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Dual effect of β-cyclodextrin (β-CD) on the inhibition of apple polyphenol oxidase by 4-hexylresorcinol (HR) and methyl jasmonate (MJ) ☆

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Abstract

Inhibition of red delicious apple polyphenol oxidase (PPO) by three non-related antibrowning agents has been studied at different substrate concentrations. The main apple polyphenol, cholorogenic acid (CA), has been used as a substrate. The three agents (β-cyclodextrin [β-CD], 4-hexylresorcinol [HR] and methyl jasmonate [MJ]) showed an inhibitory effect at every substrate concentration tested. From the inhibition constant (K_i) values, it was concluded that the inhibitors' strength decreased in the order HR > β-CD > MJ. A combination of β-CD-HR had a synergic effect, which was not observed for the combination of β-CD-MJ. A competitive-type inhibition was obtained for HR with a $K_i = 0.26$ mM. β-CD and MJ behaved as mixed-type inhibitors, although more than one inhibitory mechanism is discussed for both agents.
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1. Introduction

The demand for fresh-cut fruits and vegetables has increased steadily during the last decade, particularly in developed countries, as a consequence of busy lifestyles, increased purchasing power and epidemiological evidence that correlates a regular intake of fresh fruits and vegetables with a reduced risk of developing several chronic and degenerative diseases (Vinson, Su, & Bose, 2001). One of the main problems for the fresh-cut industry is the enzyme-catalyzed browning of their products that occurs after peeling and cutting (Hicks et al., 1996). This process is one of the main obstacles for the industrialization of fresh-cut apples, since apple polyphenol oxidase (PPO, EC 1.14.18.1;1.10.3.1) rapidly oxidizes *o*-diphenols into *o*-quinones, which condense, by a non-enzymatic pathway, with amino acids, proteins or other compounds to form brown pigments (Coseteng & Lee, 1987; Janovitz-Klapp, Richard-Forget, Goupy, & Nicolas, 1990; Sapers & Douglas, 1987).

Traditionally, sulfites had been used as antibrowning agents in the food industry; however, their use became restricted due to concerns about negative effects on human health. For that reason, several new strategies have been

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studied in order to reduce the browning of fresh-cut apple slices and juices. These strategies may be divided in four groups according to their mode of action: (a) reducing agents such as ascorbic acid (Buta, Moline, Spaulding, & Wang, 1999; Gacche, Zore, & Ghole, 2003; Özoglu & Bayindirli, 2002; Sapers et al., 1989); (b) enzyme (PPO) inhibitors (Buta et al., 1999; Gacche et al., 2003; Iyidogan & Bayindirli, 2003; Monsalve-González, Barbosa-Canovas, McEvily, & Iyengar, 1995; Sapers et al., 1989); (c) chelating and complexing agents (Fayad, Marchal, Billaud, & Nicolas, 1997; Gacche et al., 2003; Hicks et al., 1996; Özoglu & Bayindirli, 2002); and (d) modified atmosphere packaging (Soliva-Fortuny, Grigelmo-Miguel, Odriozola-Serrano, Gorinstein, & Martín-Belloso, 2001).

4-Hexylresorcinol (HR, Fig. 1a) is a GRAS resorcinol derivative that has been described as a potent inhibitor of shrimp blackspot due to PPO activity, and has been successfully used for browning control of fresh-cut apples, potatoes and avocados (Jiménez & García-Carmona, 1997; Monsalve-González et al., 1995). The combination of HR with *N*-acetylcysteine, isoascorbic acid and calcium propionate prevented browning in Red Delicious apple slices for up to 5 weeks at 5 °C (Buta et al., 1999). Kinetic studies demonstrated that HR inhibits PPO activity either by a competitive type (Jiménez & García-Carmona, 1999) or a slow-binding inhibition mechanism (Jiménez & García-Carmona, 1997), depending on the substrate.

Cyclodextrins (CD, Fig. 1b) are natural cyclic oligomers built up from 6, 7 or 8 glucopyranose units, (named α-, β- and γ-CD, respectively) linked by α-(1–4)-glycosidic bonds. They posses a hydrophobic inner cavity, in which organic molecules can be trapped forming inclusion complexes (Alvarez-Parrilla, de la Rosa, & Rodrigo-García, 2005a). Several authors have described that in the presence of β-CD, apple PPO activity is inhibited because there is a reduction of free substrate due to the complexation process between the CD and the PPO substrate (Billaud, Regaudie, Fayad, Richard-Forget, & Nicolas, 1995; Fayad et al., 1997; Hicks et al., 1996). When combined with ascorbic acid and derivatives, a synergic effect over the inhibition of PPO (Gacche et al., 2003) and lipoxygenase (LOX) (Nuñez-Delicado, Sánchez-Ferrer, & García-Carmona, 1997) has been reported. Contrary to expectations, the inhibition of banana PPO by CDs, in the presence of enzyme inhibitors (4-iodophenol and cinnamic acid) using a hydrosoluble substrate (dopamine) showed an activation of the enzyme, instead of the anticipated inhibition. This activation in the enzymatic activity was explained by considering a competitive complexation process between the substrate and the inhibitors with CDs (Mar Sojo, Nuñez-Delicado, García-Carmona, & Sánchez-Ferrer, 1999).

Methyl jasmonate (MJ, Fig. 1c) is a naturally occurring plant growth regulator that has been used to extend shelf life of fresh-cut fruits and vegetables. Even though treatments with MJ are related to a reduction of microbial growth, and prevention of chilling injury, Buta and Moline (1998) observed that MJ vapors also inhibited browning of fresh-cut celery and peppers. As far as we know, the inhibition mechanism of MJ on vegetable tissue browning has not been previously studied.

Having in mind that there is little information on the combined effect of β-CD with commonly used inhibitors over the catalytic activity of PPO, the aims of this paper were (a) to study the effect of HR and MJ, alone and combined with β-CD, on the inhibition of the oxidation of chlorogenic acid (CA, Fig. 1d) catalyzed by Red Delicious apple PPO, and (b) to determine the inhibition mechanism of β-CD and MJ.

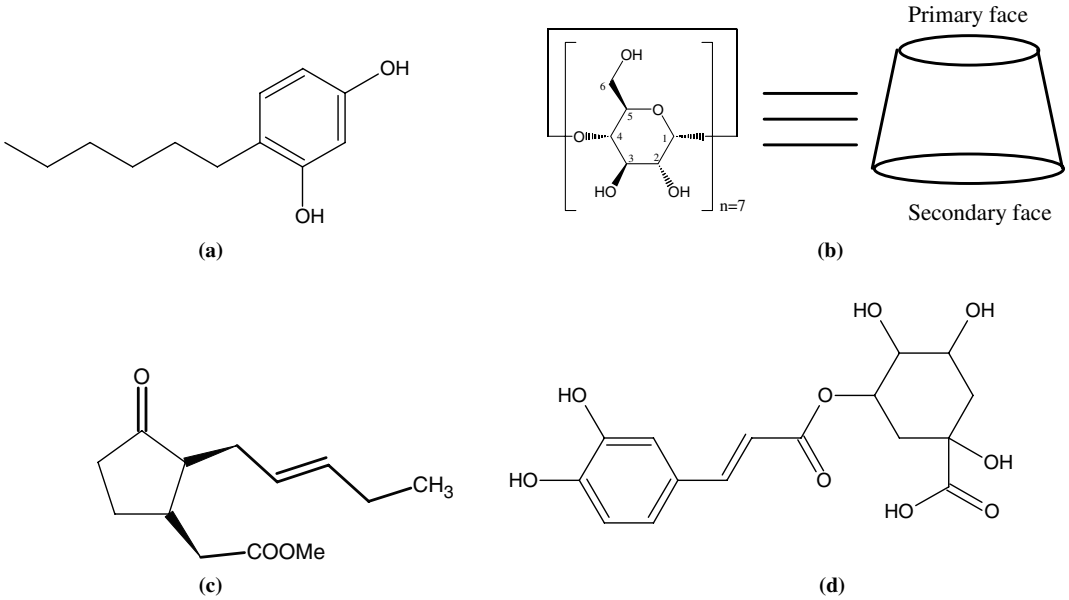

Fig. 1. Chemical structure of molecules used in this study: (a) 4-hexylresorcinol; (b) β-cyclodextrin, β-CD; (c) methyl jasmonate; (d) chlorogenic acid.

2. Materials and methods

2.1. Materials

β-Cyclodextrin (cyclomalto-heptaose, Cavamax W7 food grade, kindly supplied by Wacker Biochem, USA) was dried in a vacuum oven, and used without further purification. Chlorogenic acid (1,3,4,5-tetrahydroxycyclohexane carboxylic acid 3-[3,4-dihydroxycinnamate]) (CA), 4-hexylresorcinol (HR), methyl jasmonate (MJ), polyvinyl polypyrrolidone (PVPP), citric acid, citric acid sodium salt, Bradford reagent and bovine serum albumin (BSA) were purchased from Sigma–Aldrich, and used without further purification. Phosphate buffers were prepared from K_2HPO_4 and KH_2PO_4, both from Jalmek A.C.S. grade (San Nicolas de los Garza, N. L., Mexico). All other reagents used were A.C.S grade from Acrōs Organics (New Jersey, USA). Apples (Red Delicious) were purchased at commercial maturity at a local market, and stored at 4 °C until processed.

2.2. Enzyme extraction

Apple PPO was partially isolated according to the methodology proposed by Soliva-Fortuny et al. (2001). In order to reduce apple to apple variability, a mixture of three apple pulps was used for each extraction. Apple pieces (25 g) were homogenized during 5 min at 4 °C, with 25 mL of McIlvaine buffer pH 6.5 (acetic acid 0.03 M, K_2HPO_4 0.14 M and NaCl 1 M) and 5% (w/w) PVPP. The homogenate was centrifuged at 2500 rpm for 30 min at 4 °C (IEC NH-SII centrifuge). The supernatant solution was filtered through a Whatman No. 1 paper. Protein concentration was determined by the Bradford method, using BSA as standard, with a BioRad Benchmark Plus microplate spectrophotometer (BioRad, Mexico city, Mexico).

2.3. Enzymatic activity

PPO activity was determined spectrophotometrically, with an Agilent 8453 spectrophotometer (Agilent Technologies, Palo Alto, CA, USA), from the measurement of the oxidation of CA at 400 and 420 nm, every 15 s during 2 min, in the presence of 6 μg/mL of protein, at 25 °C pH 5 (0.1 M citrate buffer), in a 1-cm path cuvette. Protein concentration was adjusted in order to add 100 μL of enzyme to 900 μL of different CA concentration (10, 5, 2, 1 and 0.5 mM). The initial reaction rate (V_0) was estimated from the linear portion of the reaction plot at each initial substrate concentration, and expressed as $\Delta U_{abs}\,s^{-1}$. Experimental data (V_0), as a function of initial CA concentration, was fitted to the Michaelis–Menten equation:

$$V_0 = \frac{V_{max} \cdot [S]}{K_M + [S]} \tag{1}$$

using a commercial non-linear least-square fit program (Sigma Plot 8.0), in order to determine K_M and V_{max}. K_M and V_{max} were also determined by the double reciprocal Lineweaver–Burk plot (Eq. (2)), and used as initial parameters for the non-linear fitting:

$$\frac{1}{V_0} = \frac{1}{V_{max}} + \frac{K_M}{V_{max} \cdot [S]} \tag{2}$$

In order to determine the effect of the inhibitors over the PPO activity, HR (0.5 mM), MJ (2 mM), β-CD (5 mM), and mixture solutions were prepared in citrate buffer. From these inhibitor stock solutions, the different CA concentrations were prepared and the enzymatic activity determined as previously described. The effect of different β-CD concentrations (2, 6 and 10 mM) was studied determining the relative PPO activity (RA) using the following equation:

$$RA = 100 \cdot \frac{A}{A_0} \tag{3}$$

where A and A_0 are the enzyme activities in the presence and absence of β-CD.

The apparent inhibition constants (K_i) for HR were determined considering that it acts as a competitive inhibitor, using the following equation:

$$K'_M = K_M \cdot \left(1 + \frac{[I]}{K_i}\right) \tag{4}$$

where K'_M and K_M are the Michaelis constants in the presence and absence of HR, respectively. For MJ, a mixed type inhibition was considered and K_i (inhibitor constant for free enzyme) and K'_i (inhibitor constant for enzyme–substrate complex) were determined using Eqs. (5) and (6), respectively (Tipton, 1996):

$$\frac{K'_M}{V'_{max}} = \frac{K_M \cdot \left(1 + \frac{[I]}{K_i}\right)}{V_{max}} \tag{5}$$

$$V'_{max} = \frac{V_{max}}{\left(1 + \frac{[I]}{K'_i}\right)} \tag{6}$$

where K'_M and V'_{max} are the Michaelis constant and maximal rate in the presence of MJ. In the case of β-CD, a mixed type inhibition was also considered, and K_i was determined graphically from the plot of the slope of the Lineweaver–Burk vs. different β-CD concentrations, according to Qing-Xi et al. (2005).

2.4. Free substrate concentration

The free substrate ($[CA - C_{11}]$) concentration was determined using Eq. (7), considering a 1:1 stoichiometry, with a stability constant of 465 M^{-1} (Alvarez-Parrilla, de la Rosa, Torres-Rivas, Rodrigo-García, & González-Aguilar, 2005b):

$$K_{11} = \frac{C_{11}}{[CD - C_{11}] \cdot [CA - C_{11}]} \tag{7}$$

2.5. Statistical analysis

All data are reported as means ± SEM of at least four replicates. Analysis of variance and LSD analysis ($p < 0.05$) were carried out in order to determine statistical differences between treatments and control (SPSS 12.0).

3. Results and discussion

3.1. Effect of HR and β-CD on the activity of apple PPO

Apple PPO was partially isolated according to the methodology proposed by Soliva-Fortuny et al. (2001), using McIlvaine buffer pH 6.5 and PVPP (5% p/p) to eliminate endogenous polyphenols. In order to minimize variability among apples, each PPO extract was obtained from a mixture of the pulps from three apples; control and inhibition experiments were carried out with the same enzyme extract. CA was used as substrate since it is described as the mayor apple polyphenol and PPO substrate (Irwin et al., 1994; Richard-Forget, Amiot, Goupy, & Nicolas, 1995). Despite Richard-Forget et al. (1995), observation that the *o*-quinone obtained from the oxidation of CA exhibited three absorption peaks at 255, 325 and 400 nm, with a maximum at 325 nm, in the present study, the catalytic oxidation of CA was followed at 400 nm, for two reasons: (i) when the spectra obtained for the oxidation of a 10 mM CA solution in the presence of apple PPO during 10 min were analyzed, the largest absorbance variation was observed at 400 nm (data not shown); and (ii) in order to eliminate interferences from HR, since preliminary results showed that this molecule presents a peak at 325 nm, while at 400 nm the absorption was negligible.

The effects of HR (0.5 mM), β-CD (5 mM) and a mixture of both compounds (0.5 mM HR + 5 mM β-CD), in the oxidation of CA catalyzed by Red Delicious PPO are presented in Fig. 2. At the studied concentrations, both compounds inhibited, in approximately the same amount, the catalytic activity of the enzyme, being this effect less evident as substrate concentration increases. These results are in agreement with other authors that have described the inhibition of PPO by HR and β-CD. Furthermore, a synergic effect of the mixture of HR and β-CD on the inhibition of the CA oxidation by PPO is also observed in Fig. 2, being this effect more evident at higher CA concentrations. This synergic effect may be explained by having in mind that both compounds inhibit the PPO activity by different pathways. Jiménez and García-Carmona (1999) observed that HR inhibited the oxidation of quercetin by broad bean PPO by a competitive mechanism; similar results were observed for eggplant PPO (Pérez-Gilabert & García-Carmona, 2000). In contrast, the inhibitory effect of β-CD on PPO activity has been attributed to the reduction of free substrate as a consequence of the complexation process between polyphenols and β-CD (Alvarez-Parrilla et al., 2005a, & references therein). Therefore, β-CD reduces the concentration of free CA that can be oxidized while HR interacts directly with the enzyme by a competitive mechanism, and consequently, combination of the two inhibition mechanisms reduces PPO activity to a higher degree.

Similar results have been reported for the inhibition of PPO (Gacche et al., 2003) and LOX (Nuñez-Delicado et al., 1997), when β-CD was combined with ascorbic acid and derivatives. Hicks and coworkers observed a synergic effect on the prevention of browning of apple juices treated with soluble or insoluble CD derivatives in combination

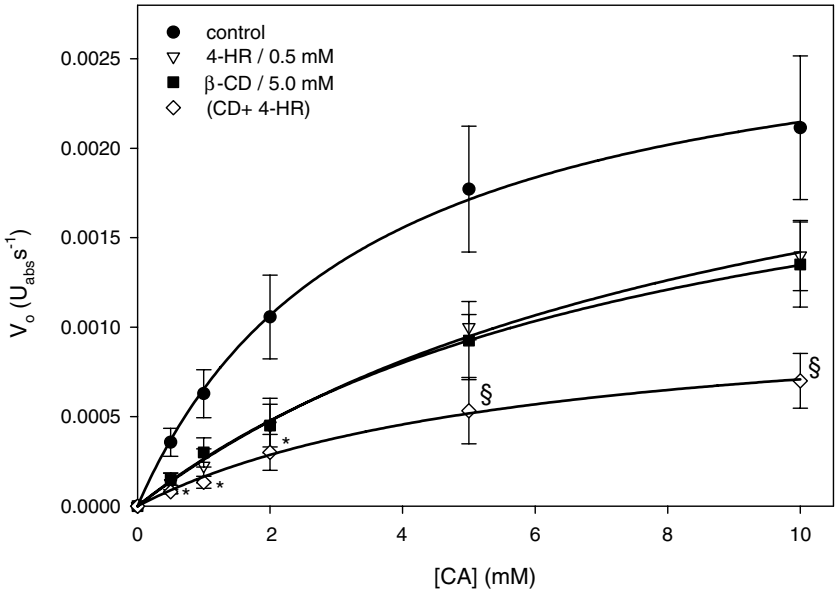

Fig. 2. Effect of HR, β-CD and mixture, on CA oxidation catalyzed by Red Delicious PPO (6 μg/mL total protein, 25 °C, 0.1 M citrate buffer pH 5, 1 mL final volume). Symbols represent experimental data (±SEM). Lines are the Michaelis–Menten curves fitted to the experimental data. * Statistical difference (LSD, $p < 0.05$) between all treatments respect to control. § Statistical difference (LSD, $p < 0.05$) between mixture and control.

with phosphates for as long as 2–3 weeks at 4 °C (Hicks, Sapers, & Seib, 1990; Hicks et al., 1996; Sapers et al., 1989). However, this same research group reported that CD was not effective in the prevention of browning of fresh-cut apples, due to the small diffusion rate of CD inside apple tissue. Considering that HR has been reported as a good browning inhibitor in fresh-cut apples (Buta et al., 1999; Monsalve-González, Barbosa-Canovas, Cavalieri, McEvily, & Iyengar, 1993; Monsalve-González et al., 1995), this synergic result observed, suggests that the combination of HR and β-CD may be an alternative to control the browning of fresh-cut apples.

The Lineweaver–Burk plots of PPO inhibition by HR and β-CD are shown in Fig. 3. The apparent kinetic parameters (K_M and V_{max}) were initially calculated by linearly fitting data plotted in Fig. 3 to Eq. (2), then, these values were used as initial parameters in the non-linear regression analysis of the experimental data to Eq. (1). Results obtained by both methods are summarized in Table 1. Control K_M value obtained in this work (3.39 mM) is somehow smaller than those reported by other authors (4.25–5.6 mM) (Fayad et al., 1997; Janovitz-Klapp et al., 1990). However, this variation may be due to cultivar, maturity and period of cold storage, as reported by Coseteng and Lee (1987).

From the intersection of the control and inhibitor plots in Fig. 3, competitive and mixed-type inhibition mechanisms are proposed for HR and β-CD, respectively. The increment of K_M, while V_{max} remained practically unchanged, confirms the competitive type mechanism proposed for HR (Jiménez & García-Carmona, 1999; Pérez-Gilabert & García-Carmona, 2000). From these data, the apparent inhibition constant (K_i) was determined using Eq. (4), and is presented in Table 1. This value is higher than that obtained for eggplant PPO, using *tert*-butylcatechol as a substrate (260 vs. 50 μM) (Pérez-Gilabert & García-Carmona, 2000).

For β-CD, a mixed inhibition pattern, in which $K_i' > K_i$ is observed in Fig. 3. Mixed inhibitors with this behavior bind to both free enzyme and enzyme–substrate complex, with a higher affinity for the free enzyme (Tipton, 1996). However, as mentioned before, β-CD has been considered to inhibit PPO only by reducing free substrate concentration and not by binding to the enzyme. To further characterize the β-CD-mediated inhibition of apple PPO, its relative activity (RA) at three CA concentrations (1, 5 and 10 mM) was determined for three β-CD concentrations

Table 1
Apparent kinetic parameters of red Delicious PPO with CA as substrate in the presence of β-CD, HR and MJ

	Control	4-HR	MJ	β-CD
V_{max}[a]	2.9	2.8	2.2	2.5
K_M[a]	3.39	9.86	4.20	8.35
V_{max}[b]	3.0	2.8	2.9	2.1
K_M[b]	3.68	10.84	6.62	6.4
K_i[c]	–	0.26	8.38	3.42[d]
K_i[e]	–	–	3.15	–
K_i'[e]	–	–	6.29	–

V_{max}: U_{abs} $s^{-1}/10^3$; K_M: mM; K_i: mM.
[a] Values obtained by non-linear fitting to Eq. (1).
[b] Values obtained by linear fitting to Eq. (2).
[c] Determined using Eq. (4).
[d] Value obtained from Fig. 6.
[e] Values calculated using Eqs. (5) and (6).

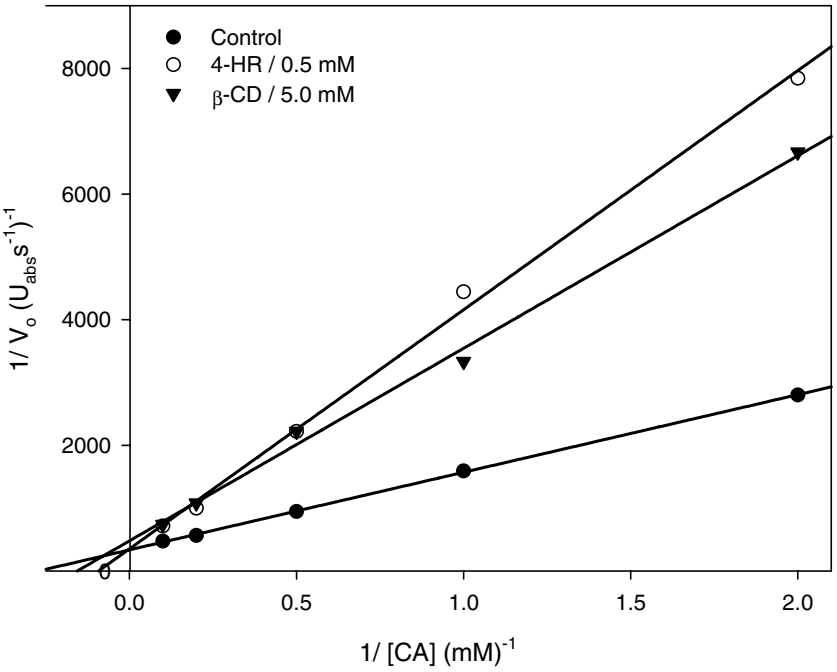

Fig. 3. Lineweaver–Burk plots for the inhibition of HR and β-CD, on the oxidation of CA by Red Delicious PPO.

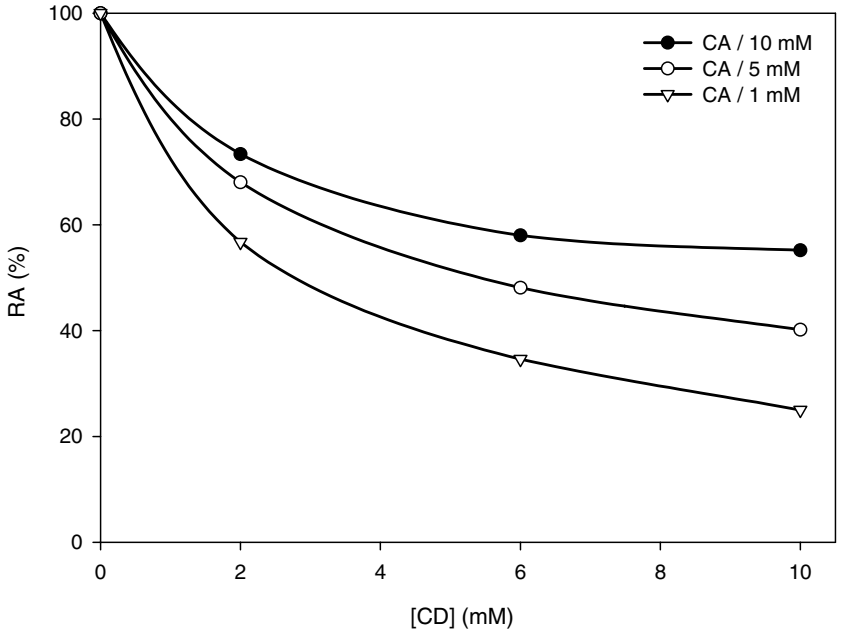

Fig. 4. Effect of different β-CD concentrations (0–10 mM) on the relative activity (RA) of Red Delicious PPO measured at three different substrate concentrations (1, 5 and 10 mM CA). Experimental conditions are the same as for Fig. 2.

(2, 6 and 10 mM). These results are shown in Fig. 4, in which it is possible to observe a decrease in enzyme activity as the concentration of β-CD increases. It is also possible to observe that this behavior is dependent on the substrate concentration, i.e. as the CA concentration increases, from 1 to 10 mM; the inhibition effect of β-CD is less evident. For this reason, β-CD ID_{50} values are dependent on substrate concentration: at 1 mM CA, β-CD $ID_{50} \approx 2.78$ mM, in contrast, at 10 mM CA $ID_{50} > 10$ mM β-CD. This can be explained in terms of the complexation of the CA, considering a 1:1 stoichiometry, as previously described (Alvarez-Parrilla et al., 2005b; Irwin et al., 1994):

$$\text{CA} + \beta\text{-CD} \overset{K_{11}}{\rightleftarrows} C_{11}$$

(i) As the concentration of β-CD increases, the equilibrium is displaced toward the formation of the complex, and consequently, there is a decrease of the free CA concentration that can be oxidized by the PPO, considering that complexed substrate can not be catalyzed, as previously reported by (Fayad et al., 1997). (ii) At the same β-CD concentration, the percentage of free CA increases as its concentration increases, and consequently the RA is higher. In this way, at a 10 mM β-CD concentration, the percentage of free CA increased from 19% to 37% when the CA concentration increased from 1 to 10 mM. These results are in agreement with those reported by other authors, suggesting that β-CD inhibitory effect is due to free substrate reduction (Fayad et al., 1997; Mar Sojo et al., 1999; Nuñez-Delicado, Mar Sojo, García-Carmona, & Sánchez-Ferrer, 2003; Nuñez-Delicado et al., 1997). These authors have also shown that the inhibition of PPO activity by β-CD-mediated complex formation is due to the fact that PPO can only work with free substrate, and not with the complex. However, in the case of mushroom tyrosinase, it was described that PPO could oxidize both free and complexed substrates, even though the activity toward the complexed substrate was lower (Fayad et al., 1997). Except in the case of mushroom tyrosinase, these authors have used this complexation-dependent inhibition mechanism in order to calculate the stability constants of the substrate–β-CD complexes, by non-linear regression of the experimental initial velocities to an equation that correlates this parameter with CD concentration. Once they have determined the stability constant, they use it to calculate the free substrate concentration, observing that when re-plotting the initial velocities vs. free substrate, the same curve was obtained for control and β-CD-containing samples, which indicates that the enzyme only catalyzes the oxidation of free substrate and, suggesting β-CD has no effect on PPO other than free substrate reduction.

In order to corroborate this inhibition mechanism, the free CA concentrations, at a 5 mM β-CD concentration, were determined considering a stability constant of 465 M^{-1} (Alvarez-Parrilla et al., 2005b), and used to replot, initial velocity data form Fig. 2 as a function of free CA concentration, as shown in Fig. 5. Control curve in Fig. 5 is the non-linear regression of control data in Fig. 2 to Eq. (1). At low CA concentrations, both systems (control and β-CD) presented similar initial velocities, however, at high CA concentrations; a β-CD inhibitory effect can be observed, since the initial velocities of the β-CD-containing system were lower than those from the control. These results suggest direct interaction of β-CD with the enzyme, and are not in agreement with those reported by the above mentioned authors that observed the same kinetics for both conditions (the absence and presence of β-CD).

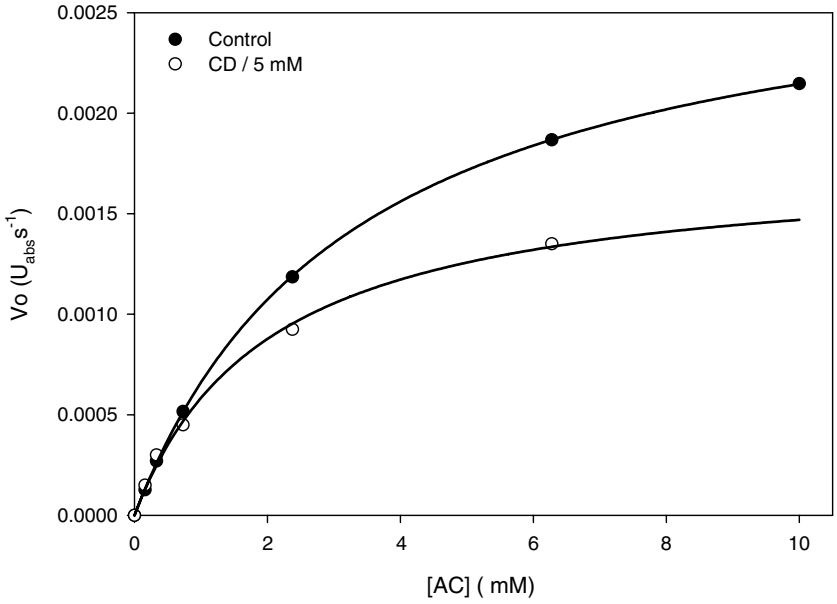

Fig. 5. Effect of β-CD (5 mM) on the oxidation of free CA. Free CA concentrations were calculated using Eq. (7), and initial velocities are those shown in Fig. 2. Experimental conditions are the same as for Fig. 2.

Therefore, using data from Fig. 4 we obtained Lineweaver–Burk plots of the inhibition of PPO by three different β-CD concentrations, in order to calculate inhibition constants (K_i' and K_i), which are inhibitor–enzyme dissociation constants (Fig. 6). This figure shows a mixed-type inhibition mechanism, since increasing β-CD concentration resulted in a series of straight lines with different slopes and intercepts (Chen, Song, Qiu, Huang, & Guo, 2005). However, the inhibition profile is not that of a typical mixed-type inhibitor.

In Fig. 6a, it is possible observe that the slope increased at higher β-CD concentrations. According to Chen et al. (2005), from the plot of the slope and the intercept vs. inhibitor concentration, the inhibition constants for the free enzyme (K_i) and enzyme–substrate complex (K_i'), can be obtained. Fig. 6b shows that the plot of the slope vs. β-CD concentration is a straight line, from which the K_i value was obtained, and is presented in Table 1. However, when the intercept of the Lineweaver–Burk plot was plotted against β-CD concentration (inset in Fig. 6b), it is possible to observe that at lower concentrations there is an increase of its value as β-CD concentration increases, but at higher β-CD concentrations, the slope steadily decreases. Therefore, it was not possible to obtain a value for K_i'.

These results may indicate that at higher concentrations, β-CD does not interact with the enzyme–substrate complex, but only with the free enzyme in order to form an enzyme–β-CD complex. If this is the case, β-CD could be considered as a competitive inhibitor, which will also explain the inhibition pattern shown in Fig. 4, where high substrate concentrations reduce β-CD inhibition. However, kinetic parameters suggest a mixed-type inhibition, and we must also bear in mind that the above data could also be explained by the complexation-dependent inhibition mechanism, described earlier. In conclusion, these contradictory results may indicate that β-CD inhibits the catalytic activity of PPO by two different mechanisms: (i) the complexation of CA, which reduces the amount of free CA able to be catalytically oxidized, and which is reversed by increasing substrate concentrations; and (ii) by interacting with some hydrophobic aminoacids from the enzyme, in order to form enzyme–β-CD complexes, with reduced catalytic activity. This second inhibition mechanism may be supported by studies from Koralewska, Augustyniak, Temeriusz, and Kanska (2004), who proposed a similar mixed type mechanism for the inhibition of Tyrosine Phenol-lyase by α-, β- and γ-CD derivatives, in which the CDs may interact directly with the enzyme.

In order to prove this hypothesis, further isothermal titration calorimetry (ITC) and fluorescence spectroscopy studies are being planed, in order to analyze the formation of the enzyme–β-CD complex, and to measure the formation of the enzyme–substrate complex in the presence and absence of β-CD.

When the apparent inhibition constants of HR and β-CD are compared, it is possible to observe that HR is a better inhibitor, since its constant is approximately 10 times smaller than that for β-CD. These results are in agreement with those observed in Fig. 2, where a β-CD concentration 10 times as high as that of 4HR, produced statistically, the same inhibition of PPO activity.

3.2. Effect of MJ and MJ–β-CD mixture on the activity of apple PPO

MJ has been used to extend the shelf life of different fresh-cut fruits and vegetables, by reducing microbial growth and chilling injury, without significantly modifying

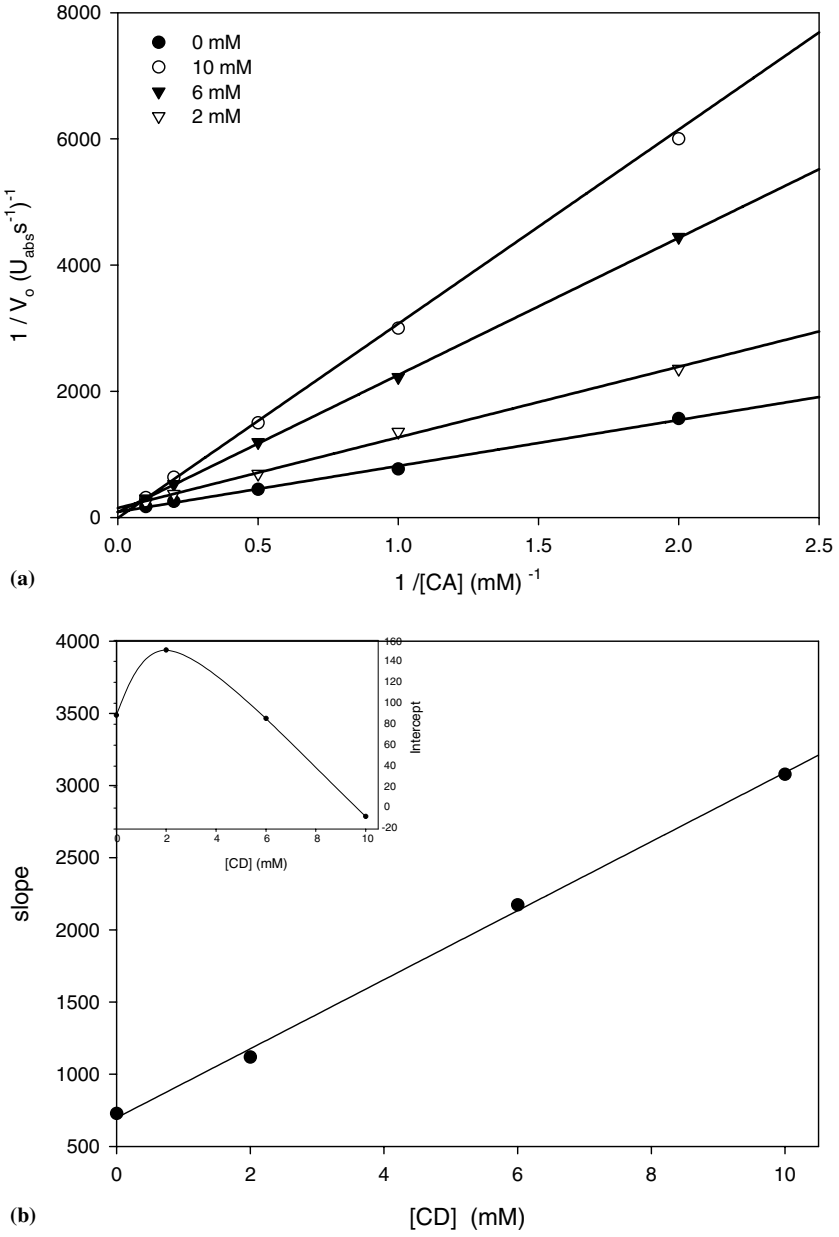

Fig. 6. (a) Lineweaver–Burk plots for the inhibition of the oxidation of CA catalyzed by Red Delicious PPO at three different β-CD concentrations (experimental conditions are the same as for Fig. 2). (b) Replot of Lineweaver–Burk slopes obtained from Fig. 6, at three different β-CD concentrations (2, 6 and 8 mM). Inset shows replot of Lineweaver–Burk intercepts.

other quality parameters (Buta et al., 1999; González-Aguilar, Buta, & Wang, 2003; Martínez-Ferrer & Harper, 2005; Wang & Buta, 2003). When fresh-cut celery was treated with MJ, browning was inhibited during the first week (Buta & Moline, 1998). González-Aguilar, Tiznado-Hernández, Zavaleta-Gatica, and Martínez-Téllez (2004) observed that when guava fruits were treated with MJ, the activity of the enzymes lipoxygenase (LOX) and phenylalanine-ammonia lyase (PAL) increased. MJ has been also used to enhance color in apple peels by activating the synthesis of anthocyanin pigments (Mattheis & Rudell, 2002). In addition, MJ-treated tomato plants showed an increased PPO activity and expression in the tomato fruits (Koussevitzky, Ne'eman, & Harel, 2004).

Having in mind that MJ increases the enzymatic activity of browning related enzymes (PAL, LOX and PPO) in whole fruits and vegetables, but reduced browning in fresh-cut celery, it seems interesting to study the effect of MJ over the oxidation of CA catalyzed by apple PPO, to better understand the mechanism of browning reduction in fresh-cut products. MJ was dissolved in water at the highest possible concentration (2 mM) in order to avoid any interference in the kinetic parameters due to the effect of organic solvents (alcohols or DMSO). The effects of MJ and MJ–β-CD mixture (2 mM MJ + 5 mM β-CD) are presented in Fig. 7a.

This figure shows that MJ inhibited CA oxidation at all substrate concentrations. When the apparent kinetic

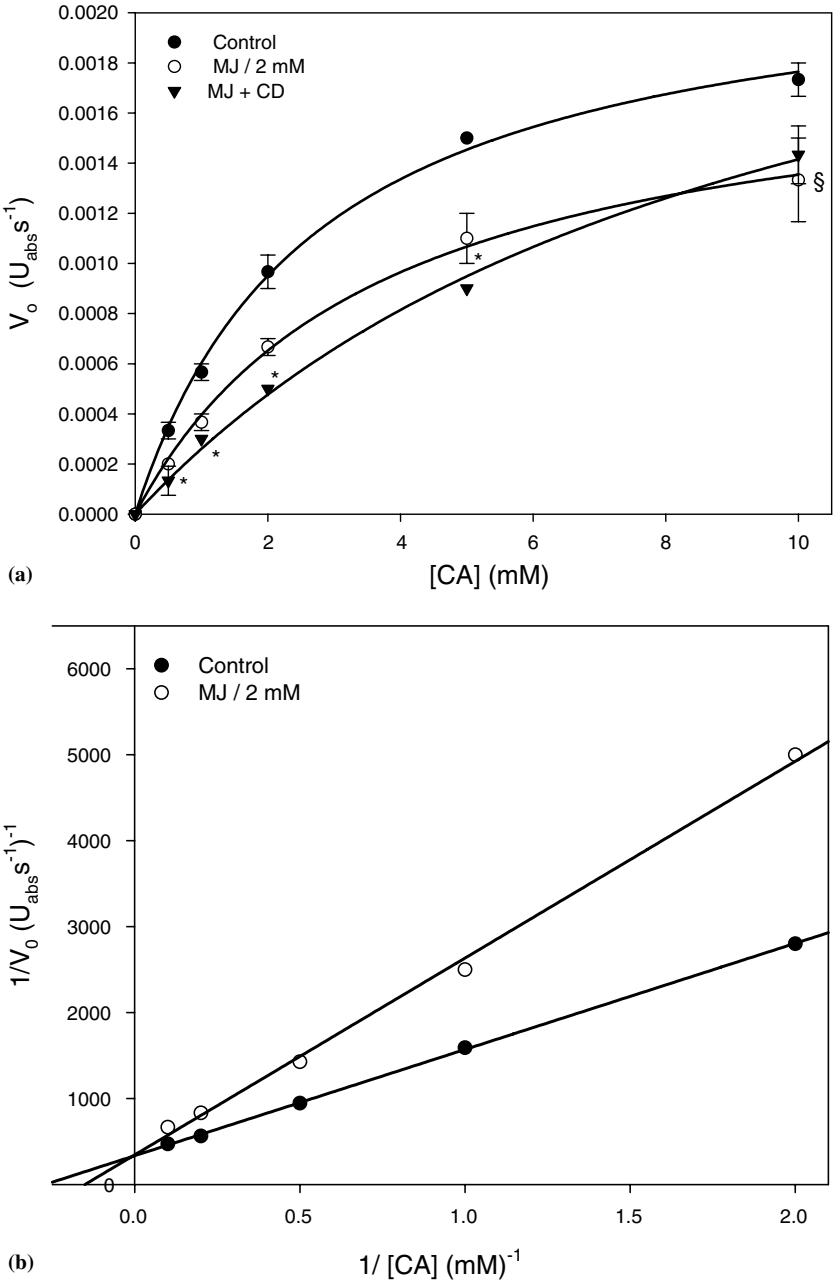

Fig. 7. (a) Effect of MJ and mixture (2 mM MJ + 5 mM β-CD), on CA oxidation catalyzed by Red Delicious PPO (experimental conditions are the same as for Fig. 2). Symbols represent experimental data (±SEM, $n = 3$). Lines are the Michaelis–Menten curves fitted to the experimental data. * Statistical difference (LSD, $p < 0.05$) between all treatments respect to control. § Statistical difference (LSD, $p < 0.05$) between MJ and control. (b) Lineweaver–Burk plots for the inhibition of MJ, on Red Delicious PPO activity.

parameters were determined fitting the experimental data to Eqs. (1) and (2) (Lineweaver–Burk plot, Fig. 7b) some differences were observed. K_M and V_{max} values obtained by linear regression (Table 1) suggested a competitive inhibition mechanism. However, from the non-linear analysis results (Table 1) a mixed type inhibition is proposed. The inhibition constants were determined considering both mechanisms, using Eqs. (4)–(6) and are summarized in Table 1. When we compare these inhibition constants with those from HR and β-CD, it is possible to conclude that even though MJ inhibits the catalytic activity of PPO, it does it in a smaller degree than the other two inhibitors. These results may suggest that the reduction of browning observed in fresh-cut celery could be partly due to PPO inhibition. As far as we know, this is the first study in which the direct effect of MJ over the catalytic activity of PPO was measured. Further studies have to be carried out in order to study the effect of MJ on the PPO activity of fresh-cut apples, in order to determine which inhibitory mechanism is actually presented by this substance, and also, to find out if the treatment with MJ will in fact reduce enzyme activity in fresh- cut fruits, or if it will increase it, as in the case of whole tomatoes.

Fig. 7a shows that β-CD had no synergic effect on the MJ-dependent PPO inhibition, in contrast with results obtained for the HR-β-CD mixture (Fig. 2). This can be explained by considering two competitive complexation processes. β-CD forms 1:1 stoichiometry complexes with both CA (Alvarez-Parrilla et al., 2005b) and MJ (unpublished results), with similar stability constants. As a consequence of this competitive equilibrium, at least two processes will occur: (i) there will be a reduction of complexed CA, and consequently an increase of free CA that can be oxidized by PPO; and (ii) part of the MJ will be complexed, reducing the amount of free MJ that can inhibit PPO activity. The combination of these two processes will give a reduction of the inhibition activity of the mixture because there will be less inhibitor (MJ) and more substrate (CA). Mar Sojo et al. (1999) observed a similar reduction on the inhibition activity of 4-iodophenol and cinnamic acid in the presence of a β-CD derivative.

From the results obtained in the present paper, it may be concluded that the three molecules studied (HR, MJ and β-CD) inhibited, in a different degree and by a different mechanism, the catalytic activity of Red Delicious apple PPO. The mixture HR-β-CD, at the studied concentrations, showed a synergic inhibition effect on the PPO activity, and finally, as far as we know, direct PPO inhibition by MJ was described for the first time.

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