Cyclodextrins as Secondary Antioxidants: Synergism with Ascorbic Acid

Estrella Núñez-Delicado, Alvaro Sánchez-Ferrer, and Francisco García-Carmona*

Department of Biochemistry and Molecular Biology-A, Faculty of Biology, University of Murcia, Campus de Espinardo, E-30071 Murcia, Spain

The preservation of the antioxidant capacity of ascorbic acid by 2-hydroxypropyl-β-cyclodextrins has been studied. For this, a model system consisting of ascorbic acid, H_2O_2, a phenolic compound, 2,2,5,7,8-pentamethylchroman-6-ol (PMC), and commercial soybean lipoxygenase was used. In this system, ascorbic acid was oxidized by PMC radicals generated by the hydroperoxidative activity of lipoxygenase, giving rise to a biphasic curve, in which the inflection point represents the time at which all the ascorbic acid has been consumed (t_{ip}). This t_{ip} depends on the enzyme, ascorbic acid, and PMC concentration. When 2-hydroxypropyl-β-cyclodextrins were included in the system, the t_{ip} increased, indicating that these cyclodextrins were acting as secondary antioxidants, so preserving the antioxidant capacity of the system. A mathematical model to explain the effect of cyclodextrins on ascorbic acid oxidation in this experimental system is described, giving a constant, K_c, which represents the preservation capacity of a particular antioxidant system produced by cyclodextrins.

Keywords: *Ascorbic acid; cyclodextrins; lipoxygenase; hydroperoxidase; PMC*

INTRODUCTION

Lipid oxidation is the cause of great concern in the food industry because it leads to the development of undesirable off-flavors (rancidity) and potentially toxic reaction products (Halliwel et al., 1995). Food antioxidants have been described as substances that function as free-radical inhibitors by interfering with the free-radical mechanism fundamental to autoxidation (Sherwin, 1972). The classical free-radical mechanism involving lipid oxidation depends on the production of free radicals (initiation phase), $R^{\bullet}$, from unsaturated lipid molecules, RH, by the action of an initiator (Simic and Taylor, 1987; Hamilton, 1989). In the propagation phase, the free radical thus generated can react with oxygen to form a peroxyl radical, $RO_2^{\bullet}$, which can further react with another lipid molecule to generate hydroperoxide, RO_2H, and another lipid radical, $R^{\bullet}$. Finally, in the termination phase two radicals react to give products that do not sustain the propagation phase. Termination also occurs when antioxidants react with the free radicals generated during propagation, thus increasing the shelf life of the product. Consequently, the manufacture of antioxidants for use in foods is an important industry.

Many types of compounds have been used as food antioxidants over the years, including those that function as free-radical scavengers, oxygen absorbers, and chelators. Some of the most widely used synthetic antioxidants are phenolic compounds such as butylated hydoxyanisole (BHA), butylated hydroxytoluene (BHT), mono-*tert*-butylhydroquinone (TBHQ), and propyl gallate, all of which are termed "primary antioxidants". Some of these antioxidants, such as BHA and BHT, are used in various combinations with resulting synergistic effects; that is, the combined effect of the two antioxidants is greater than the sum of individual effects obtained when used alone (Sherwin, 1972).

This synergistic effect should not be confused with the synergism that occurs when a primary antioxidant is added along with acid chelators or sequestrants such as citric, tartaric, oxalic, malic, phytic, or succinic acid (St. Angelo et al., 1988, 1990) which are known as "secondary antioxidants" (Porter, 1980; St. Angelo, 1996).

Because of the growing concern about the potential health hazard presented by synthetic antioxidants (Grice, 1986; Shahidi et al., 1992; Jones, 1992), there is a renewed interest in naturally occurring antioxidants, which are presumed safe. One such naturally occurring primary antioxidant that is widely used in the food industry is ascorbic acid, which, along with its esterified derivatives, functions as an antioxidant by protecting double bonds and scavenging oxygen. One important chemical characteristic of ascorbic acid is its redox properties, which enable it to fuction as a reducing agent and a free-radical scavenger, and its ability to act as a synergist by converting oxidized tocopherols back to the reduced form (Niki et al., 1984).

Cyclodextrins are a unique group of naturally occurring cyclic oligosaccharides, which are derived from starch via a bacterial degradation process. They are composed of six, seven, or eight glucose residues linked by $\alpha(1 \rightarrow 4)$ glycosidic bonds in a cylinder-shaped structure and are denominated as α-, β-, and γ-cyclodextrins, respectively. The central cavity of these molecules is hydrophobic, while the rims of the surrounding walls are hydrophilic. A wide range of organic and inorganic guest molecules can be accommodated in the hydrophobic cavity to form inclusion complexes that exhibit a high degree of water solubility and chemical stability (Lach and Pauli, 1966; Thakkar and Hall, 1968; Tomida et al., 1978; Lundberg et al., 1979; Uekama, 1979; Saenger, 1980). Modified cyclodextrins which have one or more branches of an α-D-glucopyranosyl unit or a $(1 \rightarrow 4)$-α-D-glucan at the carbon 6 site of the glucose residues have many advantages over the parent cyclodextrins. For example, they are highly soluble both in water and in organic solvents and produce a remarkable enhancement of the solubility of water-insoluble compounds by complexation (Koizumi et al., 1987; Okada et al., 1988). Among them, 2-hydroxypropyl-β-cyclodex-

* Author to whom correspondence should be addressed (fax, +34 68 364147; e-mail, alvaro@fcu.um.es).

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trin is one of the most widely used for its high solubility and low cost. These properties have been exploited commercially, especially in pharmaceutical and food fields. However, any increase in the overall antioxidant capacity of a primary antioxidant (ascorbic acid) when cyclodextrins (2-hydroxypropyl-β-cyclodextrins) are present in the media has not been studied. This paper demonstrates that cyclodextrins can act as a natural secondary antioxidant.

MATERIALS AND METHODS

Materials. Electrophoretically pure (Kulkarni and Cook, 1988a) soybean lipoxygenase-L1 (EC 1.13.11.12) type V (646 000 units/mg of protein) prepared by affinity chromatography (Allen et al., 1977) was purchased from Sigma, Madrid, Spain. PMC (2,2,5,7,8-pentamethylchroman-6-ol) was kindly provided by Eisai Co. Ltd., Tokyo, Japan. 2-Hydroxypropyl-β-cyclodextrins were kindly supplied by Amaizo, American Maize-Products Co., Hammond, IN. All other chemicals used were of analytical grade.

Phase Solubility Diagram of 2-Hydroxypropyl-β-cyclodextrins and PMC. The phase diagram was carried out according to Higuchi and Connors (1965). Excess amounts of PMC were added to aqueous solutions containing increasing concentrations of 2-hydroxypropyl-β-cyclodextrins (0−80 mM) in 100 mM phosphate buffer (pH 7.4) and shaken at 25 °C for 3 days to reach equilibrium. The aqueous solutions were then centifuged at 13 000 rpm for 10 min. The supernatant was filtered through a 0.2 μm membrane filter and diluted in 80% (v/v) ethanol−water. The PMC concentration was spectrophotometrically determined and the filtrate absorbance measured at 290 nm.

Measurement of PMC Oxidation by Lipoxygenase. The oxidation of PMC to PMC-quinone was followed spectrophotometrically in a Uvikon 940 (Kontron) or HP8452A diode array spectrometer. This was not done at the absorption maximum of the PMC-quinone ($\lambda_{max} = 272$ nm) but at 255 nm ($\epsilon_{255} = 11\,315\ M^{-1}\ cm^{-1}$), where the difference between the PMC baseline and the absorbance of PMC-quinone was very pronounced. This left shift in the wavelength was due to the high absorption maximum of PMC ($\lambda_{max} = 290$ nm, $\epsilon_{290} = 2690\ M^{-1}\ cm^{-1}$). The reference cuvette contained all of the components of the reaction medium except the enzyme. The hydrogen peroxide and lipoxygenase solutions were freshly prepared every day, and their concentrations were calculated using $\epsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$ (Nelson and Kiesow, 1972) and $\epsilon_{280} = 160\,000\ M^{-1}\ cm^{-1}$ (Spaapen et al., 1979), respectively.

Ascorbic Acid Obtained from Kiwi Fruit. A 20 g sample of fresh peeled kiwi fruit in 40 mL of phosphate buffer (100 mM, pH 7.4) was homogenized for 30 s in a high-speed blender and centrifuged at 10000*g* for 30 min at 4 °C. This supernatant was used as the ascorbic acid source. The concentration of ascorbic acid present in this supernatant was determined using a test color kit for L-ascorbic acid supplied by Boehringer Mannheim (Barcelona, Spain).

RESULTS

Lipoxygenases (EC 1.13.11.12, linoleate:oxygen oxidoreductase) are non-heme iron-containing oxidases that have been found in plants, animal tissues (including marine products), and, more recently, mushrooms and other fungi. They have two activities, dioxygenase and hydroperoxidase, which are associated with a single protein (Kulkarni and Cook, 1988b; Joseph et al., 1993; Naidu et al., 1994). By acting as dioxygenase, lipoxygenases (LOX) convert polyunsaturated fatty acids with a *cis,cis*-1,4-pentadiene unit (linoleic or arachidonic acid) to pentadienyl radical intermediates, which are then trapped by oxygen to yield *cis,trans*-conjugated diene hydroperoxides (Wiseman et al., 1988). These latter compounds act as cosubstrates for the second hydroperoxidative activity. This latter reaction is slow (Balls et al., 1943) but increases if a suitable electron donor, as cosubstrate, is included in the reaction medium (Balls et al., 1943; Kemal et al., 1987; Mansuy et al., 1988) or if the fatty acid hydroperoxide is replaced by H_2O_2 (Kulkarni and Cook, 1988a; Pérez-Gilabert et al., 1994a,b; Núñez-Delicado et al., 1996, 1997). These two LOX-catalyzed reactions are of interest to food scientists, both because of their role in the genesis of volatile flavor and aroma compounds in plant products and because of their ability to form free radicals which can then attack other constituents, such as proteins, colors, phenolics, and vitamins. In fact, Roy and Kulkarni (1996) have recently described the co-oxidation of ascorbic acid with linoleic acid.

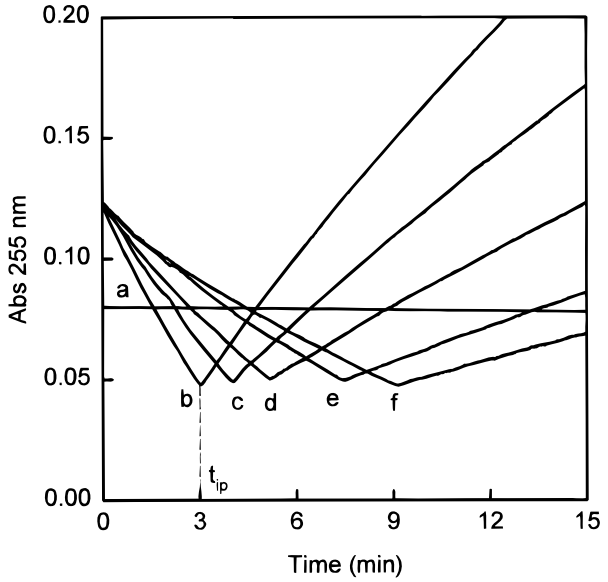


Figure 1. PMC-mediated oxidation of commercial ascorbic acid by lipoxygenase: (a) reaction medium contained 100 mM phosphate buffer, pH 7.4, 200 μM H_2O_2, 6.8 μM ascorbate, and 0.25 μM LOX; (b−f) effect of cyclodextrin concentration. The reaction medium was the same as for part a but with 81.7 μM PMC and increasing concentrations of 2-hydroxypropyl-β-cyclodextrins: (b) 0 μM CD, (c) 125 μM CD, (d) 375 μM CD, (e) 750 μM CD, and (f) 1 mM CD; t_{ip}, time of inflection point (see text for details).

However, when ascorbic acid was incubated with commercial soybean lipoxygenase in the presence of H_2O_2 in 100 mM phosphate buffer, pH 7.4, no oxidation was observed under the assay conditions (Figure 1a). However, when a poorly water-soluble tocopherol model compound, PMC (2,2,5,7,8-pentamethylchroman-6-ol) (Chart 1), was added to the latter medium, a biphasic trace was obtained at 255 nm (Figure 1b). The initial decrease in absorbance was due to the oxidation of ascorbate to dehydroascorbate by PMC radicals with the concomitant recycling of one molecule of PMC (Scheme 1, step 2). After the depletion of ascorbate, here referred to as time of the inflection point (t_{ip}), the reaction was followed by an increase in absorbance due to the appearance of PMC-quinone (Scheme 1, step 3) (at 272 nm) with a concomitant decrease in PMC ultraviolet peak at 292 nm (Figure 2A), both PMC and quinone in equilibrium with CD. The formation of an isosbestic point at 286 nm indicated that PMC was transformed into PMC-quinone at a constant ratio. Matrix analysis of the repetitive scan of Figure 2A using the method of Coleman et al. (1970) confirmed the presence of two kinetically related species: PMC and PMC-quinone (Figure 2B).

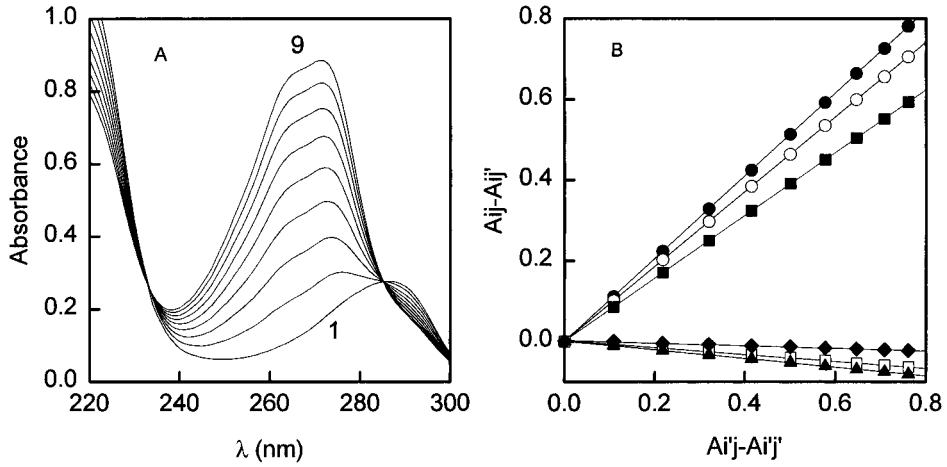
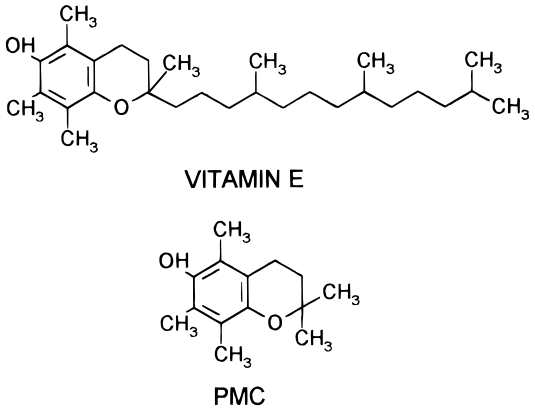


Figure 2. (A) Oxidation of PMC produced by lipoxygenase in the presence of H_2O_2. The reaction medium at 25 °C contained 100 mM phosphate buffer. pH 7.4, 100 μM PMC, 50 μM H_2O_2, and 0.25 μM lipoxygenase. The scans were obtained every 2 min. (B) Coleman's graphical analysis for two absorbing species. In this analysis, A_{ij} is the absorbance at wavelength i obtained during tracing j: i' = 272 nm, j' = first trace, i = ●(264 nm), i = ○ (274 nm), i = ■ (276 nm), i = □ (288 nm), i = ▲ (290 nm), and i = ◆ (547 nm).

Chart 1. Chemical Structures of Vitamin E and PMC

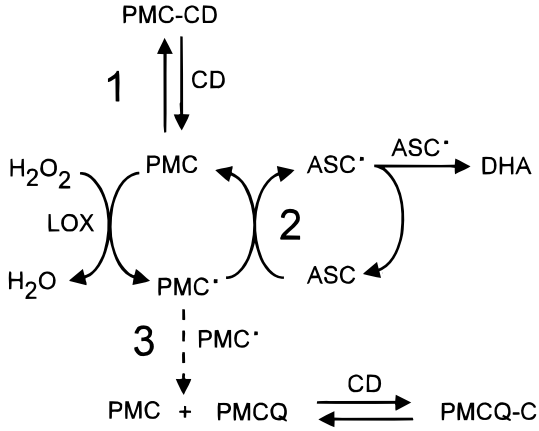


The above inflection point time was proportional to the ascorbate, PMC, and enzyme concentrations (data not shown). This recycling of PMC radicals by ascorbate was similar to that described for vitamin E or Trolox C and ascorbate during lipid peroxidation (Marquard, 1963; Niki et al., 1984; Davies et al., 1988). Similar results were obtained and explained in the horseradish peroxidase-catalyzed oxidation of PMC in the presence of ascorbate and H_2O_2 (Nakamura and Hayashi, 1992).

When increasing concentrations of 2-hydroxypropyl-β-cyclodextrins (0−1.5 mM) were added to the reaction medium, an increase in t_{ip} was observed (Figure 1c−f), compared with the control (Figure 1b). This protective effect of cyclodextrins on ascorbic acid oxidation was also confirmed in the extract of kiwi fruit supplemented with PMC as phenolic compound (Figure 3b−f), compared with the controls (Figure 3a,b). These showed an exponential relationship between cyclodextrin concentration and t_{ip} when both ascorbic acid from kiwi fruit extract (Figure 4, filled square) and commercial ascorbic acid at two different concentrations (Figure 4, open and filled circles) were used.

Since ascorbic acid is a hydrophilic compound, it cannot be included in cyclodextrins, and so, the apparent cyclodextrin-mediated protection of ascorbic acid both *in vitro* and *in vivo* is probably due to the complexation of PMC into the hydrophobic cavity of the cyclodextrins. To test this hypothesis, the oxidation of PMC by lipoxygenase in the presence and absence of ascorbic acid was studied. *In vitro,* a quadratic decrease in enzymatic activity was observed as cyclodextrin concentration was increased (Figure 5).

Scheme 1. PMC-Mediated Oxidation of Ascorbate Catalyzed by Lipoxygenase in the Presence of Cyclodextrins[a]



[a] Free PMC, in equilibrium with cyclodextrin-bound PMC (PMC−CD) is oxidized by lipoxygenase in the presence of H_2O_2 to PMC radical, which rapidly reacts with ascorbate to give ascorbate radical and recycles the PMC. At high ascorbate concentrations, the ascorbate radicals dismutate to give dehydroascorbate, until ascorbate is depleted. Then, PMC radicals dismutate to give PMC-quinone, which is in equilibrium with cyclodextrins. PMC−CD, complex between PMC and cyclodextrins; PMC•, PMC radical; ASC, ascorbate; ASC•, ascorbate radical; DHA, dehydroascorbate; PMCQ, PMC-quinone; PMCQ−CD, complex between PMC-quinone and cyclodextrins.

To understand this inverse relationship, a Michaelis−Menten velocity equation must be obtained as a function of the only two known parameters, i.e., total cyclodextrin concentration and total PMC concentration. For this, it was assumed that the enzyme works in a range of PMC concentrations below K_m, since the water solubility of PMC is in the micromolar order, while the K_m for PMC is in the millimolar order, as has been described for the hydroperoxidative oxidation of other chromanols (Trolox C) by soybean lipoxygenase (Núñez-Delicado et al., 1997). Assuming the above, v can be expressed as:

$$v = \frac{V_m}{K_m}[\mathrm{PMC}]_f \qquad (1)$$

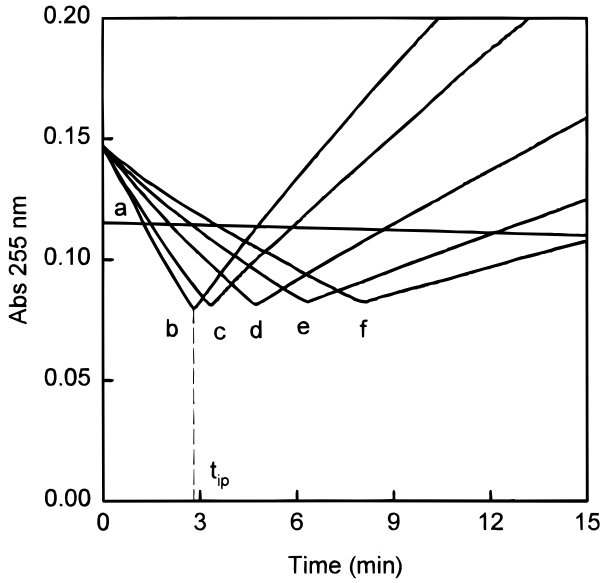


Figure 3. PMC-mediated oxidation of kiwi fruit ascorbic acid by lipoxygenase: (a) reaction medium at 25 °C contained 100 mM phosphate buffer, pH 7.4, 200 μM H_2O_2, 6 μM ascorbate extracted from kiwi fruit, and 0.25 μM LOX; (b−f) effect of cyclodextrin concentration. The reaction medium was the same as for part a but with 81.7 μM PMC and increasing concentrations of 2-hydroxypropyl-β-cyclodextrins: (b) 0 μM CD, (c) 125 μM CD, (d) 375 μM CD, (e) 750 μM CD, and (f) 1 mM CD; t_{ip}, time of inflection point (see text for details).

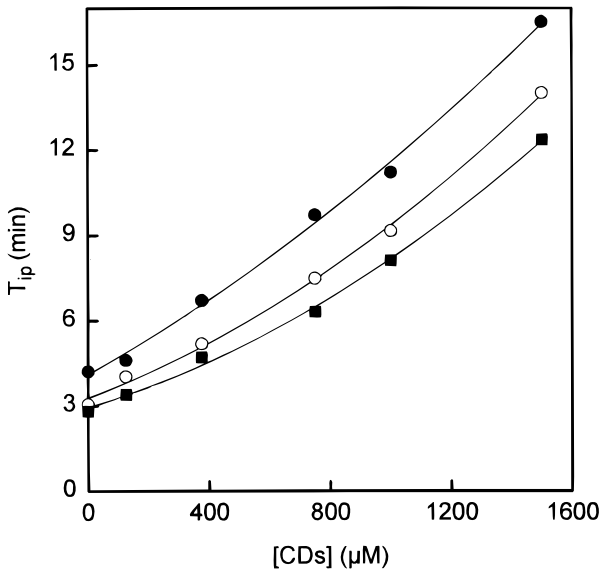


Figure 4. Effect of 2-hydroxypropyl-β-cyclodextrin concentration on t_{ip} in kiwi fruit extract and in a commercial ascorbate solution. The reaction medium at 25 °C contained 100 mM phosphate buffer, pH 7.4, 200 μM H_2O_2, 0.25 μM lipoxygenase, increasing concentrations of 2-hydroxypropyl-β-cyclodextrins (0−1500 μM), and a fixed concentration of PMC and ascorbic acid: (●) 62.37 μM PMC, 6.8 μM commercial ascorbate; (○) 81.7 μM PMC, 6.8 μM commercial ascorbate; (■) 81.7 μM PMC, 6 μM ascorbate extracted from kiwi fruit.

where subscript f refers to the concentration of the free compound, in this case, free PMC.

Then, $[PMC]_f$ can be expressed as a function of $[PMC]_t$ and $[CD]_t$, where the subscript t stands for overall compound concentration, and assuming that only one molecule of PMC may enter into a cyclodextrin molecule (stoichiometry 1:1), the equilibrium can be expressed as:

$$PMCf + CDf \underset{K_c}{\rightleftharpoons} PMC{-}CD_{complex} \quad (2)$$

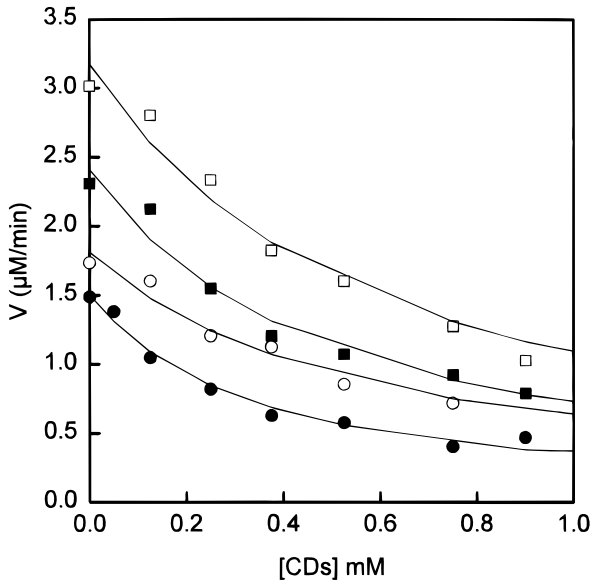


Figure 5. Effect of 2-hydroxypropyl-β-cyclodextrin concentrations on oxidation of PMC by lipoxygenase. The reaction medium at 25 °C contained 100 mM phosphate buffer, pH 7.4, 200 μM H_2O_2, 0.25 μM lipoxygenase, and increasing concentrations of 2-hydroxypropyl-β-cyclodextrins (0−1 mM). Several PMC concentrations were examined: (●) 52.7 μM, (○) 62.16 μM, (■) 81.25 μM, and (□) 106.25 μM, respectively.

where the complexation constant, K_c, is usually defined as

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

Taking into account the mass balance

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

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

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

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

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

Then, substituting these two latter equations into eq 4, the following quadratic relationship was obtained

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

From this, $[PMC]_f$ can be obtained, introduced in eq 1

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

and substituted in eq 1 to give,

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

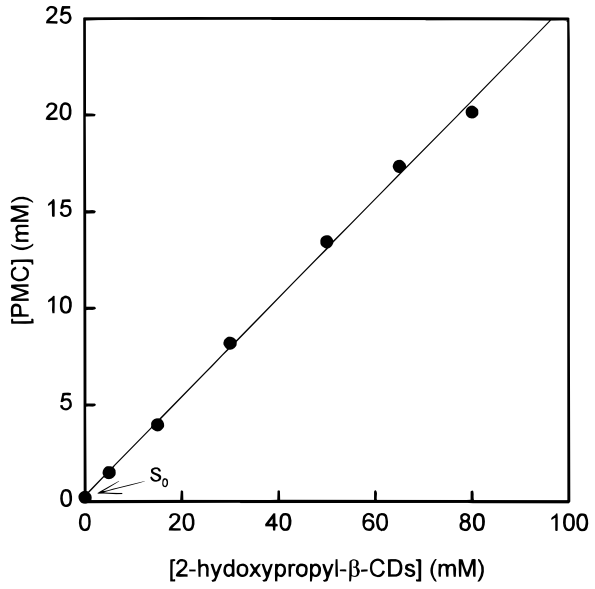


Figure 6. Phase solubility diagram of 2-hydroxypropyl-β-cyclodextrins and PMC in phosphate buffer (100 mM, pH 7.4) at 25 °C. S_0 means the solubility of PMC in water (200 μM).

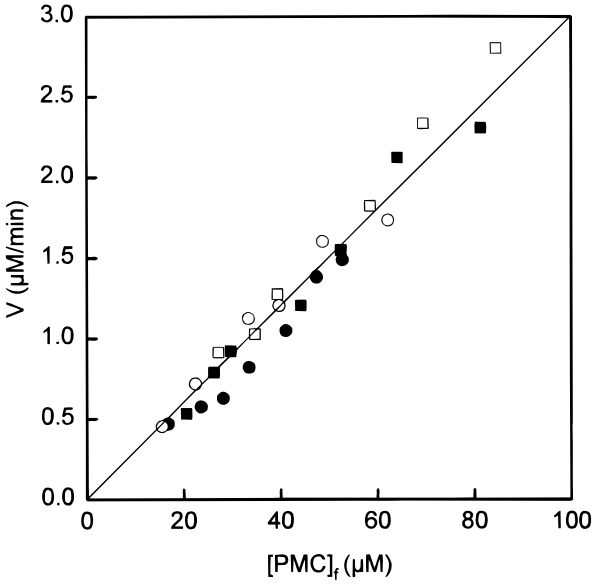


Figure 7. Effect of free PMC concentration on lipoxygenase activity. The free PMC concentrations were calculated from data shown in Figure 5, using eq 9 (see text for details).

This eq 10 shows a nonlinear relationship between v and $[CD]_t$ as in Figure 5. Fitting the data by nonlinear regression using Sigma Plot (Jandel Scientific), a value of 2.5×10^3 M^{-1} was obtained for K_c. This value is in agreement with the K_c calculated using the physical method of Higuchi and Connors (1965) and their equation

$$K_c = \frac{\text{slope}}{[S]_0(1 - \text{slope})} \qquad (11)$$

where $[S]_0$ is the solubility of a compound in water (Figure 6).

The fact that the solubility of PMC increased linearly as a function of the 2-hydroxypropyl-β-cyclodextrin concentration in the PMC phase solubility diagram indicates that the stoichiometry of complexes between PMC and 2-hydroxypropyl-β-cyclodextrins was 1:1, as is well known (Szejtli, 1994), confirming one of the assumptions of the mathematical model. The other assumption that the enzyme was working at PMC concentrations below K_m can be deduced by replotting the data of Figure 5 using eq 9 (Figure 7). It is important to note that the points on the different curves in Figure 5 which represent the same activities also have the same $[PMC]_f$, which clearly indicates that the enzyme is only working with free substrate and not with the complex PMC−CD.

DISCUSSION

There is a growing interest to find new natural antioxidants for use in food, although another approach would be to look for "preservers" of the natural antioxidant capacity of a particular food. In this approach, the use or development of new natural secondary antioxidants is a new and challenging field. In this paper, we show that natural cyclodextrins, which are used as legal additives in many countries (Japan, Sweden, Germany, United States, Argentina, and Hungry) (Szejtli, 1996), can be used to enhance the naturally occurring antioxidant capacity of a food. These cyclodextrins, which act as "secondary antioxidants", have an additional advantage in that they are very inexpensive.

The capacity of cyclodextrins to function as secondary antioxidants can be evaluated for a determined system (e.g., food, cyclodextrins, and oxidative enzyme) using the system's K_c, which can be defined as a protective constant. If K_c is high, equilibrium is displaced to form complexes, which means that the natural substrates are included in the hydrophobic cavity of the cyclodextrins and that they are not available to the oxidative enzymes. This increases the half-life of natural antioxidants in food and prolongs the time that the food can be kept.

ABBREVIATIONS USED

PMC, 2,2,5,7,8-pentamethylchroman-6-ol; LOX, lipoxygenase; CD, cyclodextrins; K_c, complexation constant.

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