Cyclodextrins as Activator and Inhibitor of Latent Banana Pulp Polyphenol Oxidase

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The effect of cyclodextrins (CDs) on *o*-diphenol oxidation catalyzed by banana polyphenol oxidase (PPO) was studied. The oxidation of dopamine, the natural substrate of banana, in the presence of cyclodextrins was unaffected, because this hydrophilic phenol does not form inclusion complexes with CDs. However, when a hydrophobic phenol such as *tert*-butylcatechol (TBC) was used, a marked inhibition was observed with *β*-, hydroxypropyl-*β*-, and maltosyl-*β*- CDs. This inhibition was due to the complexation of TBC in the CD core, demonstrating that banana pulp PPO worked only toward free substrate and not toward the complex TBC−CDs. In addition, the effect of some inhibitors in the presence of CDs and dopamine as substrate was studied. Increasing concentrations of CDs, in the presence of two inhibitors (4-iodophenol and cinnamic acid) were able to activate the inhibited enzyme to reach the noninhibited level by complexing the inhibitors in the hydrophobic core of the CDs. This dual effect of CDs as activator and inhibitor was tested in crude banana pulp extracts, with surprising activation effects never before described being observed.

Keywords: *Aqueous two-phase systemm; polyphenol oxidase; banana; Triton X-114; polyethylene glycol; latent enzyme; cyclodextrins; complex agents*

INTRODUCTION

Enzymatic browning, with a few exceptions (medlars, prunes, black raisins, dates, black figs, zapote, tea, coffee, and cocoa), is considered as product degradation, which lowers fruit quality both visually and with regard to taste and nutritional characteristics (Marquès et al., 1995).

The oxidative reactions that go with this process, independently of their cause (maturation, cold storage, wounding, crushing, resistance to fungal attack, etc.), involve contact between the oxidative enzymes and various (poly)phenol compounds previously compartmentalized in the vacuoles. The main enzyme involved in this process is polyphenol oxidase (PPO), which uses molecular oxygen to catalyze the *o*-hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and their further oxidation to colored and highly reactive *o*-quinones (diphenolase activity) [for reviews, see Mayer and Harel (1979), Mayer (1987), and Sánchez-Ferrer et al. (1995)]. These *o*-quinones readily polymerize and/or react with endogenous amino acids and proteins to form complex brown pigments.

The prevention or inhibition enzymatic browning is a major concern of the food industry all over the world, and attempts have been made to eliminate from the reaction one or more of its essential components: oxygen, enzyme, copper, or (poly)phenols (Richardson and Hyslop, 1985). Oxygen can be excluded from the reaction by immersing the fruits and vegetables in water, syrup, and brine or by exposure to vacuum or modified atmospheric packing (Langdon, 1987). However, this treatment is not definitive, because when the package is opened, oxygen is reintroduced and browning will restart. The enzyme can be effectively heat denatured by steam blanching before freezing or carrying food (McEvily et al., 1992) at the expense of adverse off-flavors and texture changes in fresh material (Langdon, 1987). In this regard, molecular biology is starting to produce antisense PPO mRNA in transgenic plants, to inhibit enzymatic browning in fresh fruits. However, development of this technology will depend on a better knowledge of PPO genes in different plant sources (Bachem et al., 1994) and must at the same time overcome the consumer's refusal of transgenic food.

The most common method for controlling browning is to use sulfites in any of their forms (sulfur dioxide, sodium or potassium metabisulfite, sodium or potassium bisulfite) (Saper, 1993). Sulfites are unique and multifaceted compounds because they are economical and can control both enzymatic and nonenzymatic browning while functioning as antimicrobials, bleaching agents, reducing agents, and antioxidants (Lambrecht, 1995). However, because of adverse health effects, the FDA has prohibited their use in fruits and vegetables served or sold raw to consumers (Taylor, 1986). Other chemical compounds such as acidifiers (citric, malic, and phosphoric acids), chelators (EDTA), and reducing agents (ascorbic acid, alone or in combination) have been used (Fayad et al., 1997). However, recent changes in lifestyle and an awareness of the relationship between food and health have led to a demand for more natural foods and safer and fewer added chemicals in processed foods.

In this respect, there has been a renewal of interest in naturally occurring antibrowning agents. Among the most promising agents are cyclodextrins (CDs), which are naturally occurring cyclic oligosaccharides derived from starch with six, seven, or eight glucose residues linked by α(1→4) glycosidic bonds in a cylindrically shaped cavity with a hydrophobic internal surface and

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a hydrophilic outer surface, designated α, β-, and γ-CDs, respectively (Szejtli, 1994). The hydrophobic cavity is able to form inclusion complexes with a wide range of organic guest molecules, including (poly)phenols (Cai et al., 1990). Such molecules have been proposed as agents in the control of the enzymatic browning of apple products caused by PPO (Sapers et al., 1989; Bachem et al., 1994, Crouzet et al., 1991; Billaud et al., 1995; Fayad et al., 1997), and their use in preventing browning has been patented (Hicks et al., 1990). Recently, the enzymatic control of browning has also been described by our group in the oxidation of phenols caused by lipoxygenase (Nuñez-Delicado et al., 1997), the CDs acting as secondary antioxidants in synergism with ascorbic acid.

The purpose of this work was to study the effect of CDs on the enzymatic phenolic oxidation of banana pulp PPO, which exhibits kinetic properties different from those of apple, endive, and mushroom PPO (Fayad et al., 1997). The study was also extended to phenol mixturess in an attempt to simulate natural conditions. In such systems, we observed a previously undescribed activation of PPO activity by 2-(hydroxypropyl)-β-cyclodextrins (2-OH-P-β-CDs).

MATERIALS AND METHODS

Materials. Bananas (*Musa acuminata* subgr. Cavendish var. Spanish Pequeña Enana) were obtained from a commercial source in Murcia (Coplaca, S.A.). After 24 h in ethylene gas in the dark, the samples were stored at 17 °C for 72 h until they were at stage 4, with the peel "more yellow than green" (Giami and Alu, 1994) and with a soluble solids content of 10%.

Biochemicals were purchased from Sigma (Madrid, Spain) and used without further purification. Triton X-114 (TX-114) was obtained from Fluka (Madrid, Spain) and condensed three times as described by Bordier (1981). Phenolic substrates [dopamine, *tert*-butylcatechol (TBC), and 4-iodophenol] were purchased from Fluka. All CDs used were kindly supplied by Cerestar USA Inc. (Hammond, IN). All other chemicals used were of analytical grade.

Purification of PPO. Banana pulp PPO was obtained by following the method of Sojo et al. (1998a). In brief, a 30 g pulp sample was homogenized with 45 mL of 4% (w/v) TX-114 in 100 mM sodium phosphate buffer (pH 7.3) for 1 min. The homogenate was centrifuged at 15000*g* for 15 min at 4 °C. The supernatant was subjected to temperature-induced phase partitioning by increasing the TX-114 concentration by an additional 6% (w/v) at 4 °C and then warming to 37 °C for 15 min. After 10 min, the solution became spontaneously turbid due to the formation, aggregation, and precipitation of large mixed micelles of detergent that contained hydrophobic protein and phenolic compounds. This solution was centrifuged at 10000*g* for 15 min at 25 °C. The detergent-rich phase was discarded, and the clean supernatant containing the banana PPO was subjected to a different aqueous two-phase system based on PEG 8000 (5% w/w)/potassium phosphate buffer (pH 7.0). After the solution had stirred for 15 min at room temperature, it was centrifuged at 10000*g* for 10 min at 25 °C. The upper PEG-rich phase was discarded, and the phosphate-rich phase containing the banana PPO brought to 15% saturation with solid $(NH_4)_2SO_4$ under continuous stirring at 4 °C. After 1 h, the solution was centrifuged at 60000*g* for 30 min at 4 °C, and the pellet was discarded. $(NH_4)_2SO_4$ was added to the clean supernatant to give 30% saturation, and the mixture was stirred for 1 h at 4 °C. The precipitate obtained between 15 and 35% was collected by centrifugation at the same rotor speed and dissolved in a minimal volume of 100 mM sodium phosphate buffer (pH 7.3). The enzyme was stored at −20 °C. This simple and fast protocol gave a 5-fold purification and 10-fold activation with sodium dodecyl sulfate (SDS) of the enzyme extract, which preserved its monophenolase activity (Sojo et al., 1998b).

Activity Determination. The oxidation of the two diphenolic substrates used was carried out spectrophotometrically, in a Uvikon 940 (Kontron Instruments, Madrid) or HP8452A diode array spectrophotometer, at their respective adsorption maxima, which are 400 nm for *tert*-butylquinone ($\epsilon = 1150$ M^{-1} cm^{-1}) and 475 nm for dopaminochrome ($\epsilon = 3300$ M^{-1} cm^{-1}). The reference cuvette contained all of the components of the reaction medium except the enzyme. The phenolic and CD solutions were freshly prepared.

Determination of Browning. Banana pulp browning was carried out by visual examination of the pulp extract after homogenization for 2 min in a Waring blender.

A 50 g banana pulp sample was homogenized with 75 mL of 100 mM sodium phosphate buffer (pH 7.3) (tube 1). A second 50 g banana pulp sample was homogenized with 10 mM 2-OH-P-β-CDs in 100 mM sodium phosphate buffer (pH 7.3) (tube 2). A third 50 g banana pulp sample was homogenized with 100 mM 2-OH-P-β-CDs in 100 mM sodium phosphate buffer (pH 7.3) (tube 3).

The three tubes were maintained at room temperature for 30 min before being photographed.

RESULTS

Bananas undergo rapid browning as a result of tissue disruption and exposure to oxygen during peeling and slicing operations prior to further processing (Jayaraman et al., 1987). The quantity of bananas used for processing is very small compared with the amount destined for fresh consumption (the second most common fresh foodstuff in volume after milk) (Cano et al., 1997). However, ∼15% of the bananas produced for export are rejected because of their size, skin stains, or other factors (Cano et al., 1990), in which case they are processed to obtain banana puree and flour as a functional ingredient (Giami and Alu, 1994). The successful prevention of banana browning is still a challenge after the banning of sodium bisulfite. In this respect, natural CDs might be an alternative. To model this study, we used partially purified banana pulp PPO from a Spanish banana variety (Pequeña Enana) isolated under mild purification conditions by using a combination of two aqueous phase systems based on Triton X-114 and PEG 8000/phosphate (Sojo et al., 1998a), dopamine as banana natural diphenolic substrate (Palmer, 1963), and three different β-CDs, β-, 2-(hydroxypropyl)-β-, and maltosyl-β-, were used.

When dopamine was assayed in the presence of the above CDs, no inhibition of the activity was found, even at 100 mM 2-OH-P-β-CD (Figure 1, solid circles). This clearly indicates that dopamine is a highly soluble and hydrophilic *o*-diphenol with little or no complexation in CDs. To confirm this, TBC, a more hydrophobic *o*-diphenol, was used. In this case, a clear decrease in activity was found when the concentration of all CDs in the reaction medium was increased, the greatest inhibition being observed with 2-OH-P-β-CD (Figure 1). In the same way, for a given CD concentration, an increase in TBC always resulted in a decrease in inhibition (Figure 2).

Taking into account the equilibrium between free TBC (TBC_f) and free CD (CD_f)

$$TBC_f + CD_f \underset{K_c}{\rightleftarrows} TBC{-}CD_{complex} \quad (1)$$

the complexation constant, K_c

$$K_c = [TBC{-}CD]/[TBC]_f[CD]_f \quad (2)$$

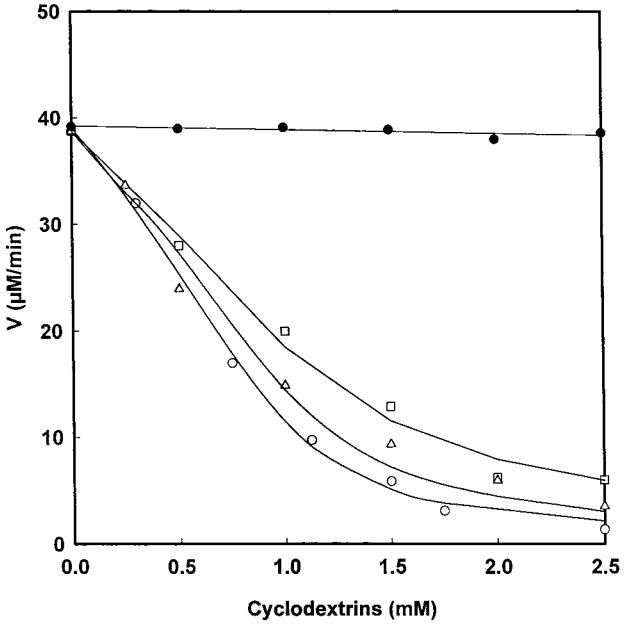

Figure 1. Effect of CDs on the activity of banana pulp PPO: (a) oxidation of 2.5 mM dopamine in the presence of 2-OH-P-β-CD by CDs (●); (b) oxidation of 1 mM TBC in the presence of 2-OH-P-β-CD (○), β-CD (△), and maltosyl-β-CD (□). The reaction medium at 25 °C contained 10 mM phosphate buffer (pH 6.5), 14 μg/mL PPO, and increasing concentrations of CDs (0−2.5 mM).

can be calculated by nonlinear regression to the following equation (López-Nicolás et al., 1997; Nuñez-Delicado et al., 1997)

$$v = \{V_m[(-([CD]_tK_c - [TBC]_tK_c + 1) + \sqrt{([CD]_tK_c - [TBC]_tK_c + 1)^2 + 4K_c[TBC]_t})/2K_c]\}/\{K_M + [(-([CD]_tK_c - [TBC]_tK_c + 1) + \sqrt{([CD]_tK_c - [TBC]_tK_c + 1)^2 + 4K_c[TBC]_t})/2K_c]\} \quad (3)$$

using graphs similar to Figure 2.

Table 1 shows the K_c values obtained with different CDs. This table clearly shows that 2-OH-P-β-CD produced maximal inhibition because its complexation constant was higher than that of the other CDs studied.

To clarify whether the banana PPO was working only with the free substrate and not with the complexed form as previously described for other PPOs, such as mushroom PPO (Fayad et al., 1997), the data of Figure 2 were replotted in Figure 3 as a function of free TBC concentration using the following expression (López-Nicolás et al., 1997; Nuñez-Delicado et al., 1997):

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

Figure 3 clearly shows that the points of the different curves in Figure 2 with the same activity also have the same $[TBC]_f$, which clearly indicates that the enzyme was working only with the free substrate and not with the complexed TBC−CD. This result is in agreement with the data observed with apple and endive PPO but in contrast with that shown with mushroom PPO, which also works with the complex (Fayad et al., 1997). The

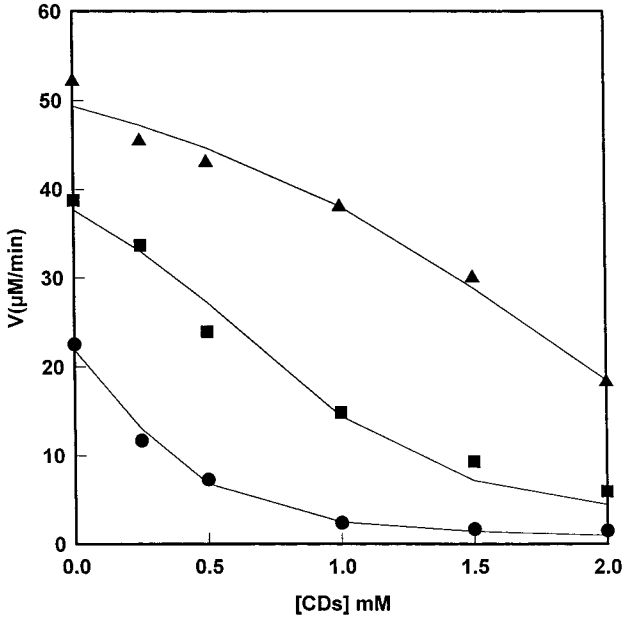

Figure 2. Effect of 2-OH-P-β-CD concentration on the oxidation of TBC by banana pulp PPO. The reaction medium at 25 °C contained 10 mM phosphate buffer (pH 6.5), 7 μg/mL PPO, and increasing concentrations of 2-OH-P-β-CDs (0−2 mM) at different TBC concentrations: (▲) 2, (■) 1, and (●) 0.5 mM.

Table 1. Complexation Constant, K_c, between TBC and CDs

CD	K_c (M^{-1})
2-(hydroxypropyl)-β-cyclodextrins	14920 ± 732
maltosyl-β-cyclodextrins	9505 ± 495
β-cyclodextrins	12010 ± 605

[a] Standard assay conditions were used with the three TBC concentrations (0.5, 1.0, and 2.0 mM) and cyclodextrins concentration ranging from 0 to 2.5 mM.

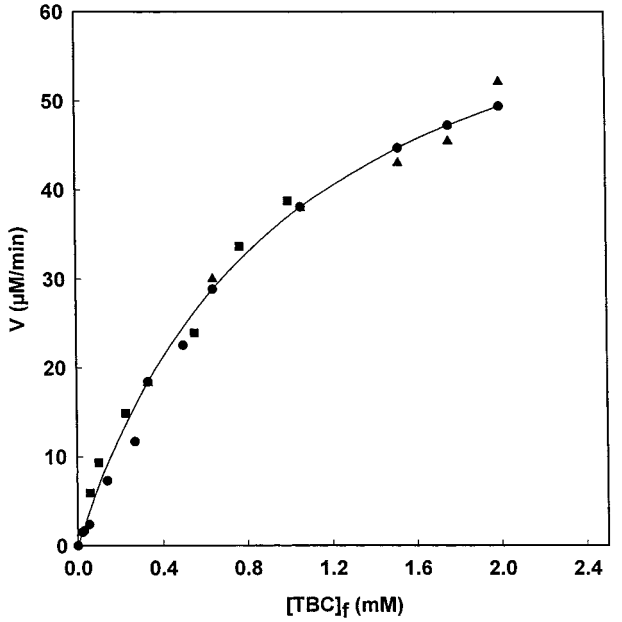

Figure 3. Effect of free TBC concentration on banana pulp PPO activity. The free TBC concentrations were calculated from the data shown in Figure 1, using eq 4 (see text for details).

fact that only commercial mushroom PPO (tyrosinase) works on the phenol−CD complex might be due to the contamination of the commercial preparation with other fungal phenol-oxidizing enzymes (laccase, peroxidase,

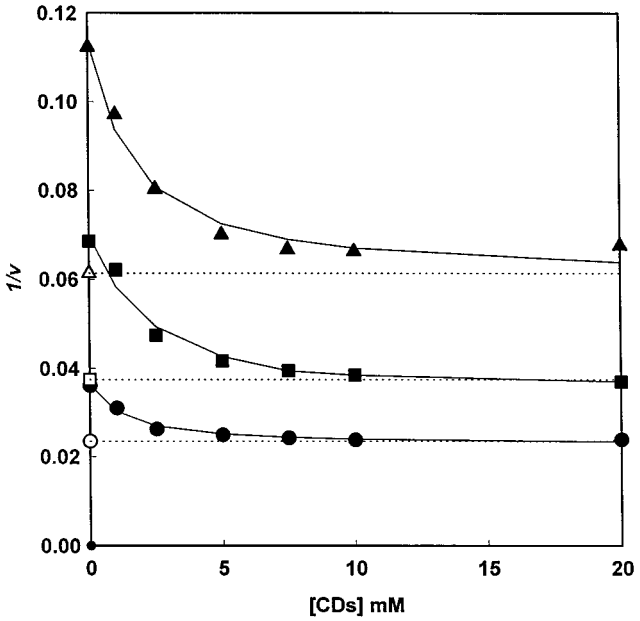

Figure 4. Effect of 2-OH-P-β-CD concentration on the oxidation of dopamine by PPO in the presence of the inhibitor, 4-iodophenol. The reaction medium at 25 °C contained 10 mM phosphate buffer (pH 6.5), 7 μg/mL PPO, 1 mM 4-iodophenol, and increasing concentrations of 2-OH-P-β-CDs (0−20 mM). Three dopamine concentrations were evaluated: (▲) 0.2, (■) 0.7, and (●) 2 mM. Open symbols represent the activity without inhibitior, and the dotted lines represent the activity level without inhibitor.

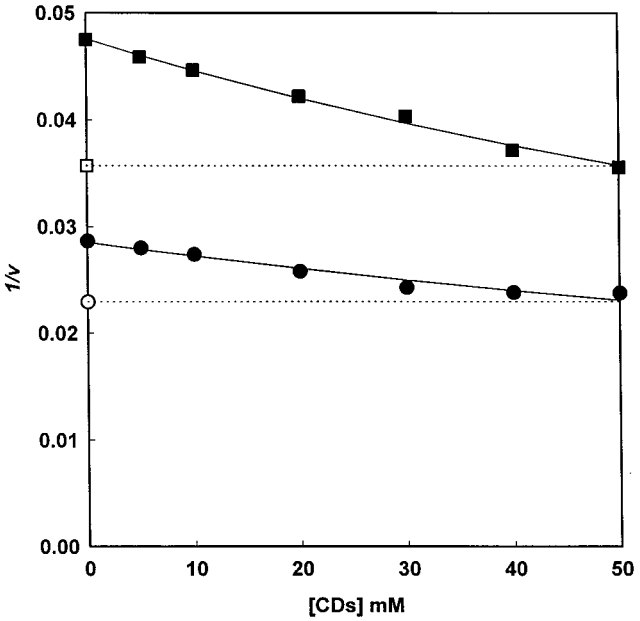

Figure 5. Effect of 2-OH-P-β-CD concentration on the oxidation of dopamine by PPO in the presence of the inhibitor, cinnamic acid. The reaction medium at 25 °C contained 10 mM phosphate buffer (pH 6.5), 7 μg/mL PPO, 0.5 mM cinnamic acid, and increasing concentrations of 2-OH-P-β-CDs (0−20 mM). Two dopamine concentrations were evaluated: (■) 0.7 and (●) 2 mM. Open symbols represent the activity without inhibitor, and the dotted lines represent the activity level without inhibitor.

etc.). Therefore, the kinetic response might be the sum of the effect of free substrate on these contaminating enzymes. Note that commercial mushroom tyrosinase represents only 3−10% of the commercial preparation (Kumar and Flurkey, 1991). The banana pulp PPO used in this paper was free of laccase and peroxidase activities (data not shown).

To complete this kinetic behavior of banana PPO in the presence of CD, the effect of an inhibitor was studied using dopamine as substrate to avoid any interference of the substrate with CDs. The competitive inhibitors used were 4-iodophenol, a monophenol that is deactivated by the presence of the iodine in para position (Duworth and Coleman, 1970) and cinnamic acid. Both compounds are very poor substrates of PPOO and avoid any cross-reactions similar to those described by Billard et al. (1995).

When the CD concentration was increased in the presence of any of the above inhibitors, a previously undescribed activation was found (Figures 4 and 5). At increasing CD concentrations, the enzyme was clearly uninhibited by 4-iodophenol, and the activity asymptotically approached the levels of activity obtained in the absence of inhibitor (Figure 4, open symbols). This activation effect of CDs was also confirmed when cinnamic acid was used (Figure 5).

To understand this behavior (Figures 3 and 4), a Michaelis−Menten defining equation for linear competitive inhibition must be obtained as a function of the only two known parameters, that is, total CD concentration and total inhibitor concentration. For this, the following scheme was assumed.

$$\begin{array}{l} E + S \leftrightarrow ES \rightarrow E + P \\ \updownarrow \leftarrow I_f + CD_f \leftrightarrow I - CD \\ EI \end{array} \qquad (5)$$

Assuming the above, the defining equation for linear competitive inhibition is

$$v = \frac{V_m[S]}{K_m\left(1 + \frac{[I]_f}{K_I} + [S]\right)} \qquad (6)$$

and thus

$$\frac{1}{v} = \frac{K_m}{V_m[S]} + \frac{1}{V_m} + \frac{K_m}{V_m[S]K_I}[I]_f \qquad (7)$$

where subscript f refers to the concentration of the free inhibitor, in this case free 4-iodophenol $[4IP]_f$ or free cinnamic acid.

$[4IP]_f$ can be expressed as a function of $[4IP]_t$ in a similar way as described above for $[TBC]_f$ (eq 4)

$$[4IP]_f = (-([CD]_tK_c - [4IP]_tK_c + 1) + \sqrt{([CD]_tK_c - [4IP]_tK_c + 1)^2 + 4K_c[4IP]_t})/2K_c \qquad (8)$$

and substituted in eq 7 to give

$$\frac{1}{v} = \frac{K_m}{V_m[S]} + \frac{1}{V_m} + \frac{K_m}{V_m[S]K_I}\{(-([CD]_tK_c - [4IP]_tK_c + 1) + \sqrt{([CD]_tK_c - [4IP]_tK_c + 1)^2 + 4K_c[4IP]_t/2K_c)}\} \qquad (9)$$

Equation 9 shows a nonlinear relationship between $1/v$ and $[CD]_t$ as in Figures 4 and 5. Fitting the data by nonlinear regression, values of 980 M^{-1}, 0.58 mM, 30.6 μM/min, and 0.49 mM were obtained for K_c, K_I, V_m, and

Figure 6. Effect of the absence/presence of 2-OH-P-β-CDs in banana pulp extract. The reaction medium at room temperature contained 50 g of banana pulp and 75 mL of 100 mM phosphate buffer (pH 7.3) (tube 1), 50 g of banana pulp and 75 mL of 10 mM 2-OH-P-β-CDs in 100 mM phosphate buffer (pH 7.3) (tube 2), or 50 g of banana pulp and 75 mL of 100 mM 2-OH-P-β-CDs in 100 mM phosphate buffer (pH 7.3) (tube 3). The pictures were taken 30 min after homogenization.

K_m, respectively, when 4-iodophenol acted as inhibitor. With cinnamic acid as inhibitor, values of 10 M^{-1}, 0.05 mM, 59 μM/min, and 0.5 mM were obtained for K_c, K_I, V_m, and K_m, respectively.

To test which of the three different browning responses (no response, inhibition, or activation) in the presence of CD are found in a banana pulp extract, 2-OH-P-β-CDs were added to this extract at two concentrations (10 and 100 mM) and compared with a control. The result (Figure 6) showed that in banana the presence of the CDs activated browning at both concentrations, indicating that the CD is acting as complexing agent for (a) possible PPO inhibitor(s).

CONCLUSION

This paper shows for the first time that CDs may activate browning in crude fruit extracts, contrary to the general assumption that they only inhibit browning. This activation phenomenon might be explained by the complexation of natural inhibiting substances present in banana. It is clear, then, that CDs have a dual effect, activating or inhibiting browning, according to the plant material used. Further studies are required to compile a complete range of fruits or raw materials for which browning is activated or inhibited by CDs before any firm conclusion about their use can be reached.

ABBREVIATIONS USED

TX-114, Triton X-114; PPO, polyphenol oxidase; SDS, sodium dodecyl sulfate; PEG 8000, polyethylene glycol Pm. 8000 Da; TBC, *tert*-butylcatechol; CD, cyclodextrins; 2-OH-P-β-CD, 2-(hydroxypropyl)-β-cyclodextrins.

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