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Cyclodextrins as resveratrol carrier system

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Abstract

The formation of resveratrol–cyclodextrin inclusion complexes in aqueous solutions has been characterized using the hydroperoxidase activity of lipoxygenase as the enzymatic system. The addition of cyclodextrins to the reaction medium had an inhibitory effect on resveratrol oxidation by lipoxygenase due to the complexation of phytoalexin into the cyclodextrin cavity, which is in equilibrium with free cyclodextrins and free resveratrol, the only effective substrate for lipoxygenase. This inhibitory effect depends on the complexation constant K_c between resveratrol and the type of cyclodextrins used. In the present work β- and G_2-β-cyclodextrins were used and their K_c were calculated by nonlinear regression of the inhibition curves obtained in the presence of cyclodextrins. The values obtained were 4317 and 5130 M^{-1} for β- and G_2-β-cyclodextrin, respectively, values which were checked and confirmed by the "cyclodextrin assay".

Keywords: Resveratrol; Cyclodextrins; Encapsulation; Lipoxygenase; Enzymatic method

1. Introduction

Resveratrol (*trans*-3,5,4′-trihydroxystilbene) is a triphenolic phytoalexin found in a variety of plant species (Frémont, 2000; Sanders, McMichael, & Hendrix, 2000), such as grapevines, mulberries and peanuts. Its synthesis is triggered by plant stress conditions such as fungal infection, UV irradiation and exposure to ozone or heavy metal ions (Frémont, 2000).

Because of its high concentration in grape skin, but not in grape flesh, white wine contains small amounts of resveratrol compared with red wine. It has been suggested that the significant amounts of resveratrol present in red wine (Goldberg, Hahn, & Parkes, 1995) might explain, at least in part, the so-called "French paradox", the apparent ability of moderate consumption of red wine to reduce the risk of cardiovascular disease (Bertelli et al., 1995; Frankel, Waterhouse, & Kinsella, 1993; Goldberg et al., 1995; Pace-Asciak, Hahn, Diamandis, Soleas, & Goldberg, 1995).

The phenolic nature of resveratrol explains its antioxidant activity. It has been shown to provide health-promoting benefits such as lowering the incidence of coronary heart disease (Frankel et al., 1993), and to possess cancer chemoprotective activity (Jang et al., 1997) and strogenic activity with varying degrees of estrogen receptor agonism (Ghem, McAndrews, Chien, & Jameson, 1997) due to its similarity in structure with the synthetic estrogen diethylstilbestrol (DES) (Ghem et al., 1997).

Resveratrol also interferes with the arachidonate metabolism by reducing the levels of leukotrienes generated by the lipoxygenase pathway and prostaglandins generated by the cyclooxigenase (COX) pathway (Jang et al., 1997; Kimura, Okuda, & Arichi, 1985; Subbaramaiah et al., 1998). It has been described as a potent competitive inhibitor of the dioxygenase activity of lipoxygenase (LOX) (Pinto, García-Barrado, & Macías, 1999).

Recently it has been concluded that resveratrol blocks the activation of the COX-2 gene (the gene that activates the COX-2 enzyme). This ingredient of functional foods was the first compound identified to both block the COX-2 gene and to inactivate the enzyme created by this

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gene (Martin, Villegas, La Casa, & De La Lastra, 2004). Some researchers believe that resveratrol may some day be viewed as an improvement over acetylsalicylic acid in fighting diseases associated with COX-2 activity, while to gain such benefits, it is either necessary to eat a few red grapes every day or take a supplement containing resveratrol.

The high hydrophobicity of resveratrol and its sensitivity to external agents such us air, light and oxidative enzymes may constitute a serious problem for its bioavailability, formulation and manipulation in the elaboration of functional foods.

Cyclodextrins (CDs) are a group of naturally occurring cyclic oligosaccharides derived from starch with six, seven or eight glucose residues linked by $\alpha(1 \rightarrow 4)$ glycosidic bonds in a cylinder-shaped structure, and denominated α-, β- and γ-cyclodextrins, respectively. The central cavity of these molecules is hydrophobic, while the rims of the surrounding walls are hydrophilic. This hydrophobic cavity forms inclusion complexes with a wide range of organic and inorganic guest molecules (Cai et al., 1990), altering their physicochemical behaviour and reducing their undesirables 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 (Buschmann & Schollmayer, 2002; Loftsson & Brewster, 1996; Szejtli, 1988). It was demonstrated that complexation can considerably increase the solubility, stability and bioavailability of the guest molecule.

Given resveratrol's many health-promoting activities, it is necessary to develop a method for increasing its bioavailability and stability.

In this paper, the *in vitro* formation of resveratrol–cyclodextrin inclusion complexes was characterized using the enzymatic system, lipoxygenase, which oxidizes resveratrol in the presence of H_2O_2. A study of this oxidation in the presence of cyclodextrins permits determination of the complexation constant between resveratrol and cyclodextrins.

2. Materials and methods

Electrophoretically pure (Kulkarni & Cook, 1988) soybean lipoxygenase (EC 1.13.11.12) Type V (701,000 Sigma units/mg protein) prepared by affinity chromatography and *trans*-resveratrol were purchased from Sigma (Madrid, Spain). Hydrogen peroxide was purchased from Aldrich (Madrid, Spain). Cyclodextrins were kindly supplied by Amaizo, American Maize-Products Company, Hammond, Indiana. All other chemicals used were of analytical grade.

The hydrogen peroxide, lipoxygenase and resveratrol solutions were freshly prepared every day, and their concentrations were calculated using $\varepsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$ (Nelson & Kiesow, 1972), $\varepsilon_{280} = 160{,}000\ M^{-1}\ cm^{-1}$ (Spaapen, Veldink, Liefkens, Vliegenthart, & Kay, 1979) and $\varepsilon_{312} = 33{,}400\ M^{-1}\ cm^{-1}$ (Pinto et al., 1999), respectively.

The activity was followed spectrophotometrically in a Shimadzu model S33 spectrophotometer at the absorption maximum of the oxidation product of resveratrol (250 nm).

Differences between the extinction coefficients ($\Delta\varepsilon$) corresponding to the difference between absorption due to substrate and reaction products were calculated for the oxidation process. The $\Delta\varepsilon$ values were calculated from a calibration curve of resveratrol (0–30 μM) quantitatively oxidized in the presence of an excess of sodium periodate, by plotting the increase in absorbance at 250 nm (from $t = 0$ to constant absorbance values) against resveratrol concentration. Taking into account the reaction stoichiometry, in which two resveratrol molecules generate one product molecule (a resveratrol dihydrodimer), the value obtained at pH 9.0 was $\Delta\varepsilon_{250} = 29{,}600\ M^{-1}\ cm^{-1}$.

Unless otherwise stated, the standard reaction medium at 25 °C contained 100 mM sodium borate buffer (pH 9.0), 400 μM H_2O_2, 30 μM resveratrol and 50 nM of soybean lipoxygenase 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 the components of the reaction medium except the enzyme served as the control.

3. Results and discussion

LOX (linoleate: oxygen oxidoreductase; EC 1.13.11.12) is a non-heme iron-containing enzyme that has a dual function: dioxygenase and hydroperoxidase (Joseph, Srinivasan, & Kulkarni, 1993; Kulkarni & Cook, 1998; Naidu & Kulkarni, 1994). This enzyme catalyzes the oxidation of resveratrol to its oxidized form in the presence of H_2O_2 in the reaction medium (Pinto et al., 1999; Pinto, García-Barrado, & Macías, 2003) (Fig. 1a). This enzyme also catalyzes the oxidation of resveratrol in the presence of cyclodextrins, producing a characteristic decrease in the resveratrol maximum at 312 nm, with a concomitant increase in absorbance at 250 nm, which can be attributed to the production of δ-viniferin (Fig. 1b). The formation of three isosbestic points at 240, 280 and 360 nm indicated that resveratrol was transformed into its oxidized form at a constant ratio in both cases (Fig. 1a and b). However, as can be seen from Fig. 1 inset, the oxidation rate decreased in the presence of cyclodextrins.

The fact that the oxidation of resveratrol by the hydroperoxidase activity of LOX decreased in the presence of cyclodextrins can be used to determine the complexation constant by means of the enzymatic method developed by our group (López-Nicolás, Bru, & García-Carmona, 1997; Núñez-Delicado, Sojo, Sánchez-Ferrer, & García-Carmona, 1999a, Núñez-Delicado, Sojo, Sánchez-Ferrer, & García-Carmona, 1999b).

The oxidation of resveratrol by the hydroperoxidase activity of lipoxygenase was carried out in the presence of two different types of cyclodextrins, β- and maltosyl-β cyclodextrins (β-CDs and G_2-β-CDs), to determine the

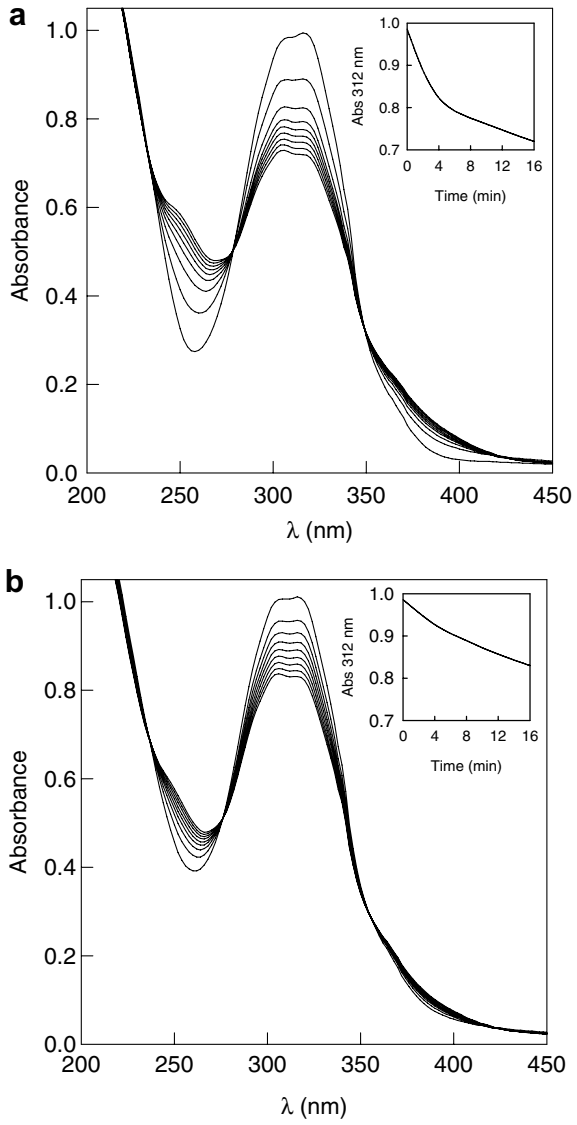

Fig. 1. Oxidation of resveratrol by lipoxygenase in the presence of H_2O_2. The reaction medium at 25 °C contained 100 mM sodium borate buffer, pH 9,0, 30 μM resveratrol, 400 μM H_2O_2, and 50 nM lipoxygenase. (a) In the absence of CDs. (Inset) Time course of the oxidation of resveratrol in the absence of CDs. (b) In the presence of G_2-β-CDs 1 mM. The scans were obtained every 2 min. (Inset) Time course of the oxidation of resveratrol in the presence of CDs.

complexation constant between resveratrol and each type of cyclodextrins.

An experiment was performed in which LOX activity was determined in the presence of increasing concentrations of β-CDs or G_2-β-CDs, while the resveratrol concentration was kept constant. As can be seen in Fig. 2, LOX activity decreased as the cyclodextrin concentration increased, depending on the type of cyclodextrin used, the inhibition being greater in the case of G_2-β-CDs than in the case of β-CDs.

These results indicated that resveratrol, a phytoalexin with three hydroxyl groups in its structure, may enter in the hydrophobic cavity of both β-CDs and G_2-β-CDs to form inclusion complexes.

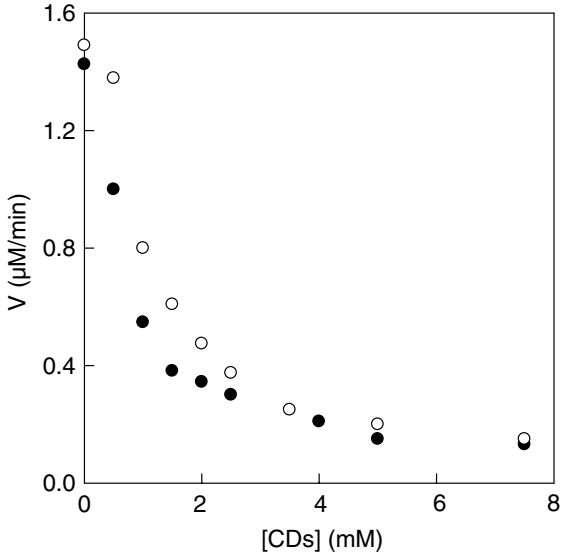

Fig. 2. Effect of cyclodextrin on the oxidation of resveratrol by lipoxygenase in the presence of H_2O_2. The reaction medium at 25 °C contained 100 mM sodium borate buffer, pH 9.0, 30 μM resveratrol, 400 μM H_2O_2, 50 nM lipoxygenase and increasing concentrations of G_2-β-CDs (●) or β-CDs (○).

When increasing concentrations of G_2-β-CDs were added to the reaction medium at different resveratrol concentrations, a clear decrease in LOX activity was observed in all cases (Fig. 3). Assuming that this inhibitory effect was due to the formation of the inclusion complexes and that free resveratrol is the only form of substrate which LOX can use, the Michaelis-Menten velocity equation could be expressed as a function of free resveratrol concentration ($[resv]_f$):

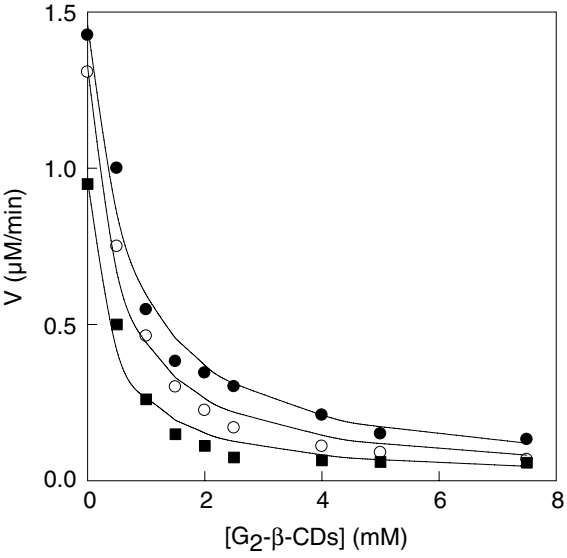

Fig. 3. Effect of G_2-β-CDs on the oxidation of resveratrol by lipoxygenase in the presence of H_2O_2. The reaction medium at 25 °C contained 100 mM sodium borate buffer, pH 9.0, 30 μM resveratrol, 400 μM H_2O_2, 50 nM lipoxygenase and increasing concentrations of G_2-β-CDs. (●) 10 μM resveratrol, (○) 20 μM resveratrol, (■) 30 μM resveratrol.

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

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

$$[\text{resv}]_f + [\text{CD}]_f \overset{K_c}{\leftrightarrow} [\text{resv–CD}] \quad (2)$$

where the complexation constant, K_c, is defined as:

$$K_c = \frac{[\text{resv–CD}]}{[\text{resv}]_f[\text{CD}]_f} \quad (3)$$

Taking into account the mass balance

$$[\text{resv}]_t = [\text{resv}]_f + [\text{resv–CD}] \quad (4)$$

$$[\text{CD}]_t = [\text{CD}]_f + [\text{resv–CD}] \quad (5)$$

and Eq. (3), $[\text{CD}]_f$ and $[\text{resv–CD}]$ can be expressed as:

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

$$[\text{resv–CD}] = K_c[\text{resv}]_f[\text{CD}]_f \quad (7)$$

Then, substituting these two later equations into Eq. (4), the following quadratic relationship is obtained:

$$K_c[\text{resv}]_f^2 + \left([\text{CD}]_tK_c - [\text{resv}]_tK_c + 1\right)[\text{resv}]_f - [\text{resv}]_t = 0 \quad (8)$$

From this, $[\text{resv}]_f$ can be obtained:

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

and substituting into Eq. (1), to give:

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

This Eq. (10) shows a nonlinear relationship between v and $[\text{CD}]_t$ as in Fig. 3. Fitting the data by nonlinear regression using Sigma Plot (Jandel Scientific), a value of $5130 \pm 421\ \text{M}^{-1}$ was obtained for K_c between resveratrol and G_2-β-CDs. This K_c value was similar to that determined by Morales, Bru, García-Carmona, Ros Barceló, and Pedreño (1998) between resveratrol and dimethyl-β-CDs ($6933\ \text{M}^{-1}$) using the physical method described by Higuchi and Connors (1965). In order to clarify whether LOX was only working with free resveratrol, the data in Fig. 3 were replotted in Fig. 4 as a function of free resveratrol, using Eq. (9). The new plot showed that the points of the different curves in Fig. 3 which represent the same activities also have the same $[\text{resv}]_f$ in Fig. 4. This clearly indicates that the enzyme was only sensitive to free resveratrol, as has been previously described for other LOX substrates included in CDs (Núñez-Delicado et al., 1999a,1999b; Núñez-Delicado, Sánchez-Ferrer, & García-Carmona, 1997). To confirm that LOX works only with free resveratrol and not with the resveratrol–CD complex ([resv–CD]) as well, as has been described for persimmon PPO (persimmon), the data of Fig. 3 were replotted as a function of the complexed resveratrol (Fig. 5):

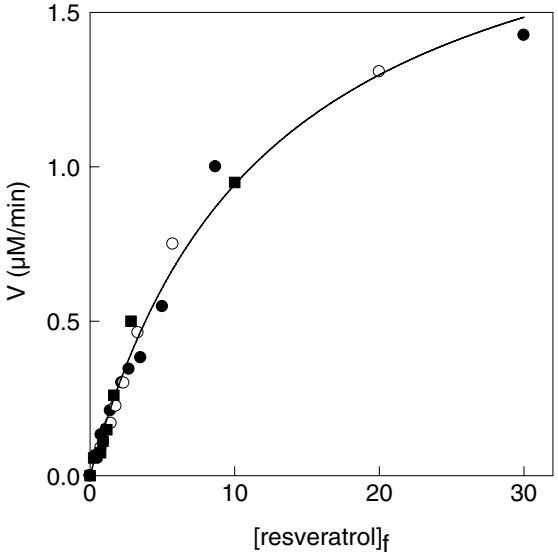

Fig. 4. Effect of free resveratrol concentration on lipoxygenase activity. The free resveratrol concentrations were calculated from data shown in Fig. 3, using Eq. (9) (see text for details). (●) 10 μM total resveratrol, (○) 20 μM total resveratrol, (■) 30 μM total resveratrol.

$$[\text{resv–CD}] = [\text{resv}]_t - [\text{resv}]_f \quad (11)$$

This clearly shows that the enzymatic activity responded to free resveratrol concentrations (Fig. 4) and not to complexed resveratrol (Fig. 5), because Fig. 4 gives a similar Michaelis-Menten representation of the data with a K_M value equal to that found in the absence of cyclodextrins (13.5 μM).

Knowing the complexation constant K_c between resveratrol and G_2-β-CDs, it is possible to control the concentration of free resveratrol and therefore the enzymatic

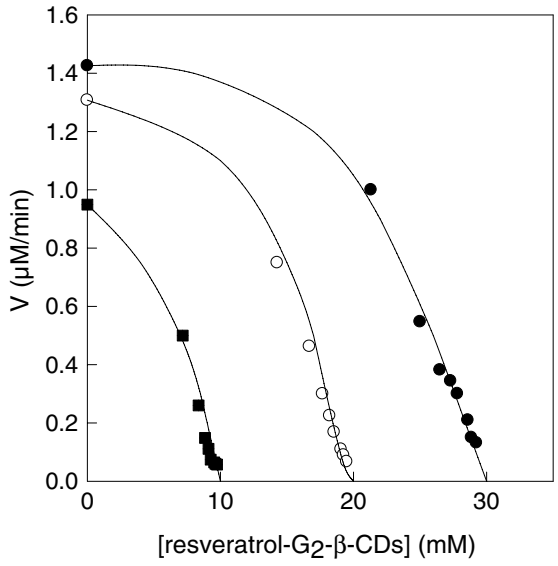

Fig. 5. Effect of resveratrol-G_2-β-CDs complex concentration on lipoxygenase activity. (●) 10 μM total resveratrol, (○) 20 μM total resveratrol, (■) 30 μM total resveratrol.

activity, by setting the appropriate concentrations of both total G_2-β-CDs and total resveratrol. To confirm this, the so-called cyclodextrin assay (López-Nicolás et al., 1997), was carried out, in which, by using the complexation constant K_c determined enzymatically ($5130 \pm 421\ M^{-1}$) and Eq. (9), total resveratrol is calculated so that free resveratrol remains constant, independent of the G_2-β-CDs concentration used. As shown in Fig. 6, LOX activity was independent of the total resveratrol concentration or total G_2-β-CDs concentration.

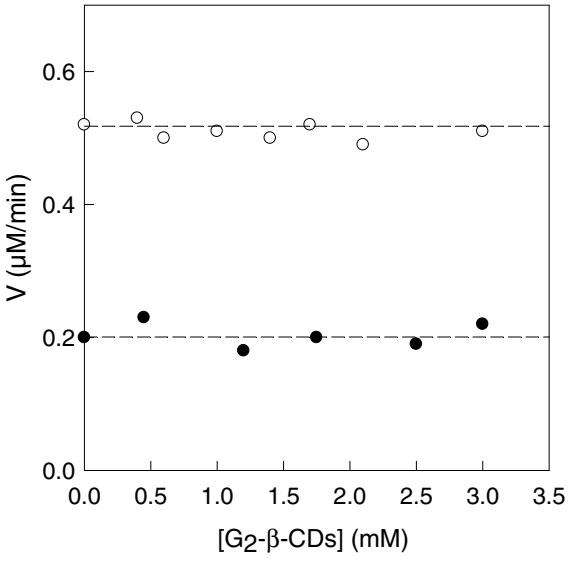

Fig. 6. Cyclodextrin assay. Lipoxygenase-catalyzed oxidation of resveratrol reaction rates at two different free resveratrol concentrations. (●) 1.5 μM resveratrol, (○) 5 μM resveratrol. The G_2-β-CDs and resveratrol concentrations were calculated using the K_c value obtained by the enzymatic method and Eq. (9) (see text for details).

Table 1
Cyclodextrin assay

$[resveratrol]_t$ (μM)	$[β\text{-CDs}]_t$ (mM)	V (μM/min)
15	0	1.25 ± 0.025
30	0.25	1.15 ± 0.125
60	0.7	1.32 ± 0.025
120	1.7	1.26 ± 0.015
250	4	1.21 ± 0.135
500	8	1.26 ± 0.015

The β-CDs and resveratrol concentrations were calculated to yield 15 μM free resveratrol using the K_c value ($4317\ M^{-1}$) determined enzymatically.

All the above experiments were also performed with β-CDs (data not shown), in which case a complexation constant K_c value of $4317 \pm 338\ M^{-1}$was obtained. This K_c value was slightly lower than that described for G_2-β-CDs, indicating that the affinity of this natural β-cyclodextrins for resveratrol was slightly lower than that shown for the modified G_2-β-CDs. These results agree with the experimental data depicted in Fig. 2, in which the inhibition presented by G_2-β-CDs was slightly higher than that presented by β-CDs.

The cyclodextrin assay was carried out using this K_c value between resveratrol and β-CDs,. In this case, as can be seen in Table 1, LOX activity remained constant, regardless of the total resveratrol concentration or total β-CDs concentration. These results were similar to those obtained in the case of G_2-β-CDs, indicating that LOX works only with free resveratrol concentration in both cases, β-CDs and G_2-β-CDs.

In conclusion, the present study demonstrates that CDs can be used as resveratrol complexation agent, to increase total resveratrol concentration in aqueous solution, while free resveratrol concentration remains constant, as do its biological activities. The delay in the resveratrol oxidation was caused by its entrapment in the internal cavity of cyclodextrins, which act as substrate reservoir in a dosage-controlled manner. Moreover, we have characterized the complexation process and demonstrated that both β-CDs and G_2-β-CDs served as resveratrol carrier systems, with similar K_c values. The cyclodextrin assay served to demonstrate that the K_c values obtained by the enzymatic method using LOX activity on resveratrol were correct for both cases, β-CDs and G_2-β-CDs.

Acknowledgements

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