J Solution Chem (2010) 39: 1168–1177
DOI 10.1007/s10953-010-9574-1

Inclusion Complexes of Cyclodextrins with Galangin: a Thermodynamic and Reactivity Study

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Received: 10 February 2010 / Accepted: 29 March 2010 / Published online: 14 August 2010

Abstract The formation of the complexes of galangin (GAL) with native β-cyclodextrin (βCD), and with its substituted counterparts such as dimethyl-βCD (DMβCD) and hydroxypropyl-βCD (HPβCD), was studied by fluorescence spectra in aqueous medium. The binding association constants (K_a) of the complexes were determined at different temperatures. The formation constants obtained have the following trend upon complex formation at the three temperatures studied: HPβCD > DMβCD > βCD. The thermodynamic data for the inclusion of GAL in DMβCD and HPβCD indicated that is mainly enthalpy driven whereas for βCD it is an entropy-driven process.

The antioxidant ability studies of GAL and CD complexes showed practically no change in its activity when the β-cyclodextrin complex is formed. The decrease in the T_{eq} observed for GAL-DMβCD and GAL-HPβCD in comparison with GAL-βCD could be due to effective protection of the phenol group in the cyclodextrin cavity, which is in agreement with molecular modeling studies.

Keywords Galangin · Cyclodextrins · Inclusion complex · ORAC-FL · Thermodynamic study · Docking

1 Introduction

Flavonoids are present in fruits, vegetables and beverages derived from plants (tea, red wine) and in many dietary supplements or herbal remedies including ginkgo biloba, soy

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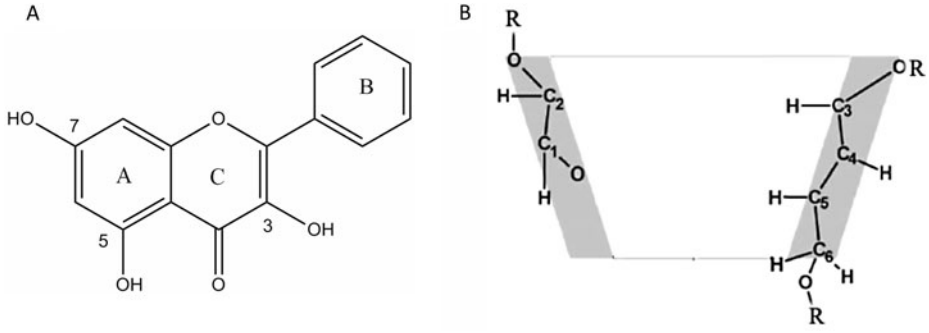

β-cyclodextrin, R = H
2-hydroxypropyl β-cyclodextrin, R = $CH_2CHOHCH_3$ or H
heptakis (2,6 *O* dimethyl) β-cyclodextrin, $R_{2,6} = CH_3$ R_3 = H

Scheme 1 (**A**) Molecular structure of galangin. (**B**) Schematic representation of β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin, and heptakis-2,6 *O* dimethyl-β-cyclodextrin

isoflavones, and milk thistle. Flavonoids have been described as health-promoting, disease-preventing dietary supplements, and have activity as cancer preventive agents [1, 2]. In addition to being potential alternatives to the commonly used synthetic phenolics for the food industry [3], they may have health benefits because epidemiological studies indicate that adequate intakes of flavonoid-rich foods may decrease the risk of coronary heart disease and certain cancers [4].

Galangin (Scheme 1A), a member of the flavonol class of flavonoid, is present in high concentrations in medicinal plants (e.g. *Alpinia officinarum*), honey and propolis, a natural beehive product. The increasing interest of this type of flavonol lies in their broad pharmacological activities (e. g., antimicrobial, spasmolytic, antiallergic, anti-inflammatory, antiviral, anticarcinogenic) [5–9]. Despite the applicable qualities, biological activities and therefore therapeutic usefulness of these substances, their bioavailability is limited because of their unfavorable physicochemical properties, especially very poor water-solubility and low oxidative stability.

On the other hand, modern nanochemistry has offered a solution to this problem by incorporation of these substances into cyclodextrins (CDs). CDs are cyclic glucose oligomers having six, seven or eight glucose units, linked by 1,4-α-glucosidic bonds and called respectively α-, β- and γ-cyclodextrin. They have the ability to form inclusion complexes with a wide variety of organic compounds, which enter partly or entirely into the relatively hydrophobic cavity of CDs simultaneously expelling the few high-energy water molecules from the inside. The size of the cavity of the CDs allows selectivity for the complexation of guest molecules, therefore acting as molecular encapsulants [10]. In the pharmaceutical, cosmetics and food industries, CDs have been used as complexing agents for various compounds, such as drugs, vitamins, and food colorants, increasing the solubility, stability, and bioavailability of the guest molecule [11, 12]. Some indications of improvement of the antioxidant activities of phenolic substances as guests of CD have been reported [13–15].

The present article is devoted to study the complexation of galangin with three different cyclodextrins, (HPβCD, DMβCD and βCD, Scheme 1B). Several thermodynamic parameters, determined from van't Hoff plots, were analyzed so as to gain information about the mechanisms involved in the association processes. Also, the effect that the cyclodextrin has on the antioxidant capacity of galangin against reactive oxygen species (ROS) was studied by the ORAC-fluorescein ($ORAC_{FL}$) methodology [16].

2 Experimental

2.1 Materials

Galangin (3, 5, 7-trihydroxyflavone), was purchased from Sigma (USA). βCD (β-cyclodextrin), DMβCD (heptakis-2,6-*O*-dimethyl-β-cyclodextrin), HPβCD (2 hydroxypropyl-β-cyclodextrin), AAPH (2,2'-azobis(2-methylpropionamidine) dihydrochloride), FL (fluorescein disodium salt) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich, Inc., St. Louis, MO. All solvents employed in the spectrophotometric analyses were spectroscopic reagent grade, from Merck.

2.2 Methods

2.2.1 Determination of the Binding Constant K_a

300 μL of GAL solution having a concentration of 5.0×10^{-4} mol·L^{-1} was added to aqueous solutions of CDs of various concentrations. The final volume of the system was kept constant to 5 mL and buffered to pH = 8 (Britton-Robinson, 0.1 mol·L^{-1}). The resulting mixture was equilibrated in a Julabo thermostatic shaking water bath for 24 h at different temperatures (298, 303 and 308 K) after which equilibrium was reached. Fluorescence emission spectra were recorded with a Perkin-Elmer LS-55 spectrofluorometer, with an excitation wavelength at 374 nm; the emission intensities were monitored at 524 nm. Excitation and emission bandwidths were set at 15 nm.

2.2.2 $ORAC_{FL}$ Assay

The ORAC analyses were carried out on a Synergy HT multidetection microplate reader, from Bio-Tek Instruments, Inc. (Winooski, VT), using 96-flat polystyrene microplates with clear bottoms, purchased from Nalge Nunc International. Fluorescence was read through the clear bottom, with an excitation wavelength of 485/20 nm and an emission filter of 520/20 nm. The plate reader was controlled by KC4, version 3.4, software. The oxygen radical absorbance capacity was determined as described by Dávalos et al., [17], with slight modifications. The reaction was carried out in 575 mmol·L^{-1} sodium phosphate buffer (pH = 7.4), and the final reaction mixture volume was 200 μL. FL (150 μL; 52 nmol·L^{-1}, final concentration) and galangin solutions in the absence or presence of CDs (70 μL), were placed in the wells of the microplate. The mixture was preincubated for 30 min at 37 °C, before rapidly adding the AAPH solution (30 μL; 19 mmol·L^{-1}, final concentration) using a multichannel pipet. The microplate was immediately placed in the reader and the fluorescence recorded every 1 min for 80 min. The microplate was automatically shaken prior to each reading. A blank with FL and AAPH using sodium phosphate buffer instead of the antioxidant solution, and eight calibration solutions using Trolox C (1, 2, 3, 4, 5 and 6 μmol·L^{-1}) as antioxidant, were also used in each assay. All reaction mixtures were prepared in triplicate, and at least three independent assays were performed for each sample. In order to avoid a temperature effect, only the inner 60 wells were used for experimental purposes, while the outer wells were filled with 200 μL of distilled water. The results were expressed as relative fluorescence with respect to the initial reading. The area under the fluorescence decay curve (*AUC*) was calculated by the equation:

$$AUC = 1 + \sum_{i=1}^{i=80} \frac{f_i}{f_0} \tag{1}$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i. The net *AUC* corresponding to the sample was calculated by subtracting the *AUC* corresponding to the blank.

2.2.3 Molecular Modeling

In silico build-up of the β-CD and its derivative forms were carried out using the Builder module of the InsightII program by adding to βCD 14 methyl groups in positions 2-, 6- (DMβCD) and 7-hydroxypropyl (MS 1) groups (HPβCD). The models obtained were then subjected to optimization using a protocol of 300 steps of conjugate gradients to avoid steric hindrance and clashes that can appear in the building process. The galangin was build using Gaussview and then it was optimized using a semiempirical method such as PM3 as implemented in the Gaussian98 package of programs [18].

Autodock3.0.5 [19] with the Lamarkian Genetic Algorithm (LGA) was used to generate the starting complexes. The parameters used for the global search was an initial population of 150 individuals, with a maximal number of energy evaluations of 1.5×10^7 and a maximal number of generations of 5×10^6 as an end criterion. An elitism value of 1 was used, and probabilities of mutation and crossing-over of 0.02 and 0.08, respectively, were used. The best solutions obtained, according to these parameters, were further refined by a local search method such as pseudo Solis and Wets "PSW".

Autodock defines the conformational space implementing grids over all of the possible solution space. With the aim of testing the ability of *Autodock* to converge into solutions that are inside of the βCD, a grid of 80 Å by side and 0.3 Å spacing between each point was set up in such a way that it covered both the external surface and the internal cavity of the βCD.

The following procedure was employed in the β-CD docking simulations: 250 runs were done for each βCD. At the end of each run, the solutions were separated into clusters according to their lowest RMSD and the best score based on a free energy function. Cluster solutions whose average score was not over 1 kcal·mol^{-1}(4.184 kJ·mol^{-1}) above the best energy obtained in the respective run were selected. Then, the solution that represented most of the complexes obtained in the run was compared with the experimental NMR data, insuring that this solution was able to reproduce it accurately.

The selected final complexes were optimized using the semiempirical PM3 method as a refining procedure with Gaussian98.

3 Results and Discussion

The stoichiometry and the binding constant, K_a, were estimated from the fluorescence data by using the modified Benesi-Hildebrand equation:

$$\frac{1}{\Delta F} = \frac{1}{\Delta F_{\max}} + \left(\frac{1}{K_a\,[\mathrm{CD}]^n}\right)\left(\frac{1}{\Delta F_{\max}}\right) \tag{2}$$

where $\Delta F = F_x - F_o$; F_x and F_o represent the fluorescence intensities of galangin in the presence and absence of total added CDs concentration respectively. $\Delta F_{\max}$ is the maximum change in fluorescence intensity, K_a is the binding constant for the galangin-cyclodextrin complex, and n represents the stoichiometry of the complex formed. A typical double reciprocal plot for $n = 1$ is shown in Fig. 1 for galangin complexed with βCD, DMβCD and HPβCD. For the three cyclodextrins the $n = 1$ plots exhibit good linearity whereas the

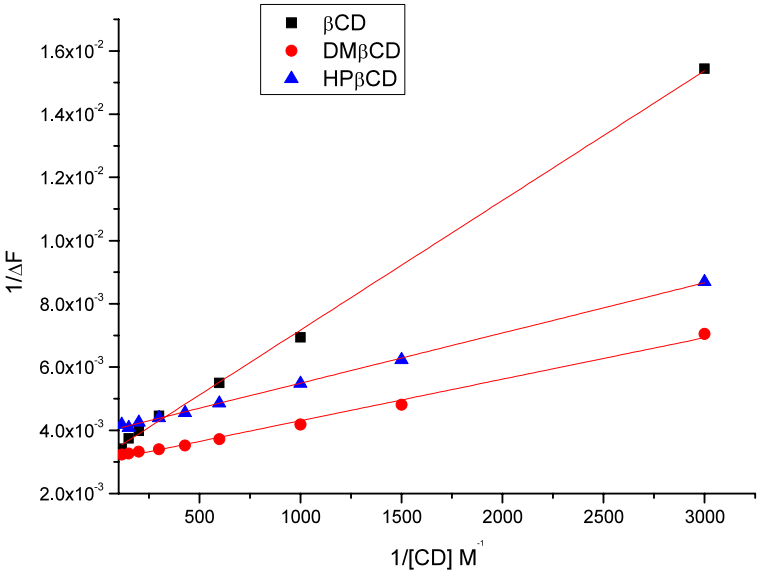

Fig. 1 Double reciprocal plots for galangin complexed with ■ βCD, ▲ HPβCD and ● DMβCD for 1:1 binding stoichiometries

$n = 2$ plots (data not shown) deviate from linearity. From these results we can infer that galangin forms 1:1 inclusion complexes with both substituted cyclodextrins and the native cyclodextrin, which is in agreement with our previous work [20].

The binding constant K_a of the complexes with this methodology at 303 K are 748, 2455 and 2273 L·mol^{-1} for βCD, HPβCD and DMβCD, respectively. This apparent binding constant was determined in an aqueous medium containing 10% methanol, which was needed to improve 'aqueous' solubility of galangin in the CD-free medium. The K_a values calculated by this spectroscopic method were found to be less than those obtained by the solubility method [20–22], due to the fact that for this spectroscopic experiment we needed to dissolve galangin in an organo-aquous medium to solubilize the flavonoid completely. Methanol was the solvent of choice due to its low affinity for binding with CD [23]

The stability constant of the complexes of galangin with the three CDs at different temperature (298, 303 and 308 K) are shown in Table 1. We note, that the stability constant for GAL-HPβCD and GAL-DMβCD complexes decrease with increasing temperature as expected for an exothermic process, which indicates that the complexation process was favorable at lower temperature. As the temperature increased, the affinity between galangin and HPβCD or DMβCD decreased. However, for GAL-βCD, the stability constant increases as the temperature rises.

Thermodynamic parameters were calculated based on the temperature dependence of the binding constant for galangin-CD binding. The thermodynamic parameters such as the enthalpy changes (ΔH) and entropy changes (ΔS) of the binding reaction are important to confirm the force of interactions of galangin with cyclodextrins. Four driving forces for the inclusion of CDs with substrates were proposed, including: hydrogen bonding between the hydroxyl groups of CDs and the guest, van der Waals interactions between host and guest molecules, hydrophobic interaction, and the release of 'high-energy water' molecules from the cavities of CDs to the bulk water. Hydrophobic interaction essentially involves favorable

Table 1 Apparent stability constant (K_a), thermodynamic parameters and antioxidant capacity (T_{eq}) of galangin forming complexes with cyclodextrin

	K_a/ L·mol^{-1}	K_a/ L·mol^{-1}	K_a/ L·mol^{-1}	ΔH/ kJ·mol^{-1}	ΔS/ kJ·mol^{-1}·K^{-1}	ΔG/ kJ·mol^{-1}	T_{eq}
T/K	298	303	308				
GAL							4.57 ± 0.37
GAL-βCD	589	748	893	31.78	0.160	−15.9	4.60 ± 0.34
GAL-DMβCD	2650	2273	1990	−21.85	−0.008	−19.47	3.40 ± 0.58
GAL-HPβCD	2818	2455	2111	−22.03	−0.008	−19.64	3.11 ± 0.26

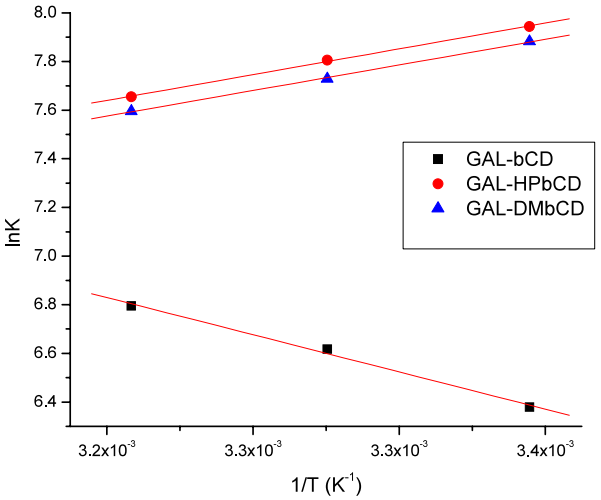

Fig. 2 Van't Hoff plot ($\ln K_a$ versus $1/T$) for galangin-cyclodextrin association

positive entropy together with a slightly positive enthalpy change, whereas the other forces involve negative ΔH and ΔS [24].

If the enthalpy change (ΔH) does not vary significantly over the temperature range studied, then its value as well as that of the entropy change (ΔS) can be determined from the van't Hoff equation:

$$\ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \tag{3}$$

where K_a is the associative binding constants corresponding to various temperatures, and R is the gas constant (8.314 J·K^{-1}·mol^{-1}). The enthalpy change (ΔH) can be calculated from the slope of the van't Hoff relationship and the Gibbs energy change (ΔG) can be estimated:

$$\Delta G = \Delta H - T\Delta S. \tag{4}$$

A plot of $\ln K_a$ versus $1/T$ for the three complexes formed were linear with correlation coefficients $R \geq 0.996$, Fig. 2. These thermodynamic parameters are listed in Table 1. By careful inspection, the following conclusions can be obtained: the negative value for the Gibbs energy (ΔG) means that the binding process is a spontaneous process and thermodynamically favored. ΔH and ΔS for GAL-HPβCD and GAL-DMβCD are negative in the experimental temperature range, which indicates that the inclusion process was exothermic

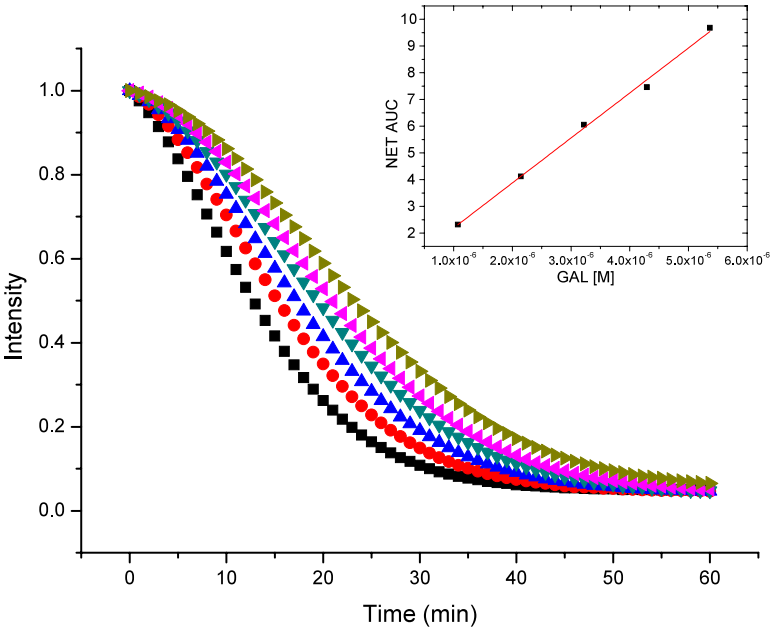

Fig. 3 FL fluorescence decay curves induced by AAPH in the presence of galangin at different concentrations. ■ blank, ● 0.13 μmol·L^{-1}, ▲ 0.27 μmol·L^{-1}, ▼ 0.40 μmol·L^{-1}, ◀ 0.54 μmol·L^{-1}, ▶ 0.67 μmol·L^{-1}. *Insets*: net *AUC* of galangin on different concentration of galangin. The net $AUC = AUC_{\text{sample}} - AUC_{\text{blank}}$; the *AUC* was calculated by Eq. 1

and an enthalpically controlled process. The negative enthalpy change arose from the van der Waal's interaction; while the negative entropy change can be explained considering that the complex causes a decrease in translational and rotational degrees of freedom of the complex molecule as compared with the free ones, giving a more ordered system. A different behavior was observed for the GAL-βCD complex, where upon complexation both positive enthalpic changes and positive entropic values are obtained, indicating that this inclusion is mainly entropically driven. Apparently, when galangin is free in solution, it seems to have a strong interaction with its solvent shell. Upon binding, this solvent shell is broken up, leading to the partly unfavorable enthalpic change. The same effect was observed [14] in the complexation of morin with native and modified cyclodextrins; complexed with DMβCD the inclusion is primarily enthalpically driven, while with βCD and HPβCD is an entropic driven process. No reason for these differences was found.

The $ORAC_{FL}$ assay expresses antioxidant activity relative to a standard (trolox) while measuring the oxidation of the fluorescent substrate by peroxy radicals generated during the reaction. This method follows a hydrogen atom transfer pathway, where the antioxidant and a peroxy radical form a stable antioxidant radical that breaks the radical chain oxidation. In order to discern any antioxidant effect of cyclodextrins per se, the disappearance of fluorescence signal of FL by the attack of the AAPH radical were measured in the presence of increasing concentrations of CDs (in the absence of galangin or trolox). In this case, no effect was observed as the CDs concentration increased indicating that CDs, at the studied concentrations, do not have an antioxidant effect per se. Figure 3 shows the FL fluorescence decay curves of galangin in the presence of AAPH. The linear relationship between the net area and antioxidant concentration was calculated at different concentrations. The regres-

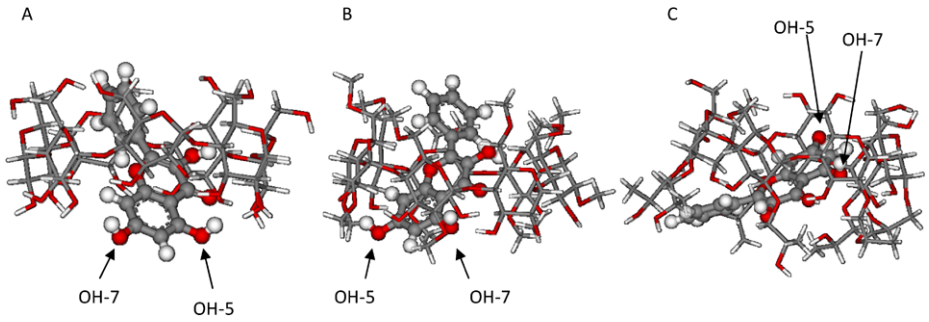

Fig. 4 Relative host-guest geometry corresponding to the minimum of the energy of the formation of (**A**) GAL-βCD complex (**B**) GAL-DMβCD complex and (**C**) GAL-HPβCD complex

sion analysis points to a linear response between the galangin concentration and the net *AUC* ($R = 0.998$). Table 1 reports the Trolox equivalent, T_{eq} for free galangin and galangin inclusion complexes with the different cyclodextrins referenced to Trolox. Practically no change in the antioxidant activity is observed when galangin complexes with β-cyclodextrin. The low T_{eq} values obtained for GAL-HPβCD and GAL-DMβCD in comparison with GAL free and complexed with βCD can be explained by the phenol group responsible for quenching peroxyl radicals penetrating into the less polar CD cavity where there may be a modification in the redox behavior of the phenol group or an effective protection of the phenol group due to the complexation. Besides increasing the solubility of flavonoids, cyclodextrins may also have an effect on their antioxidant activity. Moreover we have determined that the inclusion geometry influences the antioxidant capacity. Our result indicated that for morin complexes [14] the antioxidant activity increased due to the stabilization of the radical inside the apolar cavity. However, for luteolin complexes [25] the antioxidant activity is maintained. This must be taken into account when the antioxidant activity is determined by $ORAC_{FL}$ utilizing CD as an solubility enhancer for lipophilic compounds [26, 27]

To know more about the inclusion geometry of galangin complexes, molecular modelling and docking studies were carried for the three complexes out. Autodock3.0.5 results revealed a preferential relative orientation for all the complexes under study, in spite of their different initial configurations. The better scored complexes obtained for βCD, DMβCD and HPβCD are shown in Figs. 4A–C. All these complexes were further refined using a semiempirical methodology such as PM3. The same trends in the inclusion geometries observed by 2D-NMR [20] are noticeable here, where the inclusion in DMβCD and HPβCD occurs in an opposite way. In the case of the GAL-βCD complex, the conformation obtained has the B-ring of galangin oriented toward the primary rim, while both A and C rings remain practically exposed to the external surface by the secondary rim. The theoretical results obtained for the GAL-DMβCD complex indicated that inclusion occurs in a similar fashion to the GAL-βCD complex. In the latter, both the A- and C-rings are almost completely inserted in the interior of the DMβCD, with the OH-5 and OH-7 groups more buried than in the GAL-βCD complex. In the case of the GAL-HPβCD complex, the results indicated that the B-ring of galangin is oriented to the secondary rim, resulting in a complex where galangin is located in the opposite direction compared to the previous complexes. Figures 4B and C show clearly that the OH-5 and OH-7 groups of galangin in GAL-HPβCD and GAL-DMβCD remain protected. They are occupying the hydrophobic cavity of the CD, a situation that does not occur in the βCD. These results could account for the observed

diminution of the antioxidant activity of these complexes in comparison with free galangin and GAL-βCD.

4 Conclusions

The association constant and thermodynamic parameters for the inclusion complex were evaluated by fluorescence spectroscopy. The formation constants obtained follow the same trend under the three temperatures studied, in the order: HPβCD > DMβCD > βCD. Thermodynamic studies of cyclodextrin complexes indicated that for DMβCD and HPβCD inclusion is mainly an enthalpy driven process while for βCD it is an entropy driven process.

No changes in the antioxidant activity were observed when galangin forms the complex with βCD. However, a diminution in antioxidant activity was observed upon complexation with DMβCD and HPβCD. Molecular modelling studies showed that this behavior could be explained by the GAL-βCD complex having the A and C rings of galangin exposed to the external surface, while in the GAL-DMβCD and GAL-HPβCD complexes, the OH groups of the chromone (OH-5 and OH-7), which are responsible for the quenching of the peroxyl radicals, are protected and this could explain the diminution of antioxidant activity. Finally, the study of the inclusion geometry is necessary to explain the changes produced in antioxidant activity involving the complexes formed.

Acknowledgements Our thanks are to Fondecyt 11080038 and to Molecular Graphic Unit of the Faculty of Chemical and Pharmaceutical Sciences, University of Chile.

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