany) for 24 h at

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Figure 1. (A) Molecular structure of quercetin. (B) Molecular structures of unsubstituted β-cyclodextrin (β-CD), hydroxypropyl-β-cyclodextrin (HP-β-CD), and sulfobutyl ether β-cyclodextrin (SBE-β-CD).
four different temperatures. This equilibration time was considered sufficient for the quercetin concentration to reach saturation, since no significant changes in quercetin concentration were observed at 24, 36, 48, 72, and 120 h. To minimize photochemical degradation, the waterbath was fully covered by aluminum foil throughout the course of the experiment. After equilibration, the solution was centrifuged at 4000 rpm under controlled temperature for 5 min, and the supernatant was passed through a 0.45 μm filter to remove undissolved solid. The first 5-mL filtrate was discarded, and the next 1 mL solution was diluted with an equal volume of methanol before assay for quercetin using either HPLC (primarily for low concentration measurements) as described next or UV-VIS spectrophotometry at 370 nm. Samples equilibrated at 48°C were checked for potential degradation, and the extent of quercetin degradation observed was not more than 2%, which was within the measurement errors. Thus quercetin was deemed chemically stable at pH 3.

High Performance Liquid Chromatography (HPLC)

Gradient-elution HPLC analysis of quercetin and potential degradation products employed a Hypersil C_{18} reversed phase column (5 μm, 250 × 4.6 mm I.D., Thermo Hypersil Ltd., Cheshire, UK) and a Waters 2695 LC system equipped with a Waters 996 photodiode array detector (Waters, Milford, MA). The mobile phase, which initially consisted of 5% methanol, 25% acetonitrile, and 70% phosphate buffer (25 mM, pH 2.6), was linearly changed to 80% methanol, 0% acetonitrile, and 20% phosphate buffer during the first 15 min, and then returned to the initial eluent composition for another 5 min. The analysis was conducted at room temperature and an eluent flow rate of 1 mL/min. Twenty microliters samples were injected onto the column. Analyte detection was set at 370 nm, and fisetin was used as internal standard. The intra-day precision of the analysis was determined within the same day by five replicate measurements of a standard solution of quercetin, while the inter-day precision was estimated on the same solution for five separate days. The inter- and intra-day RSD was less than 5.2%. Calibration curves constructed with standard quercetin solutions of appropriate concentrations for solubility calculation showed excellent linearity with R^2 > 0.999.

Stability Assessment

A stock solution of quercetin containing 1 mg/mL was prepared in methanol. Twenty microliters of stock solution was added to 10-mL PBS buffer (prepared from Phosphate Buffered Saline tablets supplied by Sigma Chemical Co, and with pH adjusted to 7.4 or 9) containing different concentrations of HP-β-CD, and SBE-β-CD, and equilibrated in a Haake Swb20 thermostatic shaking waterbath (HAAKE Mess-Technik GmbH & Co.) at 37°C for 0.5 h. Immediately after the addition of the quercetin solution, the pH of the mixture was measured again to ensure its constancy using a pH meter. After equilibration, the solution was vortexed briefly, and aliquots (0.25 mL) of the solution were then withdrawn at different time intervals, adjusted to about pH 3 with 20 μL 9% v/v phosphoric acid, and mixed with an equal volume of 50% v/v aqueous methanol. One hundred twenty microliters of above diluted sample was then mixed with 30 μL of internal standard solution, and 50 μL of the resulting mixture was injected onto the HPLC for quercetin analysis as described above. Degradation products shown in the HPLC chromatogram were confirmed by comparing their retention times and UV spectra with those of the authentic compounds.

The concurrent complexation and degradation of quercetin involving β-CDs at a given pH can be depicted by Scheme I as follows: where Q is the unionized form of free quercetin; Q' is the ionized form of free quercetin; Q–β-CD is the unionized form of complexed quercetin; Q'–β-CD is the ionized form of complexed quercetin; K_{c1} is the binding constant of the complex for unionized quercetin; K_{c2} is the binding constant of the complex for ionized quercetin; K_{a1} is the binding constant of the complex for unionized quercetin; K_{a2} is the binding constant of the complex for ionized quercetin; K_a is the ionization equilibrium constant.
constant of free quercetin; \( K_{c2} \) is the ionization constant of complexed quercetin; \( k_1 \) is the apparent first-order rate constant of the unionized form of free quercetin; \( k_2 \) is the apparent first-order rate constant of the unionized form of complexed quercetin; \( k_3 \) is the apparent first-order rate constant of the ionized form of complexed quercetin; and \( k_4 \) is the apparent first-order rate constant of the ionized form of complexed quercetin. A 1:1 stoichiometry is assumed for the complexation reaction (see Discussion).

According to Scheme I, quercetin exists in four different species, viz, \( Q, Q^-, \) \( Q^-β-CD, \) and \( Q^-β-CDs \), and the rate of degradation of quercetin depends on the concentrations of these species, which in turn are governed by the extent of ionization of quercetin in both free and complexed forms (\( K_{c1} \) and \( K_{c2} \)), the extent of complexation of the neutral and ionized quercetin with \( β-CDs \) (\( K_{c1} \) and \( K_{c2} \)), and the pH of the medium. Thus the following equation can be derived to relate the overall observed degradation rate constant (\( k_{obs} \)) of quercetin to the concentration of \( β-CD \):

\[
k_{obs} = \frac{k_1+k_2K_{c1}[β-CD]+\frac{K_{c2}}{K_{c1}}(k_3+k_4K_{c2}[β-CD])}{1+K_{c1}[β-CD]+\frac{K_{c2}}{K_{c1}}(1+K_{c2}[β-CD])}
\]

(1)

where \( [β-CD] \) is the total concentration of the \( β \)-cyclodextrin, and other parameters are as defined above. It can be seen that Equation 1 contains a total of seven parameters—three equilibrium constants (\( K_{c1}, K_{c1}, K_{c2} \)) and four rate constants (\( k_1, k_2, k_3, \) and \( k_4 \)).

In the present study, \( k_1 \) and \( k_2 \) were assumed equal to zero, since the phase solubility studies at pH 3 (where the quercetin \( (pK_{a1} = 7.03)^{11} \) is essentially ionized) showed no detectable degradation, and \( K_{c1} \) could therefore be calculated directly from the phase solubility data. \( K_{c2}, k_3, \) and \( k_4 \) can be readily determined by conducting the stability study under strongly alkaline condition (pH \( pK_a ≥ 2 \)), where the quercetin exists essentially in the ionized form and the contributions from the unionized quercetin can be ignored. At such a strongly alkaline pH, Scheme I can be simplified to Scheme II as follows.

Based on Scheme II, the following equation can be derived to relate the \( k_{obs} \) of ionized quercetin to the \( β-CD \) concentration:

\[
k_{obs} = \frac{k_3+k_4K_{c2}[β-CD]}{1+K_{c2}[β-CD]}
\]

(2)

Thus, in the present study, the stability of quercetin was conducted at pH 9. \( k_3 \) was measured in the absence of \( β-CDs \) (i.e., \( [β-CD] = 0 \)), while \( K_{c2} \) and \( k_4 \) were computed by nonlinear iterative fitting of the \( k_{obs} \) data obtained at pH 9 to Equation 2 using SigmaStat (version 3.1). Results are shown in Table 2.

**1H-Nuclear Magnetic Resonance (NMR) Spectroscopy**

\( 1H \)-NMR spectroscopic analyses were conducted on a JEOL 400 MHz FT-NMR at room temperature. Quercetin and \( β-CDs \) were dissolved in 20% v/v dimethylsulfoxide-\( d_6 \) (DMSO) in deuterated aqueous phosphate buffer (0.05 M; pH 3.4). DMSO was employed as internal standard. The chemical shift displacements were calculated according to the formula: \( \Delta\delta_{obs} = \delta_{(complex)} - \delta_{(free)} \), where \( \delta_{(complex)} \) is the chemical shift of quercetin with \( β-CDs \), and \( \delta_{(free)} \) is the chemical shift of quercetin without \( β-CDs \).

The binding constant \( K_c \) of the complex was calculated from \( \Delta\delta_{obs} \) using the following Benesi-Hildebrand equation:

\[
\frac{1}{\Delta\delta_{obs}} = \frac{1}{K_c \cdot \Delta\delta_c \cdot [β-CD]} + \frac{1}{\Delta\delta_c}
\]

(3)

where \( \Delta\delta_c \) is the difference in chemical shift between free quercetin and the pure quercetin-\( β-CD \) complex; and \( [β-CD] \) is the total (starting) \( β-CD \) concentration. Plot of the reciprocal of \( \Delta\delta_{obs} \) against the reciprocal of \( [β-CD] \) will afford a linear relationship with slope \( 1/(K_c \cdot \Delta\delta_c) \) and intercept \( 1/\Delta\delta_c \). \( K_c \) can be obtained by dividing the intercept by the slope.

**Molecular Modeling Studies**

Molecular mechanics and molecular dynamics (MD) simulations were performed in the framework of the AMBER4.0 force field,\(^{13}\) using the sugar parameters proposed by Homans.\(^{14}\) A newly developed modeling algorithm, which allows a simple definition of the starting relationship
between the β-CD ring and the guest molecule, was used to construct quercetin/β-CD complexes. Details of the parameters and the construction method will be reported elsewhere (Zheng et al., in preparation). Briefly, the β-CD ring was constructed with seven identical glucose units positioned symmetrically around the x axis, such that all the O1–C5 bonds are in the y-z plane, and the O1 atom of glucose-1 is located on the z axis, 6 Å from the origin. The 2-OH and 3-OH groups of each glucose project into +x space, and the 6-OH groups project into −x space. A planar quercetin molecule (in the x-y plane) was docked into this β-CD model with the B-ring to C-ring bond coincident with the x axis (see Figure 2, in which the midpoint of this bond is positioned at x = 0, i.e., at the origin). Multiple starting positions were generated by movement of the bond along the x axis, and complexes with either the A-ring or the B-ring projecting into +x space were built. Each complex was energy minimized in vacuo, and the lowest energy complexes were used as the starting geometries for MD simulations. These complexes were solvated in a box of TIP3P water molecules, with a minimum depth of 12.5 Å of water from the complex to the edge of the water box (typically about 1430 water molecules). The system was first relaxed using 600 cycles of conjugate gradient energy minimization (100 cycles on the complex only, and then 500 cycles on the complex and water). After relaxation, the MD simulation was performed using periodic boundary conditions under constant pressure, with a time step of 0.002 ps and a 8.0 Å nonbonded cutoff. Isotropic scaling of the velocities was used to maintain the pressure at 1 atm. The temperature of the simulation was maintained at 298.15K. The dielectric constant was set to 1.0.

RESULTS AND DISCUSSION

Phase-Solubility Study

The solubility of quercetin determined at room temperature was 0.441 ± 0.0487 µg/mL. All phase-solubility diagrams of quercetin with β-, HP-β-, and SBE-β-CD within the concentration range studied at pH 3 and 24–48°C displayed a typical A\textsubscript{L} type diagram (i.e., linear increase of quercetin solubility with increasing β-CD concentration), consistent with a 1:1 molecular complex formation for all three β-CDs.\textsuperscript{10} Figure 3 shows a typical A\textsubscript{L} diagram obtained at pH 3 and 24°C. The binding constants (K\textsubscript{c1}) of the complexes were calculated from the slopes of the linear phase-solubility plots using the following formula: K\textsubscript{c1} = slope/[S\textsubscript{0} (1−slope)], where S\textsubscript{0} is the intrinsic solubility of quercetin.\textsuperscript{15} Results are summarized in Table 1.

As shown in Table 1 and Figure 3, the binding constants (K\textsubscript{c1}) and solubilities of neutral quercetin determined with the three β-CDs followed the same rank order of SBE-β-CD > HP-β-CD > β-CD, reflecting an enhancement of binding and solubility of quercetin with an increase in substitution and hydrophilicity of the β-CDs. A similar rank order of binding constants has been reported by Dollo et al. for the three β-CDs with four neutral guest 1,2-dithiole-2-thiones.\textsuperscript{16} The observed differences in binding constants among the three β-CDs were much larger for the more lipophilic thiones (which have an additional benzene ring), 5-phenyldithiole thione (K\textsubscript{c} = 10,705, 2863, and 2322/M for SBE-β-CD, HP-β-CD, and β-CD at 37°C, respectively), and anetholetrithione (K\textsubscript{c} = 12,834, 6227, and 2841/M for SBE-β-CD,
Table 1. Equilibrium Binding Constants and Thermodynamic Parameters of Quercetin-β-Cyclodextrin Complexes (n = 3)

<table>
<thead>
<tr>
<th>CD</th>
<th>297K</th>
<th>303K</th>
<th>312K</th>
<th>321K</th>
<th>$\Delta G^\circ$ (297K), kJ/mol</th>
<th>$\Delta S^\circ$ (297K), J/K/mol</th>
<th>$\Delta H^\circ$ (297K), kJ/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-CD</td>
<td>1028 (36)</td>
<td>964 (49)</td>
<td>664 (18)</td>
<td>475 (29)</td>
<td>−17.2 (0.09)</td>
<td>−32.2 (11.2)</td>
<td>−26.7 (3.5)</td>
</tr>
<tr>
<td>HP-β-CD</td>
<td>11048 (142)</td>
<td>8802 (314)</td>
<td>6156 (189)</td>
<td>4395 (85)</td>
<td>−23.0 (0.03)</td>
<td>−27.8 (6.1)</td>
<td>−31.3 (1.9)</td>
</tr>
<tr>
<td>SBE-β-CD</td>
<td>25340 (500)</td>
<td>20303 (417)</td>
<td>13703 (304)</td>
<td>9821 (147)</td>
<td>−25.1 (0.05)</td>
<td>−22.5 (2.6)</td>
<td>−31.8 (0.8)</td>
</tr>
</tbody>
</table>

Values in parentheses depict standard deviations.

![Figure 3. Phase solubility diagrams of quercetin/β-CDs systems in phosphate buffer (0.05 M, pH 3) at 24 °C (n = 3). Key: (●) β-CD, (■) HP-β-CD, (▲) SBE-β-CD.](image)

HP-β-CD, and β-CD at 37 °C, respectively. Zia et al. have also reported that neutral compounds show stronger binding to SBE-β-CD than to HP-β-CD, while also finding that negatively charged guests had weaker binding with SBE-β-CD, compared to the neutral form of the guest. The quercetin binding data are consistent with the above trends for neutral molecules, and the stronger binding observed with SBE-β-CD and HP-β-CD may be due to additional contacts with the substituted CDs, which compared to β-CD offer altered binding surfaces to the host. Although the substituted CDs are relatively more hydrophilic, the substituents also include butyl and propyl chains that provide additional hydrophobic surface area for host–guest interaction, and this may permit stronger quercetin binding. Alternatively, or perhaps additionally, hydrogen bonding interactions between hydroxyl groups of the complexed quercetin and the CD substituents might stabilize the quercetin complex. A third possibility is that the more hydrophilic outer surface of the substituted β-CDs could compete more favorably against the relatively hydrophobic quercetin (logP = 1.81 ± 0.46) for interaction with water, thereby promoting the partitioning (inclusion) of quercetin into the hydrophobic cavity of the β-CDs. It is also worth noting that the binding constants of the quercetin complexes with β-CD and HP-β-CD (1028 ± 36 and 11048 ± 142/M at 24 °C) determined in the present study are significantly higher than those (402 and 532/M at 25 °C) reported recently by Pralhad and Rajendra-kumar. This discrepancy may be explained by the uncontrolled ionization and degradation of quercetin in the unbuffered aqueous media employed in the latter study.

An increase in temperature reduced the binding constant, $K_{c1}$, without changing the type of the phase-solubility diagrams. The relevant thermodynamic parameters for the complexation process, that is, the standard free energy change ($\Delta G^\circ$), the standard enthalpy change ($\Delta H^\circ$), and the standard entropy change ($\Delta S^\circ$), were determined from the linear plot of ln $K_c$ versus 1/T (van’t Hoff plot). As revealed in Table 1, complexation of quercetin with β-CDs in aqueous media was accompanied by a negative $\Delta G^\circ$ and negative $\Delta H^\circ$, reflecting a spontaneous, exothermic process. It has been suggested that the main driving force for complex formation is the release of enthalpy-rich water from the β-CD hydrophobic cavity and the replacement of the vacated site with the low-enthalpy guest molecule. Thus, the favorable reduction of free energy observed is mostly attributable to a large negative $\Delta H^\circ$, while the contribution from $\Delta S^\circ$, which can be either positive or negative, is relatively minor. Such large enthalpy changes are mainly associated with intermolecular interactions via hydrogen bonding and van der Waals forces.

Chemical Stability Study

As demonstrated by the phase solubility studies, quercetin was chemically stable in aqueous media at pH 3 and 24–48 °C. However, as the pH increased beyond 5, degradation of quercetin became apparent (data not shown), suggesting that only the ionized form of quercetin (i.e., anion) generated at alkaline pHs is susceptible to
degradation within the temperature range studied. In order to determine the degradation rate constants (k_3 and k_4) of the free and complexed forms of the quercetin anion at 37 °C using Equation 2, the effects of the more water-soluble β-CDs (i.e., HP-β-CD and SBE-β-CD) on the degradation of quercetin were investigated in aqueous media at a strongly alkaline pH (i.e., pH 9) to ensure essentially complete ionization of quercetin. The degradation reaction was observed to follow an apparent first-order process. A plot of the observed rate constants (k_{obs}) versus the concentrations of HP-β-CD/SBE-β-CD is presented in Figure 4. The k_3 parameter determined at zero β-CD concentration together with the K_{c2} and k_4 values computed by nonlinear iterative curve fitting based on Equation 2 is summarized in Table 2.

It can be seen that the presence of HP-β-CD substantially decelerated the degradation of the quercetin anion under alkaline condition with the k_3 value of the free anion being considerably larger than the k_4 estimate of the complexed form (by 7.2-fold). However, for the SBE-β-CD, the k_4 value computed for complexed anion was statistically insignificant (p = 0.4169) with an unacceptably large standard error, suggesting that the contribution of the complexed ion to the overall degradation was negligible. To verify this point, the stability data were reanalyzed by nonlinear iteration with the k_4 parameter set to zero. The reanalysis confirmed that the contribution of k_4 was negligible, as suggested by an insignificant change (<1%) in overall R^2 value (Table 2).

Compared with the unionized (neutral) species, the quercetin anion exhibited much weaker binding with the two substituted β-CDs, as expected from its enhanced hydrophilic character. However, as suggested by the measured binding constants (Table 2), the anion bound more strongly to HP-β-CD than to SBE-β-CD, which was the reverse order to that displayed by the neutral quercetin (Table 1). The much weaker binding of the quercetin anion with SBE-β-CD can be explained by the negative charge repulsion between the quercetin and SBE-β-CD anions.\(^{17}\) It has been reported previously that the decomposition of quercetin in aqueous media possibly

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**Table 2.** Equilibrium Binding Constants and Degradation Rate Constants of Quercetin-β-Cyclodextrin Complexes in PBS Buffer (pH 9) at 37 °C (n = 3)

<table>
<thead>
<tr>
<th></th>
<th>K_{c2} (M^{-1})</th>
<th>k_3 (h^{-1})</th>
<th>k_4 (h^{-1})</th>
<th>k_3/k_4</th>
<th>R^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin anion</td>
<td>—</td>
<td>5.969 (0.528)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Q(^{-}) + HP-β-CD</td>
<td>1009 (248)</td>
<td>—</td>
<td>0.826 (0.267)</td>
<td>7.2</td>
<td>0.953</td>
</tr>
<tr>
<td>Q(^{-}) + SBE-β-CD</td>
<td>112 (26)</td>
<td>—</td>
<td>-0.410 (0.489)</td>
<td>—</td>
<td>0.956</td>
</tr>
<tr>
<td></td>
<td>137 (13)(^{a})</td>
<td>—</td>
<td>0(^{a})</td>
<td>—</td>
<td>0.951(^{a})</td>
</tr>
</tbody>
</table>

Values in parentheses depict standard deviations.

\(^{a}\)Values were computed by repeating the nonlinear iterative curve fitting of the data with k_4 set to 0.
involves the attack of hydroxyl ions on the C-ring of quercetin. The negligible $k_4$ value of the complex formed by the quercetin and SBE-$\beta$-CD anions reflects a particularly stable structure, which may be ascribed to the ability of the negative charges on the SBE-$\beta$-CD anions to repel incoming hydroxyl ions, which serve as the decomposition catalyst.

Only PCA (3,4-dihydroxybenzoic acid) could be confirmed as a degradation product of quercetin under alkaline condition at 37°C, by comparing the retention time in HPLC and the UV spectrum (UV $\lambda_{\text{max}}$ = 220, 260, 294 nm) of the compound with those of the authentic sample. This degradation product has been reported previously by Makris et al. However, other possible degradation products of quercetin, namely, 2,4,6-trihydroxybenzoic acid and 1,3,5-trihydroxybenzene, were not detected under the present experimental conditions. Addition of $\beta$-CDs, particularly SBE-$\beta$-CD, was found to significantly decelerate the formation of PCA. Incubation of quercetin at pH 7.4 and 37°C with $\beta$-CD, HP-$\beta$-CD, and SBE-$\beta$-CD separately (in a 1:333 molar ratio) for 2 h reduced the formation of PCA from 100% (i.e., when no $\beta$-CD was added) to 30%, 16%, and 0%, respectively.

$^1$H-NMR Spectroscopic Analysis

In NMR analysis, formation of $\beta$-CD inclusion complexes is normally evidenced by changes in chemical shifts of both the guest and $\beta$-CD molecules. Such chemical shift changes may provide valuable insight into the molecular conformation of the inclusion complexes. For this reason, the present study employed NMR spectroscopy to probe the modes of complexation between quercetin and various $\beta$-CDs.

Being more sensitive than carbon NMR spectroscopy, proton NMR spectroscopy has been more widely used to characterize inclusion complexes involving $\beta$-CDs. In the present study, owing to the extremely poor aqueous solubility of quercetin, water could not be used alone to dissolve quercetin, and a certain amount of DMSO cosolvent was therefore added to promote the dissolution of quercetin in sufficient concentration for the NMR analysis. Figure 5 presents the solution $^1$H-NMR spectra of pure quercetin and $\beta$-CD. The proton positions were assigned based on previous reports. There was no peak overlap between quercetin and the $\beta$-CD in the $^1$H-NMR spectrum (Figure 5). The spectra of HP-$\beta$-CD and SBE-$\beta$-CD are not presented here because of the relatively impure chemical nature and poor spectral resolution of the modified $\beta$-CDs, which are closely associated with the different degree and position of substitution on their outer cone-shaped structures. On account of this purity problem, the chemical shifts of these $\beta$-CDs could not be reliably used for the NMR characterization (including 1D NMR and 2D ROESY NMR) of their related complexes, and consequently, the present study has only considered the proton chemical shifts of the guest quercetin for establishing the mode of complexation with the $\beta$-CDs.

For the $^1$H-NMR spectrum, the presence of $\beta$-CDs caused a significant downfield shift for the proton (H8) on the A-ring of quercetin and an upfield shift for the protons (H2’, H6’, and H5’) on the B-ring, and the shifts followed in descending order of SBE-$\beta$-CD > HP-$\beta$-CD > $\beta$-CD (Figure 6). No new peak was observed when the complexes were formed, indicating that the guest molecule was in rapid exchange between the free and the complexed states.

Increasing the amount of SBE-$\beta$-CD while keeping the concentration of quercetin constant (i.e., in molar ratio of 5:1, 10:1, 20:1) brought about a progressive change in chemical shifts (either upfield or downfield) in the $^1$H-NMR spectrum of quercetin. Plot of the reciprocal of these chemical shift changes versus the reciprocal of the concentration of SBE-$\beta$-CD (Benesi–Hildebrand plot) yielded a linear relationship (see Equation 3), as shown in Figure 7. The same result was observed for HP-$\beta$-CD. The binding constants averaged from individual estimates of those protons undergoing most significant chemical shift displacement were 560 and 1200/M for the HP-$\beta$-CD and SBE-$\beta$-CD complexes, respectively, consistent with those (510 ± 30 and 1260 ± 54/M) determined by the phase solubility method. However, in comparison with the purely aqueous solvent system (Table 1), the presence of 20% v/v DMSO drastically lowered the $K_c$ values of both HP-$\beta$-CD and SBE-$\beta$-CD complexes (by ~20-fold), indicative of considerable weakening of the quercetin binding by DMSO.

Compared with H8, H2’, H6’ and H5’, the complexation-induced chemical shift change of H6 on quercetin is the smallest (Figure 6), suggesting that the part of quercetin carrying this proton may not be included in the central cavity of the $\beta$-CDs. Similar chemical shift displacements of the same protons have been reported for (+)-catechin, a water-soluble flavonoid with basically the same molecular structure as that of quercetin, but without the carbonyl oxygen at C4 and the
unsaturated π bond at C2–C3, reflecting a comparable mode of complexation with β-CDs.\textsuperscript{23} Analysis of the proton shift changes using the continuous variation method (Job’s method) further confirmed the formation of a 1:1 stoichiometric complex for (+)-catechin and β-CD in purely aqueous medium.\textsuperscript{21} It should be noted that this reported NMR work on catechin-β-CD complex required no DMSO cosolvent because the flavonoid is water-soluble, and the close similarity in β-CD-induced proton shift changes between (+)-catechin and β-CD is expected.

**Figure 5.** \(^1\)H-NMR (400 MHz) spectrum of quercetin alone (upper) or β-CD alone (lower) in 20% v/v DMSO-\(d_6\) in deuterated aqueous phosphate buffer (pD 3.4) at room temperature.

**Figure 6.** Change of \(^1\)H-chemical shifts (400 MHz) of quercetin in the presence and absence of β-CDs (molar ratio = 1:10) in 20% v/v DMSO-\(d_6\) in deuterated aqueous phosphate buffer (pD 3.4) at room temperature. Key: (□) β-CD; (■) HP-β-CD; (□) SBE-β-CD.

**Figure 7.** Benesi–Hildebrand plots based on the chemical shift changes of H6(□), H8(■), H6'(×), H2'(△), and H5' (+) of quercetin.
catechin and quercetin would imply that the added DMSO, though capable of reducing the binding strength of the quercetin-β-CD complex in water (possibly via binding competition or quercetin extraction), will not significantly alter the basic mode of interaction.24,25

Based on the results of NMR and stability studies, it can be inferred that the B-ring, C-ring, and at least part of the A-ring of quercetin (except C6) exhibit significant interaction with the hydrophobic β-CD cavity.

**Molecular Modeling**

To further probe the mode of interaction between quercetin and β-CD, a molecular modeling (molecular mechanics/molecular dynamics) study was conducted both in vacuo and in water. A proposed favorable structure for the 1:1 inclusion complex between β-CD and quercetin is shown in Figure 8. In this structure, the quercetin B-ring projects onto the 2-OH/3-OH face of β-CD, and the A-ring projects from the 6-OH face. Other possible locations of quercetin were examined using both minimization and MD simulations, but were shown to be energetically less favorable than the complex shown in Figure 8.

In this orientation (Figure 8), all of the quercetin hydroxyl groups are outside the hydrophobic β-CD cavity, while the aromatic B-ring is positioned inside this cavity. Most importantly, the simulation results are in good agreement with the experimental data. Compared to the A-ring, the B-ring is more closely associated with the β-CD cavity, consistent with the significant decrease in the total degradation rate and decreased rate of formation of one of the degradation products, PCA, which forms from the B-ring of quercetin. In the complex in Figure 8, H6 in the A-ring is situated outside the β-CD cavity, and so the effect on the NMR chemical shift of H6 caused by complexation is small, compared with other protons (Figure 6). Relatively, H8 of the A-ring is oriented towards the β-CD cavity, hence its greater chemical shift on complexation, and H6' on the B-ring is predicted to be located centrally in the β-CD cavity, which is consistent with the large chemical shift of this proton upon complex formation (Figure 6).

**CONCLUSIONS**

The present study clearly demonstrated that the aqueous solubility and chemical stability (at alkaline pH) of quercetin can be substantially augmented via complexation with β-CDs, particularly with SBE-β-CD. The equilibrium binding constants and thermodynamic parameters determined by phase solubility study as well as the significant chemical shift changes of quercetin observed with the various β-CDs in 1H-NMR analyses are consistent with the formation of inclusion complexes. The results from MD simulations are in good accord with the NMR data, and allow the proposal of a favored orientation for quercetin in the β-CD cavity.

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**REFERENCES**


