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Partial Purification of Latent Persimmon Fruit Polyphenol Oxidase

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Persimmon fruit polyphenol oxidase (PPO) was partially purified using a combination of phase partitioning with Triton X-114 and ammonium sulfate fractionation between 50 and 75%. The enzyme, which showed both monophenolase and diphenolase activities, was partially purified in a latent form and could be optimally activated by the presence of 1 mM sodium dodecyl sulfate (SDS) with an optimum pH of 5.5. In the absence of SDS, the enzyme showed maximum activity at acid pH. SDS-PAGE showed the presence of a single band when L-DOPA was used as substrate. The apparent kinetic parameters of the latent enzyme were determined at pH 5.5, the V_m value being 15 times higher in the presence of SDS than in its absence, whereas the K_M was the same in both cases, with a value of 0.68 mM. The effect of several inhibitors was studied, tropolone being the most active with a K_i value of 0.45 μM. In addition, the effect of cyclodextrins (CDs) was studied, and the complexation constant (K_c) between 4-*tert*-butylcatechol (TBC) and CDs was calculated using an enzymatic method. The value obtained for K_c was 15580 M^{-1}.

KEYWORDS: Persimmon; polyphenol oxidase; cyclodextrins; latent enzyme

INTRODUCTION

Polyphenol oxidase [PPO (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1] is a bifunctional copper-containing enzyme that uses molecular oxygen to catalyze the co-oxidation of monophenols to *o*-diphenols (monophenolase or cresolase activity) and their subsequent oxidation to *o*-quinones (diphenolase or catecholase activity) (*1*). The *o*-quinones thus generated polymerize to form colored compounds, which are responsible for losses in nutrient quality (*2*).

PPO is an oxidase widely distributed in the plant kingdom and has been detected in most fruits and vegetables (*3*). The presence of this enzyme has a large impact in the food industry because it is the main enzyme involved in the undesirable browning of fruits and vegetables during processing and storage. PPO has been partially purified from many fruits, including grape (*4*), apple (*5*), guava (*6*), peach (*7*), banana (*8*), pear (*9*), kiwi (*10*), strawberry (*11*), plum (*12*), cherry (*13*), and pineapple (*14*).

Persimmon fruit (*Diospyros kaki*) is a popular fruit in Japan, Asia, and South America. It is a good source of fiber as well as vitamins A and C (*15*) and dietary carotenoids, which probably are involved in the reduction of degenerative human diseases (*16*) because of their antioxidant and free radical scavenging properties (*17*). Due to its nutritional relevance, interest in persimmon has increased worldwide in recent years, and the fruit is considered a specialty fruit in the U.S. market (*18*).

Persimmon fruits are classified into two groups: astringent and non-astringent. Commercially astringent species cannot be eaten when firm, unless the astringency has been removed artificially. The different methods used to remove the astringency include ethanol vapor treatment of the fruit on the tree or after harvest (*19*), carbon dioxide gas treatment (*20*), immersion in warm water (*21*), and freezing and over-ripening on the tree or after harvest (*22*). Some of these methods remove the astringency of persimmon fruit by condensation of soluble tannins with the acetaldehyde produced in the flesh during the treatment but result in flesh darkening. In such cases, the degree of flesh browning is similar to that achieved when the astringency is lost naturally in the non-astringent species.

In both cases, an undesirable browning results, which has been associated with the increase in PPO activity in the flesh (*23*, *24*). It has been reported that the maximum of an unspecified oxidizing enzyme and peroxidase in persimmon coincided with the darkening of the flesh and peel (*24*). However, there have been very few studies on persimmon fruit PPO since then (*23*, *24*), and its enzyme activity has not been characterized to any great extent.

The objective of the present work was the partial purification and kinetic characterization of PPO activity in the flesh of the var. Triumph astringent persimmon fruit in an attempt to find natural inhibitors of its browning.

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Table 1. Partial Purification of Persimmon PPO

	vol (mL)	total protein (mg)	total activity[a] (units) −SDS	total activity[a] (units) +SDS[b]	specific activity[c] (units/mg)	purification (-fold)	recovery (%)	activation (-fold)	phenolic compounds (mg/mL)
crude extract	90	70	64.8	616	8.8	1	100	9.5	15.7
supernatant 4% TX-114	81	39	58.5	585	15	2	95	10	8.6
50–75% $(NH_4)_2SO_4$	4	14	37	554	40	4.5	90	15	1.6

[a] Assayed with TBC as susbtrate. [b] Assayed with 1 mM SDS. [c] Refers to the SDS-activated form.

MATERIALS AND METHODS

Plant Material. Persimmon fruit (*Diospyros kaki* L. var. Triumph) was kindly supplied at commercial maturity by Agromedina S.A.T. (Lepe, Huelva, Spain).

Reagents. Biochemicals were purchased from Fluka (Madrid, Spain) and used without further purifications. Inhibitors (cinnamic acid, L-mimosine, tropolone, ascorbic acid, L-cysteine, metabisulfite, and diethyldithiocarbamate) were from Sigma (Madrid, Spain). 2-Hydroxypropyl-β-cyclodextrins (HP-β-CDs) were kindly supplied by Amaizo, American Maize Products Co., Hammond, IN. Triton X-114 was obtained from Fluka and was condensed three times as described by Bordier (*25*), using 100 mM sodium phosphate buffer (pH 7.3). The detergent-rich phase of the third condensation had a concentration of 25% Triton X-114 (w/v).

Enzyme Purification. Persimmon fruit PPO was extracted and partially purified using the method described by our group in 1996 (*26*).

Fresh persimmons were washed and hand-peeled. A 50 g sample was homogenized with 100 mL of 6% (w/v) Triton X-114 in 100 mM sodium phosphate buffer (pH 7.3) for 1 min. The homogenate was subjected to temperature-induced phase partitioning, and the mixture was kept at 4 °C for 10 min and then warmed to 37 °C for 15 min. At this time, the solution became spontaneously turbid due to the formation, aggregation, and precipitation of large mixed micelles of detergent, which contained hydrophobic proteins and phenolic compounds. This turbid solution was centrifuged at 10000*g* for 15 min at 25 °C. After the pellet and detergent-rich phase had been discarded, the clear detergent-poor supernatant (crude enzyme extract) was subjected to additional temperature-induced phase partitioning to remove the remaining phenols. For this, fresh Triton X-114 was added to obtain a final concentration of 4% (w/v) and the mixture held at 37 °C for 15 min. After centrifugation at 25 °C at 10000*g*, the detergent-poor supernatant of 4% (w/v) Triton X-114, which contained the soluble persimmon PPO, was brought to 50% saturation with $(NH_4)_2SO_4$ 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 clear supernatant to give 75% saturation and stirred for 1 h at 4 °C. The precipitate obtained between 50 and 75% was collected by centrifugation at the same rotor speed and dissolved in a minimum volume of 100 mM sodium phosphate buffer (pH 7.3). The salt content was removed by dialysis and the enzyme stored at −20 °C.

Enzyme Activity. Diphenolase activity was determined spectrophotometrically at 400 nm (*2*) with TBC (ϵ_{400} = 1150 M^{-1} cm^{-1}). Because 4-*tert*-butylphenol is not a substrate for the monophenolase activity of PPO without H_2O_2 (*27*), this activity was measured using 4-methylcatechol (4MC) and *p*-cresol, since the quinone obtained in the redox recycle makes it possible to reach the minimum level of *o*-diphenol in the steady state. One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of *tert*-butyl-*o*-quinone per minute.

The standard reaction medium at 25 °C contained 50 μg/mL of partially purified PPO, 10 mM sodium acetate buffer (pH 5.5), and 3 mM TBC in a final volume of 1 mL.

In the SDS standard assays samples contain the above mixture with 2 μg/mL of partially purified persimmon fruit PPO and 1 mM SDS detergent in a cuvette. To determine the effect of the inhibitors, we measured PPO activity in the steady state in standard reaction media in the presence or absence of the stated concentration of inhibitors. Each sample was assayed in triplicate, and the mean and standard deviation were plotted.

Enzyme Activity in the Presence of Cyclodextrins. In the CD standard assays the reaction medium at 25 °C contained 10 mM sodium acetate buffer (pH 5.5), 50 μg/mL PPO, 1, 3, or 5 mM TBC, and increasing concentrations of HP-β-CDs (0−10 mM).

Electrophoresis. SDS−Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Angleton and Flurkey (*28*). Samples were mixed with glycerol, SDS, and bromophenol blue before being applied to 10% polyacrylamide gels. Electrophoresis was carried out for 1 h at 25 °C in a Mini protein cell (Bio-Rad). The gels were stained for PPO activity in 100 mL of 10 mM sodium acetate buffer (pH 5.5) containing 5 mM L-DOPA.

Determination of Proteins and Phenols. The protein content was determined according to Bradford's dye binding method, using bovine serum albumin (BSA) as a standard (*29*). Total phenolic compounds were determined in 80% ethanol using the Folin−Denis method (*30*).

RESULTS AND DISCUSSION

Persimmon fruit PPO was partially purified using a combination of phase partitioning in Triton X-114 and ammonium sulfate fractionation. The presence of Triton X-114 (6% w/v) in the extraction buffer avoided the enzymatic browning of persimmon flesh extract after homogenization. After centrifugation, the supernatant gave a clear solution, termed crude extract in the purification table (**Table 1**). To take full advantage of Triton X-114 in the removal of phenols and hydrophobic proteins (*2*), another 4% (w/v) of Triton X-114 was added and the solution phase-partitioned by increasing the temperature to 37 °C and holding for 15 min. This two-phase step involved a loss of proteins, a slight loss of PPO activity (5%), and a 50% reduction in the level of phenols. In the following step, the ammonium sulfate fractionation provided 4.5-fold purification with 90% recovery (**Table 1**).

Up to this ammonium sulfate fractionation step, the degree of purification was similar to that obtained for potato tuber (*2*), mushroom (*26*), and banana (*8*), using the Triton X-114 method, although recovery was higher in persimmon than in potato tuber (90 vs 18%), indicating the absence of hydrophobic forms of PPO in this fruit.

The reduction of phenolic compounds to only 10% of the original content is similar to that described in the Triton X-114 purification of potato tuber and mushroom PPO (*2*, *26*). This low percentage may represent a fraction of the highly hydrophilic compounds that are attached to sugars. The removal of phenols by Triton X-114 was sufficient to avoid browning of the enzyme solution, even after many cycles of freezing and thawing or after months of storage at −20 °C. When ammonium sulfate was used without a previous phase separation step, the partially purified enzyme turned black.

In addition, the enzyme was still present in a latent state after the ammonium sulfate step and could be activated by SDS (15-fold) (**Table 1**), unlike spinach (*31*) and broad bean PPOs (*32*), both of which are activated by ammonium sulfate. A latent state

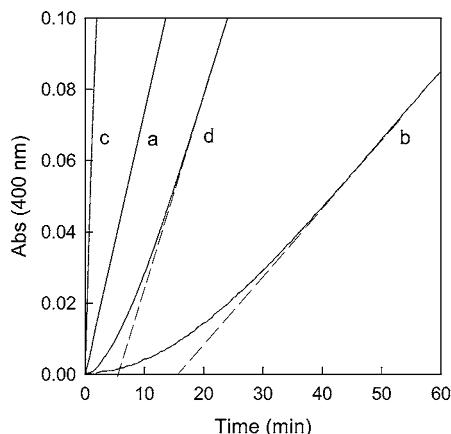

Figure 1. Enzymatic activities of persimmon fruit PPO in the presence and absence of SDS: (a, c) diphenolase activity at 25 °C [reaction medium contained 10 mM sodium acetate buffer, pH 5.5, 3 mM 4-methylcatechol, and 2 μg/mL PPO (a) without SDS and (c) with 1 mM SDS]; (b, d) monophenolase activity at 25 °C [reaction medium contained 10 mM acetate buffer, pH 5.5, 0.3 mM *p*-cresol, and 50 μg/mL PPO (b) without SDS and (d) with 1 mM SDS].

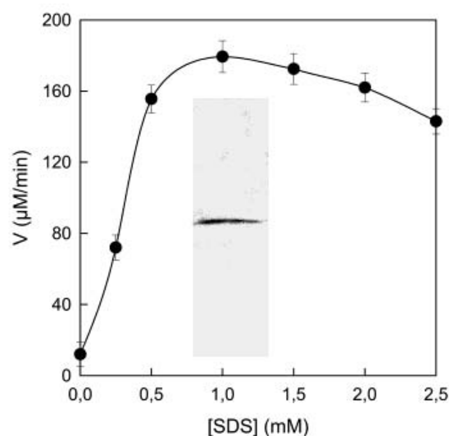

Figure 2. Effect of SDS concentration on persimmon fruit PPO activity. Reaction medium at 25 °C contained 10 mM sodium acetate buffer, pH 5.5, 3 mM TBC, 4 μg/mL PPO, and increasing concentrations of SDS (0–2.5 mM). (Inset) SDS-PAGE (10% gel) of persimmon fruit PPO stained with 5 mM L-DOPA in 10 mM sodium acetate buffer (pH 5.5).

PPO similar to that of the persimmon has been described for the peach by our group (*7*).

The latent enzyme thus obtained was a true PPO because it showed both diphenolase and monophenolase activities in the absence and presence of SDS (**Figure 1**, curves a,c and b,d, respectively). The optimum SDS concentration for activation of the enzyme was 1 mM (**Figure 2**). This activation of the latent enzyme by SDS is a common feature of other latent PPOs (*7*, *33*, *34*), which has been attributed to a conformational change in the protein (*35*). In addition, the latent enzyme is also activated by 10% SDS-PAGE, appearing as a single band when L-DOPA is used as substrate (**Figure 2**, inset).

Figure 3 shows the pH profile for the oxidation of TBC by partially purified PPO in the absence and presence of SDS. In the absence of SDS (**Figure 3**, solid circles) the activity increased at acidic pH. This low pH optimum is a result of the PPO activity being induced by acid shocking (*31*, *35*−*37*). However, the presence of SDS (**Figure 3**, open circles) eliminated the acidic pH optimum and a new maximum appeared at pH 5.5, as was previously described for other PPOs

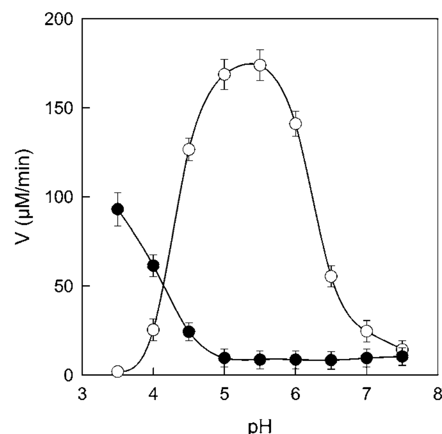

Figure 3. Effect of pH on persimmon fruit PPO activity in 10 mM sodium acetate (pH 3.5–5.5) and 10 mM sodium phosphate (pH 6.0–7.5) in the presence (○) or absence (●) of 1 mM SDS. Reaction medium at 25 °C contained 3 mM TBC and 4 μg/mL PPO.

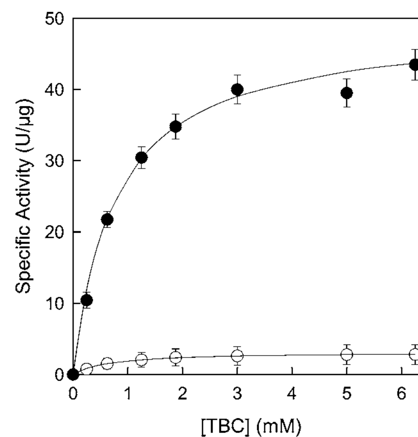

Figure 4. Effect of TBC concentration on persimmon fruit PPO activity. Reaction medium contained (○) 50 μg/mL PPO or (●) 2 μg/mL PPO and 1 mM SDS, both containing TBC concentrations ranging from 0 to 6.25 mM in 10 mM sodium acetate buffer (pH 5.5).

in fruits and plants (*29*, *30*). Thus, activation with SDS depended on the surfactant concentration (**Figure 2**) and pH (**Figure 3**) (*32*).

The apparent kinetic parameters (V_m and K_M) were calculated by nonlinear regression to the Michaelis−Menten equation using the data obtained at pH 5.5 (the maximum pH for persimmon PPO activity) in the presence and absence of SDS. **Figure 4** shows the variations in initial velocity versus substrate concentration in these conditions. The value obtained for each of the maximum velocities showed that the activation increased 15-fold with SDS ($V_m = 3.2$ μM/min/μg of protein in the absence of SDS and $V_m = 49$ μM/min/μg of protein in its presence) (**Figure 4**). This increase in V_m without changes in the K_M ($K_M = 0.68$ mM) agrees with previously described behavior with and without SDS in latent potato leaf PPO (*33*) and latent broad-bean PPO (*32*).

To further characterize the partially purified enzyme, a detailed study of its inhibition was carried out. **Table 2** shows the effect of various inhibitors on the diphenolase activity of latent persimmon fruit PPO in the presence and absence of 1 mM SDS, using TBC as substrate. All of the inhibitors, reducing agents, and substrate analogues used in this study inhibited PPO activity, the extent of the inhibition being dependent on the concentration of the compound used and the presence or absence of SDS. Among the reducing agents (**Table 2**), metabisulfite

Table 2. Percentage Inhibition of Partially Purified Persimmon Fruit PPO by Reducing Agents[a] and Substrate Analogues[a]

	−SDS				1 mM SDS			
	1 μM	10 μM	100 μM	1 mM	1 μM	10 μM	100 μM	1 mM
Reducing Agents								
ascorbic acid	0	0	10	100	13	13	26	100
L-cysteine	5	15	35	56	9	10	43	100
metabisulfite	4	12	74	83	14	21	88	100
diethyldithiocarbamate	9	16	100	100	7	12	100	100
Substrate Analogues								
tropolone	20	80	93	100	30	85	94	100
L-mimosine	0	0	9	61	0	0	5	30
cinnamic acid	0	28	44	86	0	0	29	69

[a] Assayed under the standard reaction condition with the appropriate concentration of inhibitor.

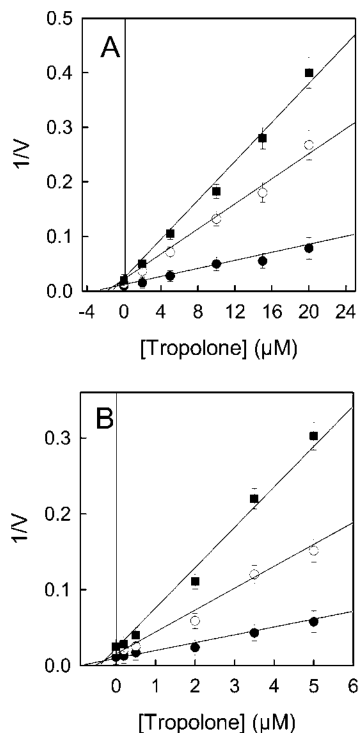

Figure 5. Dixon plot (1/*v* vs *I*) for the competitive type of inhibition presented by tropolone: (A) reaction medium contained 50 μg/mL PPO, tropolone (0–20 μM), and three different concentrations of TBC [0.625 mM (●), 1 mM (○), and 3 mM (■)]; (B) reaction medium contained 2 μg/mL PPO, 1 mM SDS, tropolone (0–5 μM), and the same TBC concentrations as (A).

and diethyldithiocarbamate appeared to be the most effective inhibitors in the presence of SDS, whereas in its absence, ascorbic acid and diethyldithiocarbamate were the most effective when added in a final concentration of 1 mM (**Table 2**). The inhibition produced by thiol compounds may be due to an addition reaction taking place with the quinones to form stable colorless products (*40*) and/or a binding to the active center of the enzyme, as in the case of metabisulfite (*41*). Ascorbate acts as an antioxidant rather than as an enzyme inhibitor because it reduces the initial quinone formed by the enzyme to the original diphenol before it undergoes the secondary reactions that lead to browning (*42*). Ascorbic acid has also been reported to cause irreversible inhibition (*43*). Finally, diethyldithiocarbamate may act by complexing the copper prosthetic group of the enzyme, as has been found for other plant PPOs (*44*).

With regard to substrate analogues (**Table 2**), tropolone was the most effective inhibitor, because it completely inhibited the enzyme at 1 mM in the presence and absence of SDS. On the other hand, inhibition by L-mimosine and cinnamic acid (**Table 2**) was SDS-dependent and more effective in the absence of SDS.

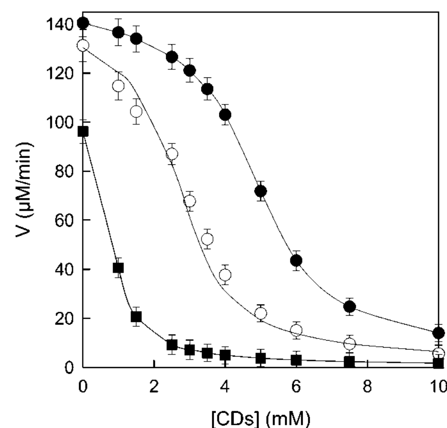

Figure 6. Effect of HP-β-CDs concentration on persimmon fruit PPO. Reaction medium at 25 °C contained 10 mM sodium acetate buffer (pH 5.5), 50 μg/mL PPO, increasing concentrations of HP-β-CDs (0–10 mM), and (●) 5 mM TBC, (○) 3 mM TBC, and (■) 1 mM TBC.

The kinetic analysis of the inhibition was made with only tropolone, because it was the most effective inhibitor of the compounds tested in **Table 2**. The inhibition was determined by Lineweaver−Burk plots of $1/v$ versus $1/s$ (data not shown) at three inhibitor concentrations and confirmed by a Dixon plot of $1/v$ versus I. Straight lines were obtained with and without SDS (**Figure 5**). This inhibitor showed competitive inhibition, and the inhibition constant, K_i, which showed a value of 0.45 μM with or without SDS, was deduced from the interception points.

To complete our study of the inhibition of persimmon fruit PPO, the effect of substrate sequestrants such as cyclodextrins was studied. Cyclodextrins (CDs) are a group of naturally occurring cyclic oligosaccharides derived from starch with six, seven, or eight glucose residues linked by $\alpha(1\rightarrow4)$ glycosidic bonds in a cylinder-shaped structure and are denominated 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 guest molecules, including (poly)phenols (*45*). Recently, it has been suggested that cyclodextrins may moderate the enzymatic browning of different fruits and vegetables (*46*−*48*), because they form inclusion complexes with the substrates of PPO, thereby preventing their oxidation to quinones and subsequent polymerization to brown pigments. This effect has also been observed

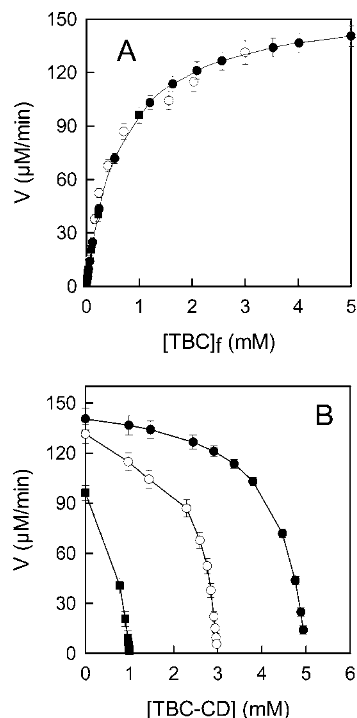

Figure 7. (A) Effect of free TBC concentration on persimmon fruit PPO. Free TBC concentrations were calculated from data shown in **Figure 6**, using eq 1 (see text for details). (B) Effect of complex TBC–CD concentration on persimmon fruit PPO. TBC–CD concentration was calculated from data shown in **Figure 6**, using eq 3 (see text for details).

by our group during the oxidation of phenols by lipoxygenase (*49*), in which CDs act as secondary antioxidants in synergism with ascorbic acid.

Because TBC is a diphenolic compound with a hydrophobic group, it enters the hydrophobic cavity to form inclusion complexes. This explains why there was a clear decrease in persimmon fruit PPO activity when increasing concentrations of HP-β-CDs were added to the reaction medium (**Figure 6**). Using the mathematical equation for the free substrate concentration in the presence of CDs (*49*, *50*)

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

the complete Michaelis−Menten equation can be expressed as

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

Equation 2 shows a nonlinear relationship between v and $[\mathrm{CD}]_t$, as is depicted in **Figure 6**. Fitting the data of this figure to eq 2 by nonlinear regression using Sigma Plot (SPSS Inc.), we obtained a value of 15580 ± 800 M^{-1} for the K_c between TBC and HP-β-CDs. This value is similar to that described for the oxidation of TBC by lipoxygenase in the presence of cyclodextrins (*51*).

To confirm that persimmon fruit PPO works only with free substrate and not with the substrate−CD complex as well, as has been described for mushroom PPO (*47*), the data of **Figure 6** were replotted as a function of free TBC concentration (**Figure 7A**) using eq 1 and as a function of the complexed TBC (**Figure 7B**):

$$[\mathrm{TBC-CD}] = [\mathrm{TBC}]_t - [\mathrm{TBC}]_f \quad (3)$$

This clearly shows that the enzymatic activity responded to free TBC concentrations (**Figure 7A**) and not to complexed TBC (**Figure 7B**), because **Figure 7A** gives a similar Michaelis−Menten representation of the data with a K_M value equal to that found in the absence of CDs.

In conclusion, the results of this paper show for the first time a detailed kinetic study of persimmon fruit PPO, which manifests itself as a single band in electrophoresis when it is isolated using Triton X-114. This enzyme was present in latent form, could be activated by the anionic detergent SDS, and was strongly inhibited by tropolone. The marked reduction in its activity in the presence of cyclodextrins opens up the possibility of a new natural control of persimmon flesh browning.

ABBREVIATIONS USED

TBC, *tert*-butylcatechol; PPO, polyphenol oxidase; SDS, sodium dodecyl sulfate; HP-β-CDs, 2-hydroxypropyl-β-cyclodextrins; CDs, cyclodextrins.

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