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Polyphenol Oxidase from Dominga Table Grape

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Dominga grape polyphenol oxidase (PPO) was extracted using phase partitioning with Triton X-114. The enzyme was obtained in latent state and could be optimally activated by the presence of 0.2% sodium dodecyl sulfate (SDS) at pH 6.0. In the absence of SDS, the enzyme showed maximum activity at acid pH. The kinetic parameters of the enzyme at pH 3.0 and 6.0 in the presence of SDS were calculated. The effect of several inhibitors was studied, tropolone being the most effective with a K_i value of 18 μM. The effect of cyclodextrins was also studied, and the complexation constant K_c between G_2-β-cyclodextrins and 4-*tert*-butylcatechol was calculated using the enzymatic method (K_c = 13960 M^{-1}). The evolution of the color parameters (L^*, a^*, b^*) of liquefied grape berries was inhibited by inhibitors of PPO activity, such as diethyldithiocarbamate, metabisulfite, and G_2-β-cyclodextrins, indicating that enzymatic browning by PPO is the main process involved in the browning of Dominga grape juice at room temperature.

KEYWORDS: Polyphenol oxidase; cyclodextrins; browning; grape juice

INTRODUCTION

Polyphenol oxidase (PPO) (monophenol, dihydroxy-L-phenylalanine:oxygen oxidoreductase, EC 1.14.18.1) is a widely distributed copper-containing enzyme associated with undesirable browning reactions in fruits and vegetables. This enzyme catalyzes two distinct reactions, each using molecular oxygen: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) (*1*). The quinones thus formed are highly reactive substances, which normally react further with other quinones, amino acids, or proteins to produce colored compounds that cause deterioration in food quality by changing their nutritional and organoleptic properties (*2*, *3*). Such reactions significantly diminish consumer acceptance, storage life, and the value of the plant products. Because of its involvement in this adverse browning effect, PPO has received much attention from researchers in the fields of plant physiology and food science.

Table grape is a fruit with a commercial quality closely related to visual appearance. The catalytic action of PPO has an enormous impact on the quality of this fruit and results in the alteration of color, flavor, texture, and nutritional value (*2*). It is a limiting factor in the handling and technological processing of crops in general.

PPO is probably present in all plants (*4*), but its activity is particularly high in those fruits and vegetables containing significant levels of phenolic compounds. Grapes constitute one of the major sources of phenolic compounds among different fruits (*5*, *6*). Moreover, it is well-known that grapes contain high polyphenol oxidase activity (*7*), and enzymatic browning occurs rapidly in damaged berries or following the crushing of fresh grapes for juice or wine production (*8*). Investigations to determine the characteristics of grape PPO and the conditions in which they are most active have been widely reported for some cultivars, including Concord (*9*), Ravat 51 and Niagara (*10*), Koshu (*11*), DeChaunac (*12*), Monastrell (*13*), Airen (*14*), Muscadine (*15*), and Mazruma (*16*).

Grape PPO activity is considerably influenced by several factors such as variety, stage of development, and environmental conditions (*17*, *18*). In this paper, latent PPO from Dominga table grapes grown under an integrated production system was isolated and kinetically characterized. A high percentage of this variety of table grape is consumed fresh, and the remaining grapes are used to produce juice. In an attempt to control the browning of the juice after grape crushing, the effects of cyclodextrins and some PPO inhibitors on color parameters were also studied.

MATERIALS AND METHODS

Sampling and Raw Material. Dominga table grape grown under an integrated production system were kindly supplied by COATO (Totana, Murcia, Spain). Grape berries, in the optimal commercial stage of maturity, as measured by the soluble solids content (SSC = 15 °Brix), were picked from three different plantations. From each plantation, 10 samples of 500 g were picked from 10 different oriented vines. All samples were mixed, transported to the laboratory, and frozen at −80 °C until they were used as PPO source.

Reagents. Biochemicals were purchased from Fluka (Madrid, Spain) and used without purification. Inhibitors (cinnamic acid, L-minmosine,

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tropolone, ascorbic acid, L-cysteine, metabisulfite, and diethyldithiocarbamate) were from Sigma (Madrid, Spain). Maltosyl-β-cyclodextrins (G_2-β-CDs) were kindly supplied by Ensuiko Sugar Refining Co., Ltd. Triton X-114 was obtained from Fluka and was condensed three times as described by Bordier (*19*), 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. Table grape Dominga PPO was extracted using the method described by Sánchez-Ferrer et al. (*20*). All extractions were made in triplicate.

Grape berries (250 g) were washed and homogenized with 100 mL of 100 mM sodium phosphate buffer (pH 7.3) containing 10 mM ascorbic acid for 1 min. The homogenate was filtered through eight layers of gauze and centrifuged at 4000*g* for 15 min. The supernatant was discarded, and the precipitate was extracted with 20 mL of 4% (w/v) Triton X-114 in 100 mM sodium phosphate buffer (pH 7.3). The mixture was subjected to temperature-induced phase partitioning and was kept at 4 °C for 15 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 centrifugation, the detergent-rich phase was discarded and the clear detergent-poor supernatant was used as enzyme source, which was stored at −20 °C.

Enzyme Activity. Diphenolase activity was determined spectrophotometrically at 400 nm (*21*) with 4-*tert*-butylcatechol (TBC) (ϵ_{400} = 1150 M^{-1} cm^{-1}). One unit of enzyme was defined as the amount of the enzyme that produced 1 μmol of *tert*-butyl-*o*-quinone per minute.

The standard reaction medium at 25 °C contained 6.5 ng/mL of partially purified PPO, 10 mM sodium acetate buffer (pH 3.0), and 16 mM TBC in a final volume of 1 mL.

In the sodium dodecyl sulfate (SDS) standard assay, samples contained the above mixture and 0.2% 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.

Enzyme Activity in the Presence of Cyclodextrins. The cyclodextrin standard medium at 25 °C contained 10 mM sodium acetate buffer (pH 3.0), 6.5 ng/mL of partially purified PPO, and increasing concentrations of G_2-β-CDs (0−25 mM) and TBC (2, 4, or 12 mM).

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Angleton and Flurkey (*22*). Samples were mixed with glycerol, SDS, and bromophenol blue before being applied to 12.6% 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 3.0) containing 5 mM 3,4-dihydroxyphenyl-L-alanine (L-DOPA) and 3 mM 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (MBTH) (*23*).

Determination of Proteins. The protein content was determined according to Bradford's dye-binding method, using bovine serum albumin (BSA) as a standard (*24*).

Thermal Stability. The enzyme was incubated at various temperatures between 30 and 100 °C for 5 min, and the residual activity was determined at pH 3.0 and 6.0 and 0.2% SDS at 25 °C.

Color Evolution Assessment. A 50 g sample of grape berries was liquefied in a Moulinex Y36 blender. The juice grape obtained was collected in a beaker containing 25 mL of distilled water alone or with enough inhibitor to produce a final concentration of 1 mM metabisulfite, 1 mM diethyldithiocarbamate (DEDTC), or 10 mM G_2-β-CDs. These mixtures were used in color determination assays, using as standard the measurement at time 0. Each sample was assayed in triplicate, and the mean and standard error were plotted.

The CIE coordinates L^* (lightness), a^* (red-green), and b^* (yellow-blue) of juice grape were determined by using a HunterLab Colorimeter ColorFlex 45/0 (Reston, VA). Three readings were obtained for each replicate to obtain uniform color measurements. Hue (H^*), chroma difference (ΔC^*), and color difference (ΔE^*) were calculated using the following equations (*25*):

$$H^* = \tan^{-1}\left(\frac{b^*}{a^*}\right)$$

$$\Delta C^* = [(\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

RESULTS AND DISCUSSION

Table grape var. Dominga was grown under integrated production in the province of Murcia (Spain). Browning is one of the factors involved in table grape quality, and the process has been associated with the presence of PPO. The darkening process in different cultivars varies, depending on the nature and quality of the enzyme present in the product (*26*−*29*).

When Dominga grape PPO was extracted using the Triton X-114 method (*20*), it was found in a latent state and free of phenols and chlorophylls, as has been previously described for other fruits and vegetables (*30*−*32*). This enzyme appeared as two clearly defined activity bands in SDS-PAGE when L-DOPA was used as substrate (**Figure 1**). These results are in agreement with those obtained for other grape PPOs, such as Airen or Monastrell, in which the same two electrophoretic bands were obtained (*33*).

In the case of latent PPO, pH is a determining factor to express enzymatic activity and, its activation by acid or basic shock has been widely described (*34*−*40*). On the other hand, latent PPOs could be activated by the anionic detergent SDS (*35*). For these reasons, the kinetics and optimal pH of the enzyme were determined in the presence and absence of SDS. In the absence of SDS the activity increased at acidic pH (**Figure 2**, solid circles) as a result of acid shocking (*36*−*38*). However, this optimum acidic pH for PPO activity was subject to change when assayed in the presence of anionic detergents, such SDS. The presence of SDS eliminated the acidic pH optimum and two new maxima appeared at pH 4.0 and 6.0 (**Figure 2**, solid squares). This activator effect of SDS has previously been described for other latent PPOs extracted from vegetal sources using the TX-114 method and measured using TBC as substrate (*32*, *38*−*40*). However, the two maxima showed by Dominga grape PPO in the presence of SDS (pH 4.0 and 6.0) have not previously been described for other plant PPOs. This effect may be related with the two electrophoretic bands shown by Dominga grape PPO in **Figure 1**.

The pH scan in the presence and absence of SDS (**Figure 2**) revealed that the highest value in the activation process was obtained at pH 6.0 (43.7-fold) (**Figure 2**, open circles). It was therefore decided to study activation by detergent at pH 6.0, although assays in the absence of SDS were carried out at pH 3.0. The degree of SDS activation obtained for Dominga grape PPO (43.7-fold) was higher than that obtained for Monastrell grape (19.2-fold) (*20*), lettuce (5-fold) (*40*), mushroom (6-fold) (*41*), peach (25-fold) (*32*), and persimmon PPO (15-fold) (*38*).

Activation with SDS depended on surfactant concentration, as shown in **Figure 3**. The optimum SDS concentration for activation of the enzyme (0.2%) was higher than that described for banana (0.06%) (*31*) or peach PPO (0.05%) (*32*). This activation of the latent enzyme by SDS is a common feature of other latent PPOs (*38*−*40*) and has been attributed to a conformational change in the protein.

Activation by SDS depended not only on the surfactant concentration and pH but also on the substrate used (*39*). For example, when TBC was used as substrate, the activation obtained for Dominga grape PPO was 43.7-fold (at pH 6.0 in the presence of 0.2% of SDS) (**Figure 2**). However, when 4-methylcatechol (4MC) was used as substrate, the activation

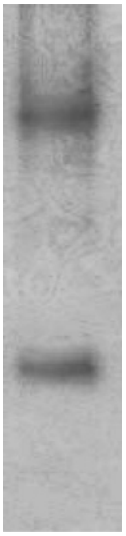

Figure 1. SDS-PAGE (12.6% gel) of Dominga grape PPO stained with 5 mM L-DOPA and 3 mM MBTH in 10 mM sodium acetate buffer (pH 3.0).

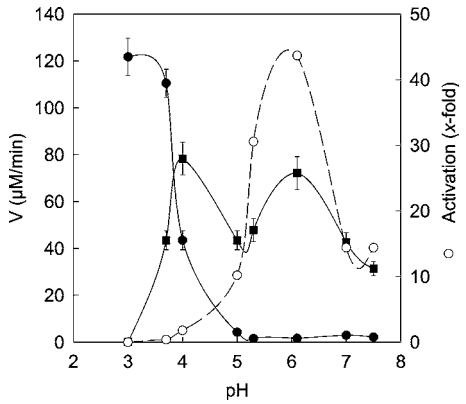

Figure 2. Effect of pH on Dominga grape PPO activity in 10 mM sodium acetate buffer (pH 3.0–5.5) and 10 mM sodium phosphate buffer (pH 6.0–7.5) in the presence (■) or absence (●) of 0.2% SDS: activation degree at different pH values (○). The reaction medium at 25 °C contained 16 mM TBC and 6.5 ng/mL PPO.

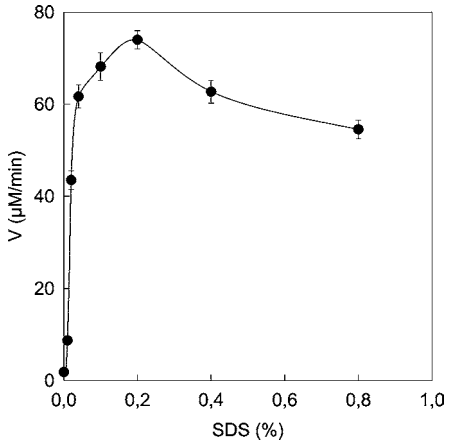

Figure 3. Effect of SDS concentration on Dominga grape PPO activity. Reaction medium at 25 °C contained 10 mM sodium phosphate buffer (pH 6.0), 16 mM TBC, 6.5 ng/mL PPO, and increasing concentrations of SDS (0–0.8%).

obtained was 21.4-fold (data not shown). These results seem to be related to the degree of hydrophobicity of the substrate used (TBC > 4MC), the conformational change produced by the

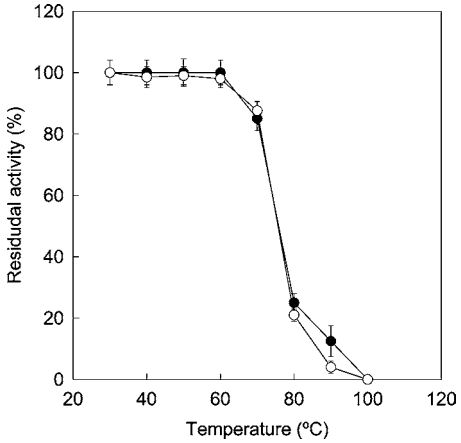

Figure 4. Thermal stability of the enzyme after heating for 5 min at temperatures between 30 and 100 °C, as determined by activity assay at 25 °C. Reaction medium contained 16 mM TBC and 6.5 ng/mL PPO in 10 mM sodium acetate buffer (pH 3.0) (●) or 10 mM sodium phosphate buffer (pH 6.0) with 0.2% SDS (○).

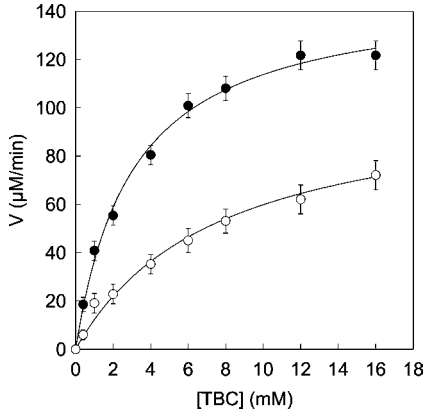

Figure 5. Effect of TBC concentration on Dominga grape PPO activity. Reaction medium at 25 °C contained 6.5 ng/mL PPO and TBC concentrations ranging from 0 to 16 mM in (●) 10 mM sodium acetate buffer (pH 3.0) or (○) 10 mM sodium phosphate buffer (pH 6.0) with 0.2% SDS.

surfactant favoring the access of the hydrophobic substrates to the active center. This effect has been previously described for potato leaf PPO (*39*).

With regard to enzyme stability and temperature, the results obtained at pH 3.0 and 6.0 in the presence of SDS 0.2% are shown in **Figure 4**. The enzyme was stable between 30 and 60 °C. From 60 to 70 °C, the residual activity decreased slightly (20%), but rapid inactivation occurred from 70 to 80 °C, and only 20% of activity remained. At 100 °C the residual activity was 0%. The temperature stability of this enzyme was relatively high in both conditions, compared to other grape PPOs (*11*, *42*).

The study of the kinetic parameters (V_m and K_M) of latent Dominga grape PPO was carried out in the absence of SDS at pH 3.0 and in the presence of 0.2% SDS at pH 6.0, using TBC as substrate (**Figure 5**). 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 3.0 and 6.0 in the presence of 0.2% SDS. **Figure 5** shows the variation in initial velocity versus substrate concentration in these conditions. The value obtained for each maximum velocity ($V_m = 125\ \mu$M/min at pH 3.0 and $V_m = 80\ \mu$M/min at pH 6.0 with 0.2% SDS) showed that the PPO was strongly activated by acid shocking. The K_M value obtained at pH 3.0 (3 mM) was lower than that

Table 1. Inhibition Percentage of Partially Purified Dominga Grape PPO by Reducing Agents[a]

	pH 3.0				pH 6.0 + 0.2% SDS			
	10 μM	0.1 mM	0.4 mM	1 mM	10 μM	0.1 mM	0.4 mM	1 mM
ascorbic acid	0	5	32	100	0	0	10	100
DEDTC	16	64	100	100	0	11	100	100
L-cysteine	0	19	66	100	0	19	100	100
metabisulfite	0	31	100	100	0	8	83	100

[a] Assayed under the standard reaction conditions with the appropiate concentration of inhibitor.

Table 2. Inhibition Percentage of Partially Purified Dominga Grape PPO by Substrate Analogues[a]

	pH 3.0				pH 6.0 + 0.2% SDS			
	10 μM	0.1 mM	0.4 mM	1 mM	10 μM	0.1 mM	0.4 mM	1 mM
tropolone	13.6	52.3	75	85.7	18.5	68.35	87.4	88.6
L-mimosine	0	0	12	16	0	4	6	15
kojic acid	5	8	10	12	6	11	15	27

[a] Assayed under the standard reaction conditions with the appropiate concentration of inhibitor.

Table 3. Changes with Time of *L**, *a**, *b**, *C**, and *H** Coordinates of Dominga Grape Juice, in the Absence and Presence of Some PPO Inhibitors[a]

reaction time (min)	Hunter values				
	*L**	*a**	*b**	*C**	*H**
			Without Inhibitor		
0	59.28 (1.51)	–2.57 (0.11)	25.81 (2.26)	25.94 (1.17)	95.77 (3.25)
5	46.83 (2.23)	4.66 (0.41)	25.52 (2.11)	25.94 (2.01)	79.65 (2.65)
10	42.48 (1.45)	6.57 (0.25)	23.94 (1.75)	24.83 (0.98)	74.66 (1.24)
20	40.45 (1.66)	7.61 (0.21)	23.78 (2.03)	24.97 (1.32)	72.26 (1.61)
			10 mM G_2-β-CDs		
0	59.28 (1.51)	–2.57 (0.11)	25.81 (2.26)	25.94 (1.17)	95.77 (3.25)
5	48.68 (2.83)	3.02 (0.26)	25.98 (2.35)	26.15 (2.19)	83.36 (2.54)
10	46.55 (1.18)	4.32 (0.17)	25.89 (1.79)	26.25 (1.67)	80.54 (1.73)
20	45.11 (1.03)	5.51 (0.32)	26.16 (2.05)	26.74 (2.35)	78.12 (1.12)
			1 mM DEDTC		
0	59.28 (1.51)	–2.57 (0.11)	25.81 (2.26)	25.94 (1.17)	95.77 (3.25)
5	52.31 (1.18)	–0.64 (0.26)	24.78 (2.01)	24.79 (1.58)	91.47 (2.15)
10	51.45 (1.35)	–0.49 (0.15)	23.82 (1.75)	23.82 (2.31)	91.18 (1.95)
20	50.03 (1.24)	0.03 (0.25)	22.48 (1.23)	22.48 (1.26)	89.93 (2.35)
			1 mM Metabisulfite		
0	59.28 (1.51)	–2.57 (0.11)	25.81 (2.26)	25.94 (1.17)	95.77 (3.25)
5	57.75 (1.34)	–3.52 (0.08)	22.41 (1.06)	22.68 (1.63)	98.92 (2.65)
10	56.81 (2.06)	–3.44 (0.23)	21.64 (1.75)	21.91 (1.35)	99.02 (3.05)
20	56.23 (1.16)	–3.43 (0.19)	20.95 (1.16)	21.23 (0.98)	99.31 (1.95)

[a] Data are the average of three measurements (standard deviations are give in parentheses).

obtained at pH 6.0 in the presence of SDS (7.6 mM). The V_m/K_M ratios were 0.04 min^{-1} at pH 3.0 and 0.01 min^{-1} at pH 6.0 with 0.2% SDS, indicating that latent Dominga grape PPO presents more affinity for TBC at acid pH that at pH 6.0 in the presence of SDS.

To further characterize latent Dominga grape PPO, a detailed study of its inhibition was carried out. All inhibitors used in this study inhibited PPO activity, the extent of the inhibition being dependent on the concentration of the compound used. Among reducing agents (**Table 1**), metabisulfite, L-cysteine, and DEDTC appeared to be the most effective inhibitors at pH 3.0 and 6.0 in the presence of 0.2% SDS. However, the action mechanism differs according to reducing agent used. The inhibition by thiol compounds may be due to an addition reaction with the quinones to form stable colorless products (*43*) and/or a binding to the active center of the enzyme as in the case of metabisulfite (*44*). 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 reaction that leads to browning (*45*). Ascorbic acid has also been reported to cause irreversible inhibition (*46*). Finally, DEDTC may act by complexing the copper prosthetic group of the enzyme, as has been seen for other plant PPOs (*47*).

Of the substrate analogues (**Table 2**), tropolone was the most effective inhibitor, inhibiting the enzyme by >50% at 0.1 mM and almost completely at 1 mM at pH 3.0 and 6.0 in the presence of 0.2% SDS. This result is in accordance with other studied PPOs, such as persimmon (*38*), banana (*31*), or potato (*21*). Surprisingly, neither L-mimosine nor kojic acid (**Table 3**) had much of an inhibiting effect in any of the conditions used.

The inhibition of tropolone was determined by Lineweaver–Burk plots of $1/v$ versus $1/S$ at three inhibitor concentrations (data not shown) and confirmed by a Dixon plot of $1/v$ versus I (**Figure 6**). Straight lines were obtained both at pH 3.0 (**Figure 6A**) and pH 6.0 in the presence of SDS 0.2% (**Figure 6B**). The inhibition constant, K_i, was deduced from the points of interception of the plots. Tropolone showed a competitive inhibition, with a K_i value of 18 μM for both studied conditions (**Figure 6**).

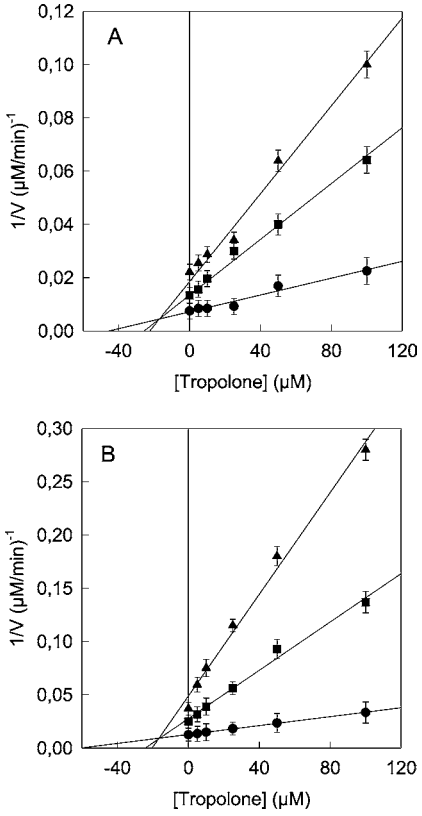

Figure 6. Dixon plot (1/*V* versus *I*) for the competitive type of inhibition presented by tropolone: (**A**) reaction medium at 25 °C contained 10 mM sodium acetate buffer (pH 3.0), 6.5 ng/mL PPO, tropolone (0–120 μM), and three different concentrations of TBC [2 mM (▲), 4 mM (■), and 16 mM (●)]; (**B**) reaction medium at 25 °C contained 10 mM sodium phosphate buffer (pH 6.0) with 0.2% SDS, 6.5 ng/mL PPO, tropolone (0–120 μM), and three different concentrations of TBC [2 mM (▲), 4 mM (■), and 16 mM (●)].

To complete the study of latent Dominga grape PPO, the enzyme responsible for the enzymatic browning that occurs rapidly following the crushing of fresh grapes for juice production, the effect of substrate sequestrants such as cyclodextrins (CDs) was studied. Cyclodextrins 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, designated 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 (*48*). It has been suggested that CDs may moderate the enzymatic browning of different fruits and vegetables (*49*−*51*), because they form inclusion complexes with the substrates of PPO, thereby preventing their oxidation to quinones and subsequent polymerization to brown pigments. This effect was also observed by our group during the oxidation of phenols by lipoxygenase (*52*), in which CDs act as secondary antioxidants in synergism with ascorbic acid.

TBC is a diphenolic compound with a hydrophobic group, which could enter hydrophobic cavity of the CDs, forming an inclusion complex. This explains why there was a clear decrease in Dominga grape PPO activity when increasing concentrations of food grade G_2-β-CDs were used in the reaction medium (**Figure 7**). Using the following mathematical equation for

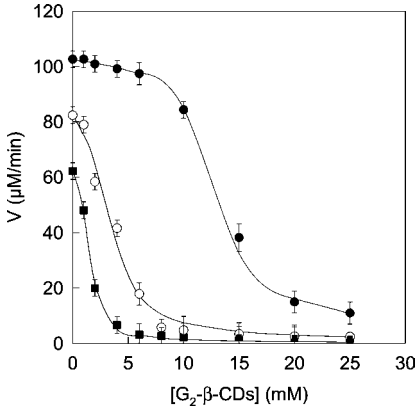

Figure 7. Effect of G_2-β-CDs concentration on Dominga grape PPO. Reaction medium at 25 °C contained 10 mM sodium acetate buffer (pH 3.0), 6.5 ng/mL PPO, increasing concentrations of G_2-β-CDs (0–25 mM), and (■) 2 mM TBC, (○) 4 mM TBC, or (●) 12 mM TBC.

determining the free substrate concentration in the presence of CDs (*52*, *53*)

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

the complete Michaelis−Menten equation can be expressed as

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

Equation 2 shows a nonlinear relationship between v and total CD concentration ($[CD]_t$), as is depicted in **Figure 7**. Fitting these data to eq 2 by nonlinear regression using Sigma Plot (SPSS Inc.), a value of 13960 ± 500 M^{-1} for the inclusion constant (K_c) between TBC and G_2-β-CDs was obtained. This value is close to that described for the K_c between TBC and hydroxypropyl-β-cyclodextrins (*38*, *54*), indicating that no steric hindrance occurs between the maltosyl group bound to the β-CD ring and the *tert*-butylcatechol side chain of TBC.

Food organoleptic properties such as color are closely connected to the phenolic composition and PPO activity (*55*, *56*). The relationship between color and PPO was studied in Dominga grape juice in the absence and presence of different PPO inhibitors. Dominga grape juice has solids in suspension; thus, its browning was determined by measuring CIELAB coordinates. The inhibitors assayed were DEDTC, metabisulfite, and G_2-β-cyclodextrins.

The evolution with time of the L^*, a^*, and b^* coordinates was measured in grape berries liquefied in the absence and in the presence of inhibitor (**Table 3**). The total color difference (ΔE^*) was also calculated. The ΔE^* is a single value that takes into account the differences between L^*, a^*, and b^* of the sample and standard. When the values presented in **Table 3** were integrated as color differences (ΔE^*), an increase with time was observed (**Figure 8**), indicating that color had evolved as a result of the browning process in the juice. This process was saturable and showed the greatest variation in the first 10 min in all cases.

A significant difference was observed in the color evolution of grape juice depending on whether the grapes were liquefied in the absence or presence of inhibitors. In the absence of

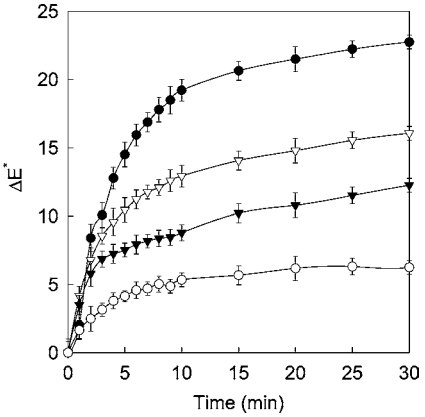

Figure 8. Evolution of color difference (ΔE^*) in Dominga grape juice at 25 °C in the absence of inhibitor (●) and in the presence of 10 mM G_2-β-CDs (▽), 1 mM DEDTC (▼), or 1 mM metabisulfite (○).

inhibitors (**Figure 8**, solid circles), ΔE^* reached a value of 19 in 10 min, whereas in the presence of different browning inhibitors, the values of ΔE^* were significantly lower at 10 min: $\Delta E^* = 13$ for 10 mM G_2-β-CDs (**Figure 8**, open squares), $\Delta E^* = 8$ for 1 mM DEDTC (**Figure 8**, open triangles), and $\Delta E^* = 5$ for 1 mM metabisulfite (**Figure 8**, open circles). These results clearly show that the inhibition of Dominga grape PPO by reducing agents or by substrate sequestrant agents has an antibrowning effect on the color evolution of grape juice. These results also indicate that enzymatic browning by PPO is the main process involved in the browning process of Dominga grape juice at room temperature. In the case of CDs, these results agree with those described for other fruits and vegetables (*57*, *58*). Food grade G_2-β-CDs could be an interesting alternative compared to restricted-use compounds, such as metabisulfite. However, the use of this CD has to be checked in each fruit, because the opposite effect was detected in mashed banana, where a sequestrating effect of a natural PPO inhibitor was described (*51*).

ABBREVIATIONS USED

CDs, cyclodextrins; DEDTC, diethyldithiocarbamate; L-DOPA, 3,4-dihydroxyphenyl-L-alanine; K_c, inclusion constant; G_2-β-CDs, maltosyl-β-cyclodextrins; MBTH, 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate; 4MC, 4-methylcatechol; PAGE, polyacrylamide gel electrophoresis; PPO, polyphenol oxidase; SDS, sodium dodecyl sulfate; TBC, 4-*tert*-butylcatechol.

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LITERATURE CITED

(1) Sánchez-Ferrer, A.; Rodríguez-López, J. N.; García-Cánovas, F.; García-Carmona, F. Tyrosinase: a comprehensive review of its mechanism. *Biochim. Biophys. Acta* **1995**, *1247*, 1−11.

(2) Vamos-Vigyazo, L. Polyphenol oxidase and peroxidase in fruits and vegetables. *Crit. Rev. Food Sci. Nutr.* **1981**, *15*, 49−127.

(3) Martínez, M. V.; Whitaker, J. R. The biochemistry and control of enzymatic browning. *Trends Food Sci. Technol.* **1995**, *6*, 195−200.

(4) Zawitowski, J.; Biliaderis, C. G.; Eskin, N. A. M. Polyphenol oxidase. In *Oxidative Enzymes in Foods*; Robinson, D. S., Eskin, N. A. M., Eds.; Elsevier Science Publishing: New York, 1991; pp 217−273.

(5) Maxcheix, J. J.; Fleuriet, A.; Billot, J. The main phenolics of fruit. In *Fruit Phenolics*; CRC Press: Boca Raton, FL, 1990; pp 1−98.

(6) Cantos, E.; Espín, J. C.; Tomás-Barberán, F. A. Varietal differences among the polyphenol profiles of seven table grape cultivars studied by LC-DAD-MS-MS. *J. Agric. Food Chem.* **2002**, *50*, 5691−5696.

(7) Radler, R.; Torokfalvy, E. The affinity for oxygen of polyphenol oxidase in plants. *Z. Lebensm. Unters. Forsch.* **1973**, *152*, 38−42.

(8) Kidron, M.; Harel, M.; Mayer, A. M. Catechol oxidase activity in grapes and wine. *Am. J. Enol. Vitic.* **1978**, *219*, 30−35.

(9) Cash, J. N.; Sistrunk, W. A.; Stutte, C. A. Characteristics of Concord grape polyphenol oxidase involved in juice colour loss. *J. Food Sci.* **1976**, *41*, 1398−1402.

(10) Wisseman, K. W.; Lee, C. Y. Characterization of polyphenol oxidase from Ravat 51 and Niagara grapes. *J. Food Sci.* **1981**, *46*, 506−508.

(11) Nakamura, K.; Amano, Y.; Kagami, M. Purification and some properties of a polyphenol oxidase Koshu grapes. *Am. J. Enol. Vitic.* **1983**, *34*, 122−127.

(12) Lee, C. Y.; Smith, N. L.; Pennesi, A. P. Polyphenol oxidase from DeChaunac grapes. *J. Sci. Food Agric.* **1983**, *34*, 987−991.

(13) Sánchez-Ferrer, A.; Bru, R.; Cabanes, J.; García-Carmona, F. Caharacterization of catecholase and cresolase activities of Monastrell grape polyphenol oxidase. *Phytochemistry* **1988**, *27*, 319−321.

(14) Valero, E.; García-Carmona, F. Characterization of polyphenol oxidase from Airen grapes. *J. Food Sci.* **1988**, *53*, 1482−1485.

(15) Lamikanra, O.; Kirbi, S. D.; Musingo, M. N. Muscadine grape polyphenol oxidase: Partial purification by high-pressure liquid chromatography and some properties. *J. Food Sci.* **1992**, *57*, 686−689.

(16) Yilmaz, H.; Sakiroglu, H.; Küfrevioglu, I. Polyphenol oxidase from Mazruma grape. *Agrochimica* **2003**, *47*, 21−27.

(17) Mayer, A. M.; Harel, E. Polyphenol oxidases in plants. *Phytochemistry* **1979**, *18*, 193−215.

(18) Sapis, J. C.; Macheix, J. J.; Cordonnier, R. E. The browning capacity of grapes. 1. Changes in polyphenol oxidase activities during development and maturation of fruits. *J. Agric. Food Chem.* **1983**, *31*, 342−345.

(19) Bordier, C. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* **1981**, *256*, 1604−1607.

(20) Sánchez-Ferrer, A.; Bru, R.; García-Carmona, F. Novel procedure for extraction of a latent grape polyphenoloxidase using temperature-induced phase separation in Triton X-114. *Plant Physiol.* **1989**, *91*, 1481−1487.

(21) Sánchez-Ferrer, A.; Laveda, F.; García-Carmona, F. Partial purification of soluble potato polyphenol oxidase by partitioning in aqueous two-phase system. *J. Agric. Food Chem.* **1993**, *41*, 1219−1224.

(22) Angleton, E. A.; Flurkey, W. H. Activation and alteration of plant and fungal polyphenol oxidase isoenzymes in sodium dodecyl sulphate electrophoresis. *Phytochemistry* **1984**, *23*, 2723−2725.

(23) Rodriguez-López, J. N.; Escribano, J.; García-Cánovas, F. A continuous spectrophotometric meted for the determination of monophenolase activity of tyrosinase using 3-methyl-2-benzothiazolinone hydrazone. *Anal. Biochem.* **1994**, *216*, 205−212.

(24) Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of proteins utilising the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(25) Soliva-Fortuny, R. C.; Biosca-Biosca, M.; Grigelmo-Miguel; Martín-Belloso, O. Browning, polyphenol oxidase activity and headspace gas composition during storage of minimally processed pears using modified atmosphere packaging. *J. Sci. Food Agric.* **2002**, *82*, 1490−1496.

(26) Whitaker, J. R.; Lee, C. Y. Recent advances in chemistry of enzymatic browning: an overview. In *Enzymatic Browning and Its Prevention*; ACS Symposium Series 600; American Chemical Society: Washington, DC, 1995; pp 2−7.
(27) Murata, M.; Tsurutani, M.; Tomita, M.; Homma, S.; Kaneko, K. Relationship between apple ripening and browning: changes in polyphenol content and polyphenol oxidase. *J. Agric. Food Chem*. **1995**, *43*, 1115−1121.
(28) Hernández, T.; Bernalte, M. J.; Sabio, E.; Vidal-Aragon, M. C. Actividad peroxidasa y polifenoloxidasa de dos variedades de cereza durante la maduración. *Alimentaria* **1997**, *283*, 45−48.
(29) Amiot, M. J.; Tacchini, M.; Aubert, S. Y.; Oleszek, W. Influence of cultivar, maturity stage, and storage conditions on phenolic composition and enzymatic browning of pear cultivars. *J. Agric. Food Chem.* **1995**, *43*, 1132−1137.
(30) Fraignier, M.-P.; Marques, L.; Fleuriet, A.; Macheix, J. J. Biochemical and immunological characteristics of polyphenol oxidase from different fruits of *Prunus*. *J. Agric. Food Chem*. **1995**, *43*, 2375−2380.
(31) Sojo, M. M.; Núñez-Delicado, E.; García-Carmona, F.; Sánchez-Ferrer, A. Partial purification of a banana polyphenol oxidase using Triton X-114 and PEG 8000 for removal of polyphenols. *J. Agric. Food Chem.* **1998**, *46*, 4924−4930.
(32) Laveda, F.; Núñez-Delicado, E.; García-Carmona, F.; Sánchez-Ferrer, A. Reversible sodium dodecyl sulfate activation of latent peach polyphenol oxidase by cyclodextrins. *Arch. Biochem. Biophys.* **2000**, *379*, 1−6.
(33) Sánchez-Ferrer, A.; Bru, R.; Valero, E.; García-Carmona, F. Changes in pH-dependent grape polyphenol oxidase activity during maturation. *J. Agric. Food Chem.* **1989**, *37*, 1242−1245.
(34) Kenten, R. H. Latent phenolase in extracts of broad-bean (*Vicia faba* L.) leaves. 1. Activation by acid and alkali. *Biochem. J.* **1957**, *19*, 379−385.
(35) Flurkey, W. H. Polyphenoloxidase in higher plants. *Plant Physiol.* **1986**, *81*, 614−618.
(36) Sánchez-Ferrer, A.; Villalva, J.; García-Carmona, F. Triton X-114 as a tool for purifying spinach polyphenol oxidase. *Phytochemistry* **1989**, *28*, 1321−1325.
(37) Moore, B. M.; Flurkey, H. Sodium dodecyl sulphate activation of a plant polyphenoloxidase. *J. Biol. Chem*. **1990**, *265*, 4982−4988.
(38) Núñez-Delicado, E.; García-Carmona, F.; Sánchez-Ferrer, A. Partial purification of latent persimmon fruit polyphenol oxidase. *J. Agric. Food Chem.* **2003**, *51*, 2058−2063.
(39) Sánchez-Ferrer, A.; Laveda, F.; García-Carmona, F. Substrate-dependent activation of latent potato leaf polyphenol oxidase by anionic surfactants. *J. Agric. Food Chem.* **1993**, *41*, 1583−1586.
(40) Chazarra, S.; Cabanes, J.; Escribano, J.; García-Carmona, F. Partial purification and characterization of latent polyphenol oxidase in iceberg lettuce (*Lactuca sativa* L.) *J. Agric. Food Chem*. **1996**, *44*, 984−988.
(41) Núñez-Delicado, E.; Bru, R.; Sánchez-Ferrer, A.; García-Carmona, F. Triton X-114-aided purification of latent tyrosinase. *J. Chromatogr.* **1996**, *680*, 105−112.
(42) Yokotsuka, K.; Makino, S.; Singleton, V. L. Polyphenol oxidase from grapes: precipitation, resolubilization and characterization. *Am. J. Enol. Vitic.* **1988**, *39*, 293−301.
(43) Ikediobi, C. O.; Obasuyi, H. N. Purification and some properties of *o*-diphenolase from white yam tubers. *Phytochemistry* **1982**, *21*, 2815−2820.
(44) Valero, E.; Varón, R.; García-Carmona, F. Kinetic study of effect of metabisulfite on polyphenol oxidase. *J. Agric. Food Chem.* **1992**, *40*, 904−908.
(45) Whitaker, J. R. Polyphenol oxidase. In *Food Chemistry*; Fennema, O. R., Ed.; Dekker: New York, 1996; pp 492−494.
(46) Golan-Goldhirsh, A.; Whitaker, J. R. Effect of ascorbic acid, sodium bisulfite and thiol compounds on mushroom polyphenol oxidase. *J. Agric. Food Chem.* **1984**, *32*, 1003−1009.
(47) Anosike, E. O.; Ayaebene, A. O. Purification and some properties of polyphenol oxidase from a new variety of potato. *Food Chem.* **1981**, *20*, 2625−2628.
(48) Cai, Y.; Gaffney, S. H.; Lilley, T. H.; Magnolato, D.; Martin, R.; Spencer, C. M.; Haslam, E. Polyphenol interactions. Part 4. Model studies with caffeine and cyclodextrins. *J. Chem. Soc., Perkin Trans. 2* **1990**, 2197−2209.
(49) Hicks, K. B.; Sapers, G. M.; Seib, P. A. Process for preserving raw fruit and vegetables juices using cyclodextrins and composition thereof. U.S. Patent 4,975,293, 1990.
(50) Fayad, N.; Marchal, L.; Billaud, C.; Nicolas, J. Comparison of β-cyclodextrin effect on polyphenol oxidation catalysed by purified polyphenol oxidase from different sources. *J. Agric. Food Chem*. **1997**, *45*, 2442−2446.
(51) Sojo, M. M.; Núñez-Delicado, E.; García-Carmona, F.; Sánchez-Ferrer, A. Cyclodextrins as activator and inhibitor of banana pulp polyphenol oxidase. *J. Agric. Food Chem.* **1999**, *47*, 518−523.
(52) Núñez-Delicado, E.; Sánchez-Ferrer, A.; García-Carmona, F. Cyclodextrins as secondary antioxidants: synergism with ascorbic acid. *J. Agric. Food Chem.* **1997**, *45*, 2830−2835.
(53) López-Nicolás, J. M.; Bru, R.; García-Carmona, F. Kinetic characteristics of the enzymatic conversion in presence of cyclodextrins: study of the oxidation of polyunsaturated fatty acids by lipoxygenase. *Biochim. Biophys. Acta* **1997**, *1347*, 140−150.
(54) Núñez-Delicado, E.; Sojo, M. M.; Sánchez-Ferrer, A.; García-Carmona, F. Hydroperoxidase activity of lipoxygenase in the presence of cyclodextrins. *Arch. Biochem. Biophys*. **1999**, *367*, 274−280.
(55) Carvajal-Millán, E.; Carvallo, T.; Orozco, J. A.; Martínez, M. A.; Tapia, I.; Guerrero, V. M.; Rascón-Chu, A.; Llamas, J.; Gardea, A. A. Polyphenol oxidase activity, color changes and dehydratation in table grape Rachis during development and storage as affected by *N*-(2-chloro-4-pyridyl)-*N*-phenylurea. *J. Agric. Food Chem*. **2001**, *49*, 946−951.
(56) Soliva-Fortuny, R. C.; Grigelmo-Miguel, N.; Odriozola-Serrano, I.; Gorinstein, S.; Martín-Belloso, O. Browning evaluation of ready-to-eat apples as affected by modified atmosphere packaging. *J. Agric. Food Chem.* **2001**, *49*, 3685−3690.
(57) Ozoglu, H.; Bayindirli, A. Inhibition of enzymatic browning in cloudy apple juice with selected antibrowning agents. *Food Control* **2004**, *13*, 213−221.
(58) Billaud, C.; Regaudie, E.; Fayad, N.; Richard-Forget, F.; Nicolas, J. Effect of cyclodextrins on polyphenol oxidation catalysed by apple polyphenol oxidase. In: *Enzymatic Browning and Its Prevention*; ACS Symposium Series 600; American Chemical Society: Washington, DC, 1995; pp 295−312.

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