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Encapsulation of Quercetin and Myricetin in Cyclodextrins at Acidic pH

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The in vitro formation of quercetin− and myricetin−cyclodextrin inclusion complexes in acidic medium has been characterized using the enzymatic system horseradish peroxidase, which oxidizes those flavonols in the presence of H_2O_2. The presence of cyclodextrins (CDs) in the reaction medium inhibited flavonol oxidation due to the complexation of the flavonol in the hydrophobic cavity of CDs. This inhibitory effect depends on the complexation constant K_c between flavonol and the CD type used. The K_c for quercetin and myricetin with the different types of CD used was calculated by nonlinear regression of the inhibition curves obtained in the presence of CDs. In both cases (quercetin and myricetin), the K_c values obtained followed the order hydroxypropyl-β-CDs > maltosyl-β-CDs > β-CDs, reflecting the greater affinity of modified cyclodextrins for the studied flavonols compared with their parental β-CDs. Moreover, the complexation efficiency (CE) values for HP-β-CDs and quercetin or myricetin were calculated (267.4 and 5.3, respectively), indicating that HP-β-CDs are more efficient for the complexation of quercetin than myricetin in the studied conditions, despite of the K_c values being very similar in both cases.

KEYWORDS: Quercetin; myricetin; cyclodextrin; enzymatic method; complexation constant

INTRODUCTION

Polyphenols are secondary plant metabolites that have received much attention because of their potential health benefits (*1–3*). Flavonoids are the major group of polyphenols found in fruits and vegetables. The health-related beneficial effects of these antioxidant compounds have been associated with antitumor, antimutagenic, and antiaging activities (*4*). The consumption of plants and plant products rich in flavonoids, such as cocoa, wine, tea, and berries, has been related with protective effects against cardiovascular disease and certain forms of cancer (*3, 5, 6*).

Flavonols constitute a major group within the flavonoids present in several foodstuffs, such as apples, cherries, citrus fruits, tea, onions, broccoli, and other green vegetables. The structure of these compounds is a key determinant of their antioxidant activity, which has also been associated with the chelation of metal ions (*7*), the inhibition of oxidative enzymes (*8*), and activation of antioxidant defenses (*9, 12*). Flavonols have been a subject of increasing research concerning their potential for use as natural antioxidants in food systems. They have been seen to act as free radical scavengers and have been widely studied for their antioxidant activity in vitro (*13–15*).

The flavonols quercetin and myricetin efficiently inhibit lipid oxidation (*16, 17*) and are considered to be better antioxidants then butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (*18, 19*). These flavonols are extremely hydrophobic and despite their wide potential health benefits, their use in fuctional foods is limited by their low solubility in water.

In recent years, cyclodextrin complexation has been successfully used to improve solubility, chemical stability, and bioavailability of a number of poorly soluble compounds. Cyclodextrins (CDs) are a group of naturally occurring cyclic oligosaccharides derived from starch with six, seven, or eight glucose residues linked by α(1→4) glycosidic bonds in a cylinder-shaped structure, denominated by α-, β-, and γ-cyclodextrins, respectively. The central cavity of these molecules is hydrophobic, whereas the rims of the surrounding walls are hydrophilic. This hydrophobic cavity forms inclusion complexes with a wide range of organic and inorganic guest molecules (*20*), altering their physicochemical behavior and reducing their undesirable effects. In the pharmaceutical, cosmetics, and food industries, cyclodextrins have been used as complexing agents to increase the water solubility of various compounds, such as drugs, vitamins, and food colorants (*21–23*). It has been demonstrated that complexation can considerably increase the solubility, stability, and bioavailability of the guest molecule.

Given the many health-promoting activities of quercetin and myricetin, a method for increasing their bioavailability and stability was thought to be necessary. To this end, in this paper, the in vitro formation of quercetin− and myricetin−cyclodextrin inclusion complexes in acidic medium has been characterized

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using the enzymatic system, horseradish peroxidase, which oxidizes those flavonols in the presence of H_2O_2. A study of this oxidation process will permit the complexation constant between flavonols and different types of cyclodextrins to be determined. Moreover, the complexation constants were calculated by phase solubility studies, and the results obtained with both methods are compared.

MATERIALS AND METHODS

Horseradish peroxidase (246 units/mg) and flavonols (myricetin and quercetin) were purchased from Sigma (Madrid, Spain). Hydrogen peroxide was purchased from Aldrich (Madrid, Spain). Cyclodextrins were purchased from TCI (Europe). All other chemicals used were of analytical grade.

The hydrogen peroxide solution was freshly prepared every day, and its concentration was calculated using $\varepsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$ (*24*).

The activity was followed spectrophotometrically in a Shimadzu model UV-1063 spectrophotometer at the absorption maximum of the oxidation product of flavonols (300 nm).

Unless otherwise stated, the standard reaction medium at 25 °C contained 100 mM sodium acetate buffer (pH 4.5), 400 μM H_2O_2, 60 μM quercetin or myricetin, and 0.037 unit of horseradish peroxidase in the case of quercetin or 0.1 unit of horseradish peroxidase in the case of myricetin in a final volume of 1 mL. The steady state was calculated from the linear zone of the product accumulation curve. A reference cuvette containing all of the components of the reaction medium except the enzyme served as the control.

Enzymatic Method. Assuming that the inhibitory effect in the flavonol oxidation rate, observed in the presence of increasing concentrations of CDs, was due to the formation of the inclusion complexes and that free flavonol (quercetin or myricetin) is the only form of substrate that peroxidase can use, the Michaelis–Menten velocity equation could be expressed as a function of free flavonol concentration ($[flavonol]_f$):

$$v = \frac{V_m[flavonol]_f}{K_M + [flavonol]_f} \quad (1)$$

This $[flavonol]_f$ could be expressed as a function of the only two known parameters $[flavonol]_t$ and $[CD]_t$, where the subscript t stands for overall compound concentration. Assuming that only one molecule of flavonol can enter a cyclodextin molecule (stoichiometry 1:1), the equilibrium may be expressed as

$$[flavonol]_f + [CD]_f \overset{K_c}{\leftrightarrow} [flavonol{-}CD] \quad (2)$$

where the complexation constant, K_c, is defined as

$$K_c = \frac{[flavonol{-}CD]}{[flavonol]_f[CD]_f} \quad (3)$$

Taking into account the mass balance

$$[flavonol]_t = [flavonol]_f + [flavonol{-}CD] \quad (4)$$

$$[CD]_t = [CD]_f + [flavonol{-}CD] \quad (5)$$

and eq 3, $[CD]_f$ and $[flavonol{-}CD]$ can be expressed as

$$[CD]_f = \frac{[flavonol]_t - [flavonol]_f}{K_c[flavonol]_f} \quad (6)$$

$$[flavonol{-}CD] = K_c[flavonol]_f[CD]_f \quad (7)$$

Then, substituting these two equations into eq 4, the following quadratic relationship is obtained:

$$K_c[flavonol]_f^2 + ([CD]_tK_c - [flavonol]_tK_c + 1)[flavonol]_f - [flavonol]_t = 0 \quad (8)$$

From this, $[flavonol]_f$ can be obtained

$$[flavonol]_f = \{-([CD]_tK_c - [flavonol]_tK_c + 1) + \sqrt{([CD]_tK_c - [flavonol]_tK_c + 1)^2 + 4K_c[flavonol]_t}\}/2K_c \quad (9)$$

which can be substituted into eq 1 to give

$$v = \frac{V_m[(-([CD]_tK_c - [flavonol]_tK_c + 1) + \sqrt{([CD]_tK_c - [flavonol]_tK_c + 1)^2 + 4K_c[flavonol]_t})/2K_c]}{K_M + [(-([CD]_tK_c - [flavonol]_tK_c + 1) + \sqrt{([CD]_tK_c - [flavonol]_tK_c + 1)^2 + 4K_c[flavonol]_t})/2K_c]} \quad (10)$$

Physical Method. Phase solubility diagrams were constructed according to the method of Higuchi and Connors (*25*). Excess amounts of flavonol (quercetin or myricetin) were added to aqueous solutions of increasing concentrations of α-, β-, γ-, maltosyl-β-, and hydroxypropyl-β-cyclodextrins up to 100 mM (15 mM in the case of β-CDs, its solubility limit), in 10 mL of water or 100 mM sodium acetate buffer (pH 4.5) at 25 °C. The samples were maintained in an ultrasonic bath for 60 min to reach equilibrium. The aqueous solutions were filtered through a 0.2 μm membrane filter and diluted in 80% ethanol/water. The quercetin ($\varepsilon_{362} = 12790\ M^{-1}\ cm^{-1}$) or myricetin ($\varepsilon_{378} = 22370\ M^{-1}\ cm^{-1}$) concentrations were spectrophotometrically determined.

RESULTS AND DISCUSSION

The fact that the oxidation of the quercetin and myricetin by horseradish peroxidase in acidic medium can be measured in the presence of cyclodextrins was used to determine the complexation constant using the enzymatic method described by our group (*26*).

An experiment was performed in which peroxidase activity was determined at pH 4.5 in the presence of different types of CDs at different concentrations while the flavonol concentration remained constant.

When quercetin was oxidized by peroxidase in the presence of increasing concentrations of natural α-, β-, and γ-cyclodextrins, a decrease in peroxidase activity was observed with β- and γ-cyclodextrins, whereas the presence of α-cyclodextrins had no effect on enzymatic activity (**Figure 1A**) because its hydrophobic cavity is smaller than those of the others. This inhibitory effect of β- and γ-cyclodextrins was studied at different quercetin concentrations, and a clear decrease in peroxidase activity was observed in all cases (**Figure 1B**).

In the case of myricetin, its oxidation by peroxidase at pH 4.5 in the presence of increasing concentrations of natural α-, β-, and γ-cyclodextrins was also studied. In this case, β-CDs had a low inhibitory effect on enzymatic activity (less than that obtained in the case of quercetin), whereas α- and γ-cyclodextrins had no effect on enzymatic activity (**Figure 2A**). Therefore, the effect of β-CDs was studied at different myricetin concentrations (**Figure 2B**).

Equation 10 (Materials and Methods) shows a nonlinear relationship between v and $[CD]_t$ as in **Figures 1B** and **2B**. When the data were fit by nonlinear regression using Sigma Plot (Jandel Scientific), the values presented in **Table 1** were obtained for the K_c between quercetin with β- and γ-CDs and myricetin with β-CDs. As can be seen in **Table 1**, the K_c value obtained for quercetin and β-CDs (426 M^{-1}) was higher than that obtained for myricetin and β-CDs (125 M^{-1}). The K_c value for quercetin and β-CDs was similar to that described by Pralhad and Rajendrakumar (*27*), calculated by phase solubility studies in water (402 M^{-1}), but was slightly lower than that described by Alvarez-Parrilla et al. (*28*), calculated by fluorescence measurements (1138 M^{-1}).

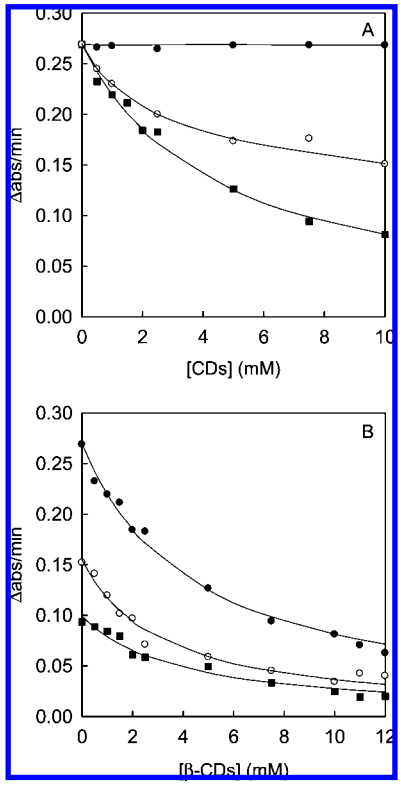

Figure 1. Effect of different types of cyclodextrin concentration on quercetin oxidation by peroxidase: (**A**) effect of different types of cyclodextrin [(●) α-CD, (■) β-CD, and (○) γ-CD]; (**B**) effect of β-CDs concentration [(●) 60 μM quercetin, (○) 30 μM quercetin, and (■) 15 μM quercetin]. The reaction medium at 25 °C contained 100 mM sodium acetate buffer, pH 4.5, 60 μM quercetin, 400 μM H_2O_2, 0.037 unit of horseradish peroxidase, and increasing concentrations of cyclodextrins.

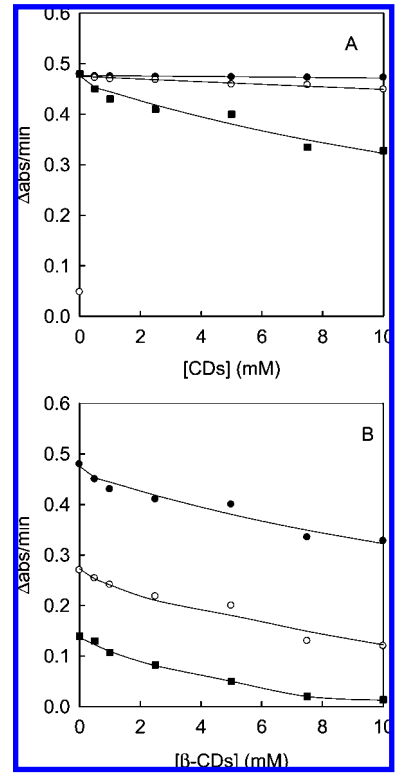

Figure 2. Effect of different types of cyclodextrin concentration on myricetin oxidation by peroxidase: (**A**) effect of different types of cyclodextrin [(●) α-CDs, (■) β-CDs, and (○) γ-CDs]; (**B**) effect of β-CDs concentration [(●) 60 μM myricetin, (○) 30 μM myricetin, and (■) 15 μM myricetin]. The reaction medium at 25 °C contained 100 mM sodium acetate buffer, pH 4.5, 60 μM myricetin, 400 μM H_2O_2, 0.1 unit of horseradish peroxidase, and increasing concentrations of cyclodextrins.

Because unmodified and unsubstituted β-CDs show a poor degree of water solubility, the inclusion of quercetin and myricetin in modified cyclodextrins was studied. Modified cyclodextrins have many advantages over the parent cyclodextrins; for example, they are highly soluble in both water and organic solvents and enhance the solubility of otherwise water-insoluble compounds by complexation (*29–32*). Among them, 2-hydroxypropyl- (HP-β-CDs) and maltosyl-β-CDs (G_2-β-CDs) are widely used because of their high solubility and low cost.

When the oxidation of quercetin and myricetin by peroxidase was carried out in the presence of HP-β-CDs or G_2-β-CDs, an inhibitory effect similar to that described with their parental β-CDs was observed (**Figure 3**), and by fitting the data by nonlinear regression their complexation constants were calculated (**Table 1**). In both cases, quercetin and myricetin, the K_c values obtained for HP-β-CDs or G_2-β-CDs were higher than those obtained for their parental β-CDs. The K_c values obtained followed the order HP-β-CDs > G_2-β-CDs > β-CDs, reflecting the greater affinity of modified cyclodextrins for the studied flavonols than their parental β-CDs. In the case of HP-β-CDs, the strong binding may be due to the assistance offered by the substitute groups.

To clarify whether peroxidase was only working with free flavonols (quercetin or myricetin) the data in **Figures 3** were replotted in **Figure 4** as a function of free flavonol, using eq 9. The new plot showed that the points of the different curves in **Figure 3**, which represent the same activities, also have the same $[\text{flavonol}]_f$ in **Figure 5**. This clearly indicates that the enzyme was sensitive to only free flavonol, as has been previously described for the oxidation of different lipoxygenase substrates included in CDs (*32–34*).

Table 1. Stability Constants (K_c) for Complexes Formed between Different Types of Cyclodextrins and the Two Flavonols under Study in Sodium Acetate Buffer (100 mM, pH 4.5), Obtained by the Enzymatic Method

guest	α-CDs K_c (M^{-1})	β-CDs K_c (M^{-1})	γ-CDs K_c (M^{-1})	HP-β-CDs K_c (M^{-1})	G_2-β-CDs K_c (M^{-1})
quercetin		426	233	1195	650
myricetin		125		830	335

One interesting property of cyclodextrins is that they can be saturated with flavonols, increasing the total concentration of flavonol in solution, whereas the free flavonol concentration remains constant. To study this effect, solubility studies of quercetin and myricetin were made at acidic pH, as described by Higuchi and Connors (*25*).

Phase solubility diagrams of quercetin and myricetin at pH 4.5 with HP-β-CDs or G_2-β-CDs are shown in **Figure 5**. In all cases phase solubility diagrams showed a linear relationship between the amounts of flavonol solubilized and the concentration of cyclodextrin in solution (A_L type), indicating that the stoichiometry of complexes was 1:1 in all cases. Using the equation described by Higuchi and Connors (*25*)

$$K_c = \frac{\text{slope}}{S_0(1 - \text{slope})} \tag{11}$$

where S_0 is the solubility of flavonoid in water, the K_c values were calculated (**Table 2**). As can be seen in **Table 2**, the K_c values obtained using the solubility method were similar to those

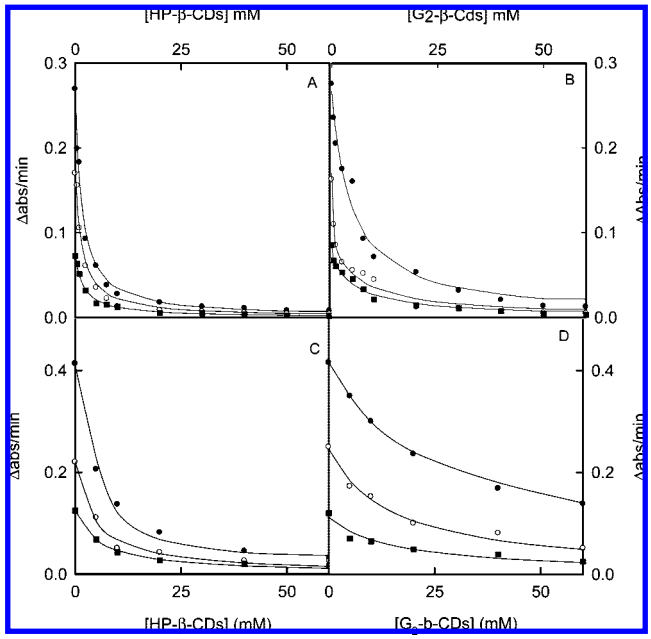

Figure 3. Effect of modified cyclodextrin concentration on flavonol oxidation by peroxidase: (**A**) oxidation of quercetin in the presence of HP-β-CDs; (**B**) oxidation of quercetin in the presence of G_2-β-CDs; (**C**) oxidation of myricetin in the presence of HP-β-CDs; (**D**) oxidation of myricetin in the presence of G_2-β-CDs; (●) 60 μM flavonol; (○) 30 μM flavonol, (■) 15 μM flavonol. The reaction medium at 25 °C contained 100 mM sodium acetate buffer, pH 4.5, 400 μM H_2O_2, 0.037 unit of horseradish peroxidase for quercetin oxidation and 0.1 unit of horseradish peroxidase for myricetin oxidation, and increasing concentrations of CDs.

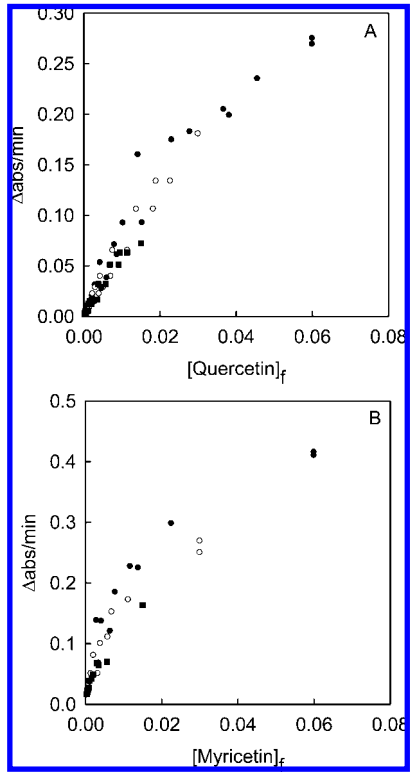

Figure 4. Effect of free flavonol concentration on peroxidase activity: (**A**) quercetin; (**B**) myricetin. The free flavonol concentrations were calculated from the data shown in **Figure 3**, using eq 9 (see text for details).

obtained with the enzymatic method (**Table 1**), indicating the validity of both methods.

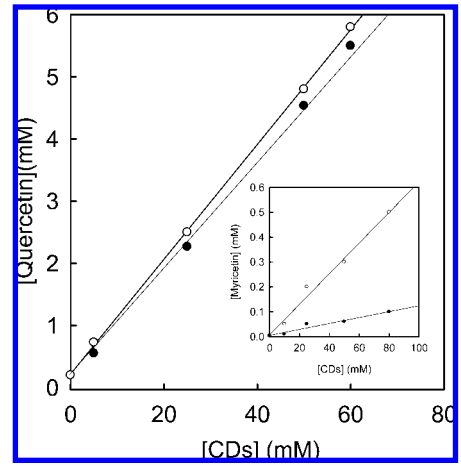

Figure 5. Phase of solubility diagram of quercetin with HP-β-CDs (○) and G_2-β-CDS (●) in sodium acetate buffer (100 mM, pH 4.5) at 25 °C. (Inset) Phase of solubility diagram of myricetin with HP-β-CDs (○) and G_2-β-CDS (●) in sodium acetate buffer (100 mM, pH 4.5) at 25 °C.

Table 2. Stability Constants (K_c) for Complexes Formed between Different Types of Cyclodextrins and the Two Flavonols under Study in Sodium Acetate Buffer (100 mM, pH 4.5), Obtained by the Physical Method

guest	β-CDs K_c (M^{-1})	HP-β-CDs K_c (M^{-1})	$G_2\beta$-CDs K_c (M^{-1})	S_0 (mM)	CE HP-β-CDs
quercetin	398	950	917	0.1281	121.7
myricetin	110	850	380	0.00625	5.3

However, it is important to note that when cyclodextrin or complexation saturating conditions are selected, it is more convenient to compare the complexation efficiency (CE) than K_c values. For 1:1 complexes, the CE can be calculated from the slope of the phase solubility diagram:

$$\mathrm{CE} = \frac{[\text{flavonol} - \text{CD}]}{[\text{CD}]_t} = S_0 \times K_c \qquad (12)$$

The CE value takes into account both the K_c value and the water solubility of the flavonol (S_0) in those conditions.

Therefore, in the case of HP-β-CDs, the K_c values obtained for quercetin and myricetin at pH 4.5 were very similar (950 and 850 M^{-1}, respectively), but, as can be seen in **Figure 5**, the level of total quercetin was always higher than the level of total myricetin. This result is in accordance with the CE value obtained for the complexation of quercetin and myricetin by HP-β-CDs at pH 4.5 (121.7 and 5.3, respectively) (**Table 2**), indicating that HP-β-CDs are more efficient for the complexation of quercetin than myricetin in the studied conditions, despite the K_c values being very similar in both cases.

In conclusion, this paper clearly shows that the flavonols quercetin and myricetin can be included in cyclodextrin at acidic pH to inhibit their enzymatic oxidation. This inhibition permitted us to calculate the complexation constants between flavonols and cyclodextrins. HP-β-CDs were the most effective, complexing both flavonols. It is important to note that cyclodextrins can be used not only to decrease the free flavonol concentration while the total flavonol concentration remains constant (in which case the free flavonol concentration depends only on the complexation constant) but also to increase total flavonol concentration while the free flavonol (S_0) concentration remains constant. In the latter case, the level of total flavonol depends on the complexation efficacy.

The complexation of polyphenols has proven to be useful in both enhancing the solubility and their bioavailability and also

protecting them from enzymatic oxidation. β-CDs and their derivates may be useful in improving the dissolution and the bioavailability of quercetin and myricetin in functional beverages.

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