JFS C: Food Chemistry and Toxicology

Ascorbic Acid and 4-Hexylresorcinol Effects on Pear PPO and PPO Catalyzed Browning Reaction

E. Arias, J. González, R. Oria, and P. Lopez-Buesa

ABSTRACT: The effects of ascorbic acid (AA) and 4-hexylresorcinol (4-HR) on pear polyphenoloxidase (PPO) activity and stability have been investigated *in vitro*. AA does not interact directly with PPO but prevents browning by reducing oxidized substrates. The 4-HR exerts a dual role on PPO. If no substrates are present, it interacts preferably with the *deoxy* form of PPO inactivating it. If substrates and 4-HR are both present they compete for the catalytic site. The 4-HR behaves then as a canonical enzyme inhibitor, binding to the *met* form of PPO. Simultaneous addition of 4-HR and AA has synergistic inhibition or inactivatory effects depending on the presence or the absence of PPO substrates.

Keywords: 4-hexylresorcinol, ascorbic acid, enzymatic browning, fresh-cut pear, polyphenoloxidase

Introduction

Consumption of fresh-cut products has increased at an annual rate of approximately 10% since 1995; the market for fresh-cut vegetables and fruits is estimated at 10 to 12 billion dollars annually (IFPA 2000). The economic success of these products is a result of 2 properties: their fresh-like appearance and ease of consumption, achieved by, for example, peeling and cutting. Such handling, however, can compromise the fresh-like appearance of the product because it triggers enzymatic browning reactions (McEvily and Iyengar 1992; Artés and others 1998; Soliva-Fortuny and others 2001) and accelerates respiratory pathways (Cantwell and Trevor 1992; Izumi and others 1996; Watada and others 1996). Control of respiratory pathways in fresh-cut products is achieved mainly by using modified atmospheres (Zagory 1998; Poubol and Izumi 2005; Del Nobile and others 2006) and low temperatures whereas enzymatic browning reactions, mostly catalyzed by polyphenol oxidase isoenzymes, are slowed down by a combination of low temperatures and the use of specific enzyme/browning inhibitors (Monsalve-Gonzalez and others 1993; Luo and Barbosa-Cánovas 1997; Dong and others 2000; Abbott and Buta 2002; Gorny and others 2002; González-Aguilar and others 2004; Fan and others 2005).

The selection of appropriate cultivars and the degree of ripeness are also very important for successful fresh-cut product elaboration (Gorny and others 1998a; Gorny and others 1998b; Gorny and others 1999; Gorny and others 2000; Buta and Abbott 2000; Kader 2002; Soliva-Fortuny and others 2002; Calderón-López and others 2005). We have recently performed a study on several pear cultivars and have found that *Conferencia* pears at commercial ripeness stage and treated with a combination of ascorbic acid (AA), 4-hexylresorcinol (4-HR), and Cl_2Ca are the most appropriate for fresh-cut elaboration (unpublished results). Cl_2Ca is used to improve texture due to its ability to interact with pectines, thereby increasing interchain bridges, 4-HR and AA are used to prevent enzymatic catalyzed browning reactions.

Enzymatic browning reactions are mainly catalyzed by polyphenoloxidase isoenzymes. Polyphenoloxidase is a complex enzyme with 2 copper ions in its active center. It is able to catalyze 2 reactions: the hydroxylation of monophenols and the oxidation of diphenols to quinones, which subsequently polymerize to yield brown pigments (Taylor and Clydesdale 1987; McEvily and Iyengar 1992). The most widely accepted reaction mechanism model involves several steps in which the oxidation state (*oxy*, *deoxy*, or *met*) of the copper ions in the active center changes during binding and transformation of pear polyphenoloxidase (PPO) substrates, molecular oxygen, and phenolic substances.

Antibrowning effects of both AA and 4-HR have been found, in PPOs of diverse origins, to be regulated by different mechanisms (Whitaker and Lee 1995; Luo and Barbosa-Cánovas 1996; Sapers and Miller 1998; González-Aguilar and others 2004; Pilizota and Sapers 2004). These can be due to their antioxidant capacity, which allows them to react with oxidized substrate molecules, or to their direct interaction with the enzyme. In the latter case several ways of interaction have been described, ranging from classical noncovalent enzyme inhibition to irreversible covalent inhibition (Weemaes and others 1999; Golan-Goldhirsh and Whitaker 1984; Osuga and others 1994). In the present work we investigate *in vitro* the mechanisms by which AA and/or 4-HR delay browning caused by PPO in order to better understand, and therefore to better control, browning processes in fresh-cut products.

Material and Methods

Materials

Pears (*Pyrus Communis L.* cv. Conferencia) used in this study were harvested at maturation stage at an experimental orchard in La Almunia (Zaragoza, Spain) and stored at 4 °C for 1 d until used. All chemicals were purchased from Sigma-Aldrich (Spain).

Extraction of PPO

Two hundred grams of peeled and cut pear tissue were homogenized with 300 mL of chilled 0.2 M sodium phosphate buffer (pH 6.8) containing 1% polyvinylpolypyrolidone (PVPP) in a Waring blender for 3 min. The homogenate was filtered through cheese cloth and the filtrate was centrifuged at 20000 × *g* for 30 min at 4 °C. The supernatant was brought first to 40% and subsequently to 80% $(NH_4)_2SO_4$ saturation with solid $(NH_4)_2SO_4$. The 80% $(NH_4)_2SO_4$ pellet was separated by centrifugation at 20000 × *g* for 30 min, redissolved in a small amount of 0.05 M phosphate buffer (pH 6.8), and dialyzed at

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doi: 10.1111/j.1750-3841.2007.00484.x

4 °C in the same buffer in cellulose dialysis tubing (mol. wt. cutoff 12000 to 14000 Da). The dialysis buffer was changed 3 times at 8-h intervals. The dialyzed sample was used as the PPO enzyme source in the following experiments.

Spectrophotometrical enzyme assay

Enzyme activity was determined by measuring the increase in absorbance at 420 or 475 nm (for chlorogenic acid and catechol or for DL-DOPA, respectively) with a spectrophotometer (UNICAM UV 500) as described by Lopez and others (1994). The dialyzed enzyme solution was diluted 1/10 (v/v) with 0.1 M sodium phosphate buffer, pH 6.8. The sample cuvette contained 900 μL of substrate (DL-DOPA, catechol or chlorogenic acid) buffer and 100 μL of enzyme solution. Enzyme activity was calculated from the linear portion of the curve. One unit of enzymatic activity is defined as an absorption increase of 0.1 absorbance units/min at 30 °C (units of enzymatic activity: units/g of fruit).

Polarographic enzyme assay

The determination was based on the method used by Golan-Goldhirsh and Whitaker (1984). O_2 consumption was measured using a Clark-type polarographic electrode from Strathkelvin Instruments Ltd. (1302 Microcathode Oxygen Electrode) connected to a computer by a 928 six-channel interface. The assays were performed at 30 °C and were started by addition of PPO. To calibrate the oxygen electrodes, a zero oxygen solution was used and at the high-end air-saturated water. Zero oxygen solution can be produced by adding a small amount of sodium sulphite to distilled water in a beaker and swirling to dissolve. To obtain air-saturated water, bubble air was bubbled through it for 15 to 20 min. This must be done at exactly the same temperature (30 °C) as the solution to be measured in the experiment, since the electrode is temperature sensitive. The sample glass tube contained 4500 μL of substrate (chlorogenic acid) dissolved in sodium phosphate buffer (pH 4.5), AA, and 4-HR at different concentrations as inhibitors, and 500 μL of enzyme solution. Enzyme activity was calculated from the linear portion of the curve (O^2 consumption compared with time).

Effect of pH

Appropriate buffers (0.1 M citrate pH 3 to 5 and 0.2 M phosphate pH 5 to 7) were used to determine the optimum pH of PPO activity. The effect of pH was tasted during the spectrophotometrical assay with catechol and chlorogenic acid and in the 4-HR inactivation study.

Enzyme kinetics

V_{max} and K_m were determined using nonlinear regression, by fitting the experimental data obtained to the Michaelis–Menten equation using the Enzfitter software program (Leatherbarrow 1987). The initial reaction rate was estimated by the assay described in the "spectrophotometrical enzyme assay" section. The measurements were performed in triplicate.

Inactivation effects of ascorbic acid and 4-hexylresorcinol

Incubation of PPO with AA and/or 4-HR in the absence of substrate was performed at 30 °C at different concentrations of AA or 4-HR, in a final volume of 1 mL of different pH (see effect of pH). Aliquots (100 μL) were taken at various times, and PPO activity was assayed immediately as described above. Activities at time zero were taken as 100%.

O_2 removal

All solutions were deaerated thoroughly under vacuum using a pump (EYELA Tokio Rikakikai Co., LDT ASPIRATOR A-3S) during 45 min at 25 °C, before mixing under continuous N_2 flux (Golan-Goldhirsh and Whitaker 1984). The polarographic electrode was used to check if the O_2 was completely removed.

Results and Discussion

Preliminary enzyme characterization

The activity of pear PPO was tested using 3 substrates: DL-DOPA, catechol, and chlorogenic acid. Very low activity levels were observed with DL-DOPA, whereas similar PPO-activity levels were found in both catechol and chlorogenic acid (5.4, 351, and 630 units/g fruit using 10 mM DL-DOPA, catechol and chlorogenic acid, respectively) (see Figure 1A and B). However,

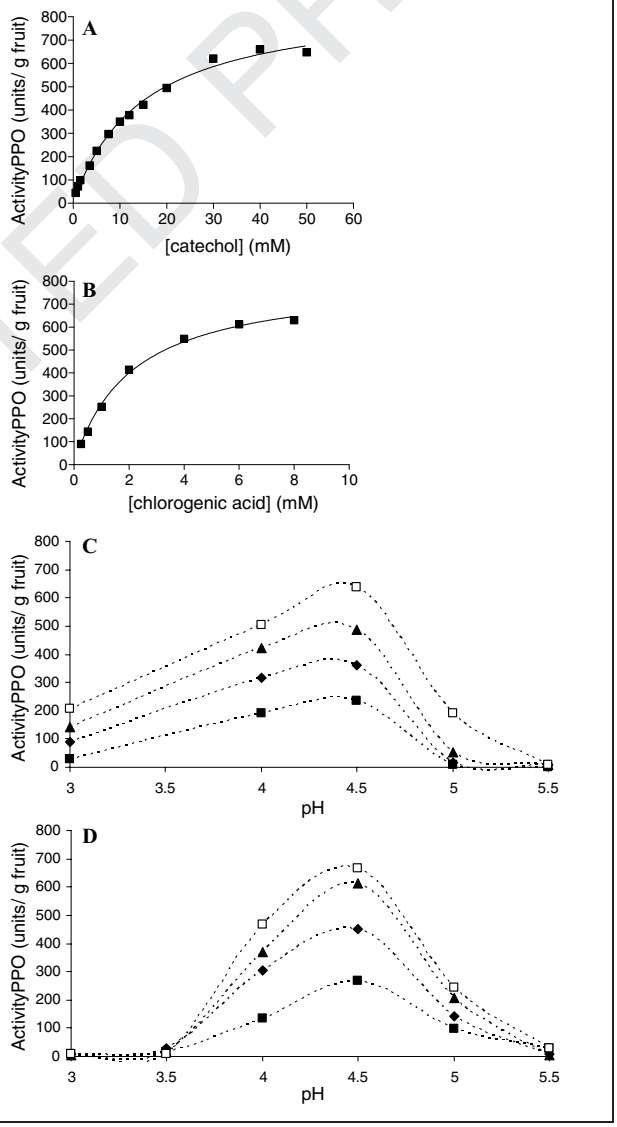

Figure 1—(A) Effect of catechol concentration on pear pear polyphenoloxidase (PPO) activity. (B) Effect of chlorogenic acid concentration on pear PPO activity. (C) Effect of pH on pear PPO activity at different catechol concentrations: (■) 5, (♦) 10, (▲) 20, and (□) 50 mM. (D) Effect of pH on pear PPO activity at different chlorogenic acid concentrations: (■) 0.5, (♦) 2, (▲) 4, and (□) 8 mM.

maximum activity was achieved at much lower chlorogenic acid than catechol concentrations. This, together with the fact that chlorogenic acid is the most abundant phenolic compound in pears (Spanos and Wrolstad 1992; Galvis-Sánchez and others 2003), led us to choose chlorogenic acid for our standard PPO assay. We then investigated the pH optimum of the enzyme and found that it has a maximum activity at pH 4.5, which matches approximately the pH value of pear juice (see Figure 1C and D). Such a low optimum pH is in sharp contrast with the widespread view that the optimum pH of plant PPOs is around 6 to 7 (McEvily and Iyengar 1992; Osuga and others 1994; Whitaker and Lee 1995; Artés and others 1998). However, our result is in agreement with previous works describing PPOs with different acid pH optimum (around 4 to 5) such as apple, eggplant, potato, pear, olive, and grape (Murata and others 1992; Sanchez Ferrer and others 1998; Fujita and Tono 1998; Pérez-Gilabert and García-Carmona 2000).

Effects of ascorbic acid on PPO activity with chlorogenic acid as a substrate

PPO activity in the presence of increasing concentrations of AA (see the slopes in Figure 2A) remains constant. However, the lag phase increases exponentially with the AA concentration (see inset Figure 2A). This is what could be expected if AA acts solely as an antioxidant compound. Antioxidants reduce oxidized substrate molecules back to their original state, therefore, impairing their polymerization to brown pigments (McEvily and Iyengar 1992; Laurila and others 1998). The extent of the lag phase correlates with the AA concentration: more time is needed to see browning at higher AA concentrations. This is due to the higher availability of AA to reduce the product of the enzymatic reaction. This acid does not stop the enzymatic reaction itself but it delays the subsequent polymerization events, which are purely chemical reactions. This can be demonstrated by measuring oxygen consumption in the reaction (see Figure 2B). No decrease in PPO activity can be observed, neither can any lag phase be detected. Accordingly, neither K_m nor V_{max} are affected by the presence of AA (see Figure 2C). We can therefore conclude that under the conditions shown in Figure 2, AA interacts directly with the product of the PPO catalyzed reaction but not directly with PPO. However, several authors report the inactivation of PPO by direct interaction with AA in PPOs from other sources (Golan-Goldhirst and Whitaker 1984; Golan-Goldhirsh and others 1992; Whitaker and Lee 1995). To find out if AA could interact directly with pear PPO, we incubated the enzyme with AA at 3 different pH (4.5, 6, and 7) before substrate addition. No decrease in activity could be observed at any pH (data not shown). We therefore we conclude that, under the conditions of the experiment, pear PPO does not interact directly with AA but with oxidized substrate molecules.

Effects of 4-hexylresorcinol on PPO activity

PPO assays in the presence of 4-HR show no lag phase but do reveal a reduction in activity (see slopes in Figure 3A). 4-HR appears to be a mixed enzyme inhibitor (see Figure 3C) that influences both the PPOs K_m (1.61 ± 0.21 mM compared with 5.07 ± 0.97 mM in the absence and presence of 0.2 mM 4-HR, respectively) and V_{max} (833.7 ± 32.6 units/g fruit compared with 632.2 ± 55.2 units/g fruit). 4-HR also accordingly inhibits also O_2 consumption as is shown in the polarographic assay (see Figure 3B).

Inactivation of PPO from other sources (apple, avocado, shrimp) by direct interaction with 4-HR has been described by other authors (Mc Evily and others 1991; Otwell and others 1992; Dawley and Flurkey 1993; Monsalve-Gonzalez and others 1993; Weemaes and others 1999; Iyidogan and Bayindirli 2004). Incubation of pear PPO with 4-HR in the absence of substrates also results in a time and pH-dependent inactivation effect (see Figure 4A). This effect is stronger at low pH and is also dependent on 4-HR concentration (see Figure 4B). It is not surprising to find pH-dependent inactivation effects given that pH-dependent inhibition effects have been already described (Ferrar and Walker 1996). To check if the inactivation effect is reversible, we dialyzed samples apparently inactivated by 4-HR. The aim of this experiment was to exclude the possibility that 4-HR could behave as a tight PPO inhibitor, that is, an inhibitor with very high affinity towards PPO (Morrison 1982; Valero and others 1992; Jimenez and García-Carmona 1997). If 4-HR acted as a tight inhibitor of pear PPO, a dialysis step would help to dissociate PPO from 4-HR and this would result in an increase in PPO activity. However, such an increase in activity was not detected after dialysis

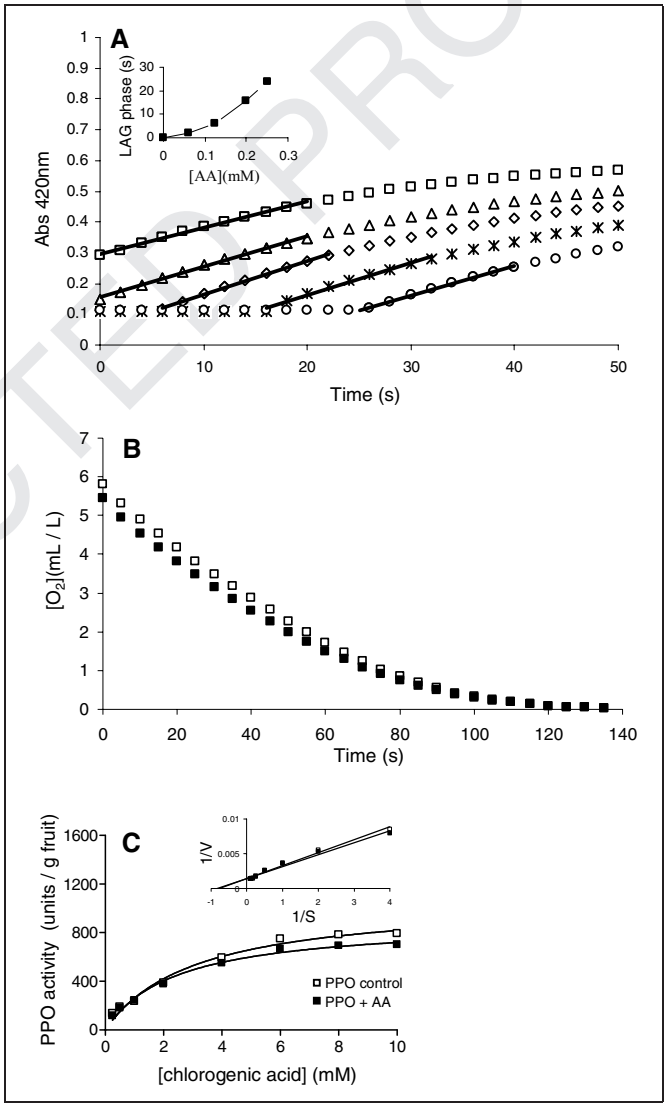

Figure 2—Ascorbic acid (AA) effects on pear pear polyphenoloxidase (PPO) activity. (A) PPO activity measured by monitoring absorbance at 420 nm at different AA concentrations: (□) 0, (△) 0.06, (◇) 0.125, (∗) 0.2, and (○) 0.25 mM. Inset: Effect of AA concentration ([AA]) on PPO lag phase. (B) PPO activity measured following O_2 consumption in the presence of 0.2 mM AA (■) or in the absence of AA (PPO control) (□). (C) The effect of 0.2 mM AA on PPO activity at different chlorogenic acid concentrations. Inset: Lineweaver–Burk plot showing the effect of AA on pear PPO using chlorogenic acid as substrate.

(see Figure 4C). Moreover, incubation of PPO with Cu^{2+} after 4-HR treatment, which other authors have used to restore PPO activity after inactivation or inhibition (Son and others 2000), did not result in any PPO activity recovery either (data not shown). Therefore, it seems that 4-HR acts as an irreversible inhibitor of pear PPO. The kinetics of the inactivation reaction is also typical of a bimolecular reaction. If the logarithm of the residual activity is plotted against time (see Figure 4D), a nonlineal kinetic of inactivation is observed because the reaction is dependent on the concentration of the reactants. It is faster at the beginning, when the concentration of the reactants is higher.

Irreversible inhibition of PPO by 4-HR, AA, oxalic acid, and hydrogen peroxide has already been described by other authors (Dawley and Flurkey 1993; Weemaes and others 1999; Son and others 2000; Kim and Uyama 2005). It has been hypothesized that the inhibition is mediated by a direct interaction of these molecules with the active center of the enzyme. Cu^{2+} atoms in the PPO active center change their oxidative states during the PPO catalytic cycle (a *deoxy* state

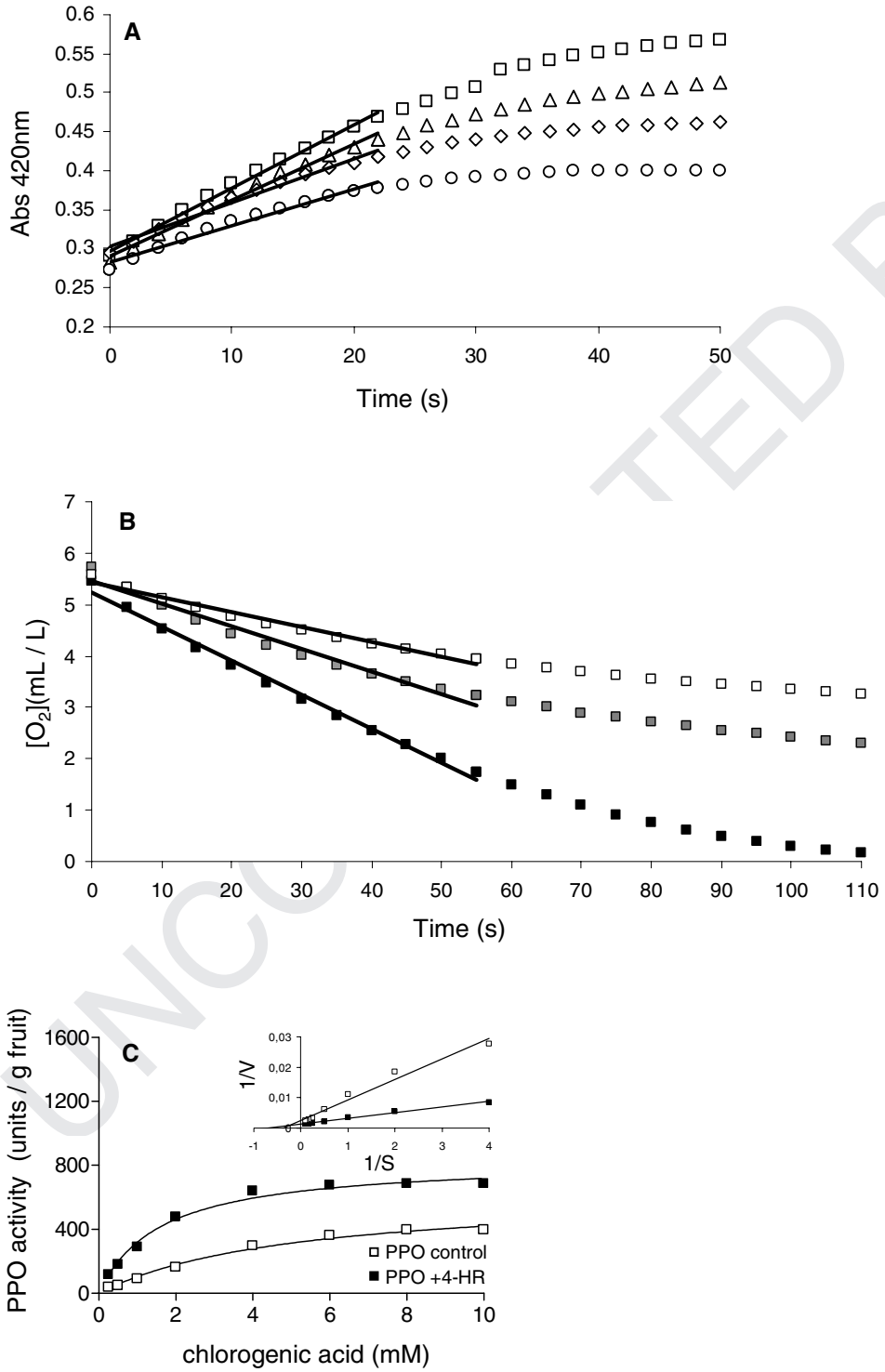

Figure 3—The 4-hexylresorcinol effects on pear pear polyphenoloxidase (PPO) activity. (A) PPO activity measured by monitoring absorbance at 420 nm in the presence of different 4-HR concentrations: (□) 0, (△) 0.05; (◇) 0.1, and (○) 0.2 mM. (B) PPO activity measured following O_2 consumption in the presence of 0.1 mM 4-HR (■),0.2 mM 4-HR (□) or in the absence of 4-HR (PPO control) (■). (C) The effect of 0.2 mM 4-HR on PPO activity at different chlorogenic acid concentrations. Inset: Lineweaver–Burk plot showing the inhibition of pear PPO by 4-HR, using chlorogenic acid as substrate.

with 2 cuprous ions, an *oxy* state with 2 cupric ions forming a complex with dioxygen, and a *met* state with 2 cupric ions binding an hydroxide ion). The sensibility of mushroom PPO to inactivation by AA is dependent on the oxidative state of the PPO (Osuga and others 1994). We have investigated if this possible dependence is also true for 4-HR acting on pear PPO by using high vacuum conditions to favor the *deoxy* form of the enzyme in spite of the *oxy* form. In Figure 5 we show that the *oxy* form is more resistant than the *deoxy* to inactivation by HR. The different behavior during inactivation by 4-HR of 2 forms of the enzyme that differ only in the oxidation state of the active center points precisely to a direct interaction of 4-HR with the active center.

In this paper, we present data supporting the idea that 4-HR can interact in 2 ways with PPO: there can be both inhibitory (Figure 3) and inactivatory interactions (Figure 4). Assuming this to be the case, it is necessary to show that 4-HR can bind to 2 different sites in the enzyme or, in our opinion more probable for structural reasons, to 2 different forms of the active site. If only PPO and 4-HR are present in the assay, 4-HR will probably react with the *deoxy* form of the enzyme inactivating it by a covalent modification of no known mechanism; we think that 4-HR reacts faster with the *deoxy* form of the enzyme because PPO inactivation of this *deoxy* form is faster (see Figure 5). If PPO, 4-HR, and phenolic substrates are present simultaneously, 4-HR and phenolic substrates would compete for PPO active site. Which one reacts with the enzyme under such conditions will depend on their respective reaction rates, which are not known, and on their concentrations. High phenolic substrate concentrations would impair a direct interaction of 4-HR with the *deoxy* form and would favor the beginning of a catalytic cycle. 4-HR could, however, inhibit the catalytic cycle after the release of the 1st quinone molecule by competing with the binding of the 2nd phenolic substrate. Again, reaction rates and concentrations of both 4-HR and phenolics would determine which one reacts with the enzyme. The reaction rates with the *met* form of the enzymes are probably very different from those with the *deoxy* and *oxy* forms at this stage, as occurs with the ability of copper chelators to interact with dicopper centers of PPOs (Rescigno and others 2002), which depend also on their oxidative state. In our model, the interaction of 4-HR with

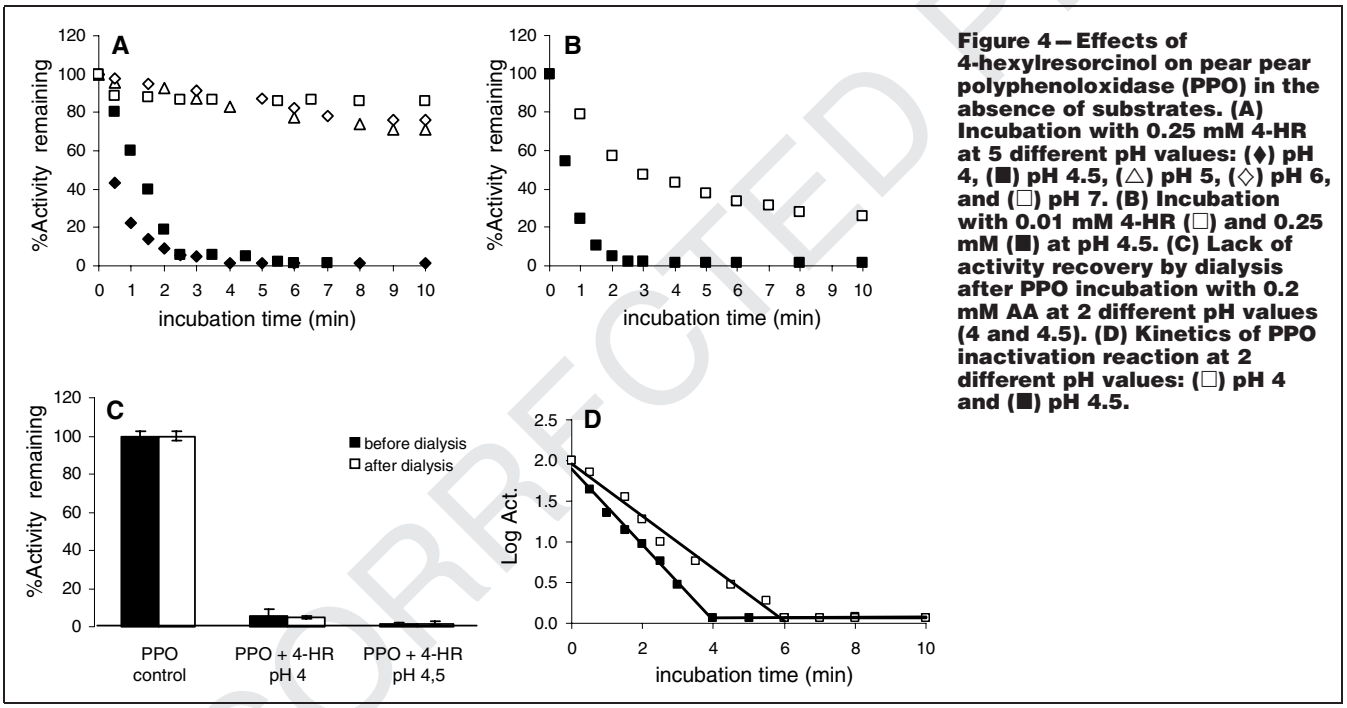

Figure 4—Effects of 4-hexylresorcinol on pear pear polyphenoloxidase (PPO) in the absence of substrates. (A) Incubation with 0.25 mM 4-HR at 5 different pH values: (♦) pH 4, (■) pH 4.5, (△) pH 5, (◇) pH 6, and (□) pH 7. (B) Incubation with 0.01 mM 4-HR (□) and 0.25 mM (■) at pH 4.5. (C) Lack of activity recovery by dialysis after PPO incubation with 0.2 mM AA at 2 different pH values (4 and 4.5). (D) Kinetics of PPO inactivation reaction at 2 different pH values: (□) pH 4 and (■) pH 4.5.

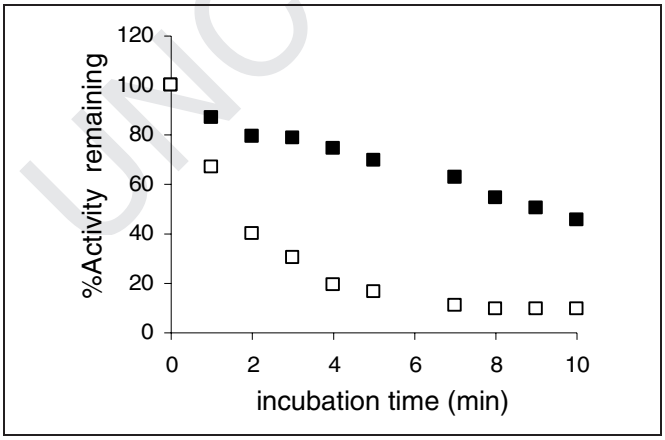

Figure 5—The effect of O_2 concentration on pear polyphenoloxidase inactivation by 4-HR: (■) saturating O_2 concentration; (□) reduced O_2 concentration.

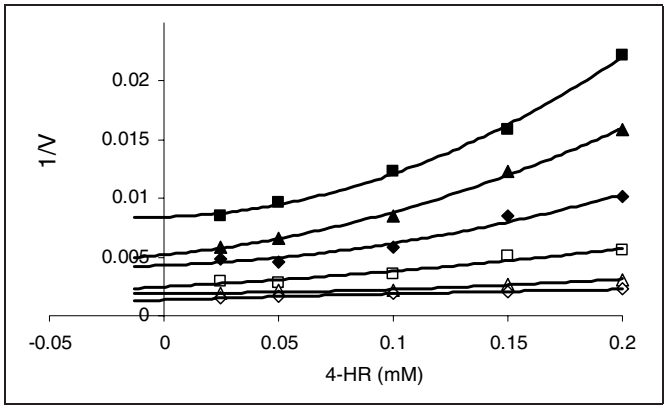

Figure 6—Dixon plot of pear pear polyphenoloxidase inhibition by 4-HR at different chlorogenic acid concentrations: (■) 0.25, (▲) 0.5,(♦) 1, (□) 2, (△) 4, and (◇) 8 mM.

Table 1 — Correlation coefficients to fit to a 1st- and 2nd-order polynomial equation

	r^2	
AC (mM)	1st-order polynomial	2nd-order polynomial
0.25	0.94	0.99
0.5	0.95	0.99
1	0.95	0.97
2	0.96	0.95
4	0.96	0.95
8	0.97	0.95

the *met* form does not result in any covalent modification with an inactivatory effect, but in a classical enzyme inhibitory effect. A closer analysis of Figure 3C shows that in the presence of 4-HR the shape of the curve is slightly hyperbolic, typical of cooperative kinetics, instead of a Michaelis–Menten one ($n = 1$, no cooperativity in the Hill equation, in the absence of 4-HR and $n = 1.37$ in the presence of 4-HR). However, this does not mean that PPO follows a cooperative kinetics. It is quite probable that at low substrate concentrations, as 0.2 mM, 4-HR competes successfully with phenolics for binding the *deoxy* form of the enzyme inactivating it. At higher phenolic substrate concentrations most of the PPO initiates the oxidation of phenolics instead of reacting directly with 4-HR. However, the latter could interfere with the binding of the 2nd phenolic substrate.

Inhibition studies using Dixon plots obtained at several fixed substrate concentrations agree with this model (Figure 6). The plots are not linear but show an upward curvature derived from less activity than expected at higher 4-HR concentrations. The quantification of this curvature can be measured comparing their fit to a 1st- and 2nd-order polynomial equation and show that the curvature is stronger (higher correlation to a 2nd-order polynomial equation, (see Table 1) at low substrate concentrations, where 4-HR can compete more successfully with substrates to bind and inactivate to the *deoxy* form of the enzyme. Preferential, or even exclusive, binding of inhibitors to 1 of the 3 oxidative states of PPO have already been described: using an enzyme kinetics approach benzoic acid has been shown to bind the *oxy* form of mushroom tyrosinase (Resigno and others 2002) and, even more convincingly, phenylthiourea, an inhibitor of sweet potato PPO binds only, as shown by crystallographic studies, to the *met* form of PPO after oxidation of a 1st phenolic substrate molecule (Klabunde and others 1998), a behavior consistent with the model we propose here for 4-HR. Q4

Combined effect of ascorbic acid and 4-hexylresorcinol

The simultaneous addition of 4-HR and AA has a synergistic effect on both PPO activity (Figure 7A) and PPO stability (Figure 7B). PPO shows a longer lag phase and less activity if both 4-HR and AA are present in the assay tube. This effect is synergistic (see inset

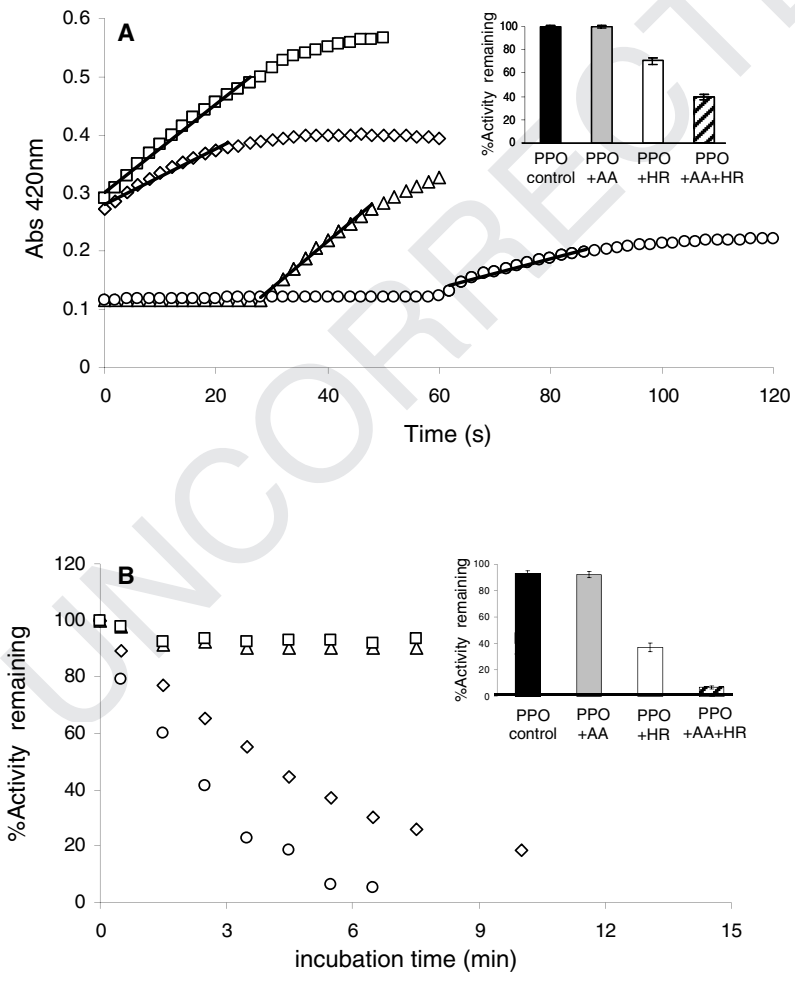

Figure 7 — Effect of simultaneous addition of 4-HR and AA on pear pear polyphenoloxidase (PPO) activity and stability. (A) PPO activity measured by monitoring absorbance at 420 nm in the presence of 0.2 mM 4-HR (◇), 0.25 mM AA (△), 0.2 mM 4-HR, and 0.25 mM AA (○) and PPO control (□) (Straight lines represent initial activities). Inset: Initial activities. (B) PPO activity after enzyme incubation with 0.2 mM 4-HR (◇), 0.25 mM AA (△), 0.2 mM 4-HR, and 0.25 mM AA (○) and PPO control (□). Inset: Activity recovery of pear PPO after 5 min incubation with AA, 4-HR, AA + HR and PPO control.

in Figure 7A) because it is higher than the simple additive effect of AA and 4-HR, especially because AA has no significant effects on PPO maximum activity. The synergistic effect of 4-HR and AA preventing PPO activity has already been described by other authors (Monsalve-Gonzalez and others 1995; Luo and Barbosa-Cánovas 1996; Buta and Abbott 2000; Dong and others 2000). PPO stability in the presence of both 4-HR and AA shows the same trend: the inactivation effect is synergistically higher when both molecules are incubated together with PPO than when acting alone (see inset in Figure 7B), again because the AA effect on PPO stability is negligible. Monsalve-Gonzalez and others (1995) suggested that the synergistic effect is due to the reducing effect of AA on 4-HR. Other authors support a similar hypothesis to explain the synergistic effect of AA and cinnamic acid acting on PPO (Sapers and others 1989). These results are consistent with antibrowning effects of the combination of 4-HR and AA on fresh-cut pears (Sapers and Miller 1998; Buta and Abbott 2000; Dong and others 2000; Abbott and Buta 2002), which are much higher than the addition of their individual antibrowning effects.

Comparison of 4-HR and AA effects on pear and mushroom PPO

Whatever the mechanism or mechanisms by which AA and 4-HR inhibit or inactivate PPO, the behavior of these antibrowning agents acting on pear PPO is completely different to their behavior acting on mushroom PPO (unpublished results). Mushroom PPO is irreversibly inactivated if incubated with AA whereas 4-HR behaves only as a canonical enzyme inhibitor. This results also in synergistic inhibitory effects of AA and 4-HR acting on mushroom PPO. It is remarkable that the inactivation of mushroom PPO by AA is also dependent on the oxidative states of the active center, the most sensitive form being the *oxy* state, contrary to what happens with pear PPO and 4-HR. Clearly, structural reasons would account for these differences. The primary structure of pear PPO is not known, but it is known from several other plant and fungal sources (Marusek and others 2006). A comparative analysis of PPO from both sources shows that they differ in the access of substrates, or competitive inhibitors, to the catalytic site (Marusek and others 2006). The catalytic site is buried in an hydrophobic cavity the access to which is protected by 1 phenylalanine-free residue in most vegetable PPOs, but not in fungal isozymes. This could explain the interaction with inactivatory effects of AA, a charged molecule with no hydrophobic part, with the catalytic site in fungal isozymes only.

However, it is complicated to correlate structural features with the effects of AA and 4-HR on mushroom and pear PPO because subtle differences in the 3-dimensional structure of PPO can account for large functional differences. An example of this is what happens with PPO and arthropod hemocyanin: they are homologous proteins both having dicopper centers and very similar tertiary structure (Gerdemann and others 2002) but whereas PPO uses O_2 to oxidize phenolic substrates, hemocyanin is an O_2 transporter with residual oxidase activity; however, the oxidase activity of hemocyanin can be greatly enhanced by a single aminoacid substitution very far away from the active site (Jackman and others 1998), or by SDS addition (Jaenicke and Decker 2004), which causes small conformational changes.

The results presented here highlight the complexity of the interaction of PPO with substrates and inhibitors. Due to the economic and scientific relevance of this topic, more detailed studies of the effects of 4-HR and AA on PPO are needed. These should include structural studies, especially of the inactivating effects of both AA and 4-HR, and also extending the experiments described here to other PPOs of fungal and vegetable origins.

Acknowledgments

This study was supported by CICyT (Project AGL 2001-1766) and by MEC, which provided a fellowship to E. Arias to carry out this investigation.

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Q11

Queries

Q1 Author: The year for reference Cantwell and Trevor has been changed from 2002 to 1992, as given in the list. Please check.

Q2 Author: Please provide the location of manufacturer 'Strathkelvin Instruments Ltd.'.

Q3 Author: Please provide the location of manufacturer 'EYELA Tokio Rikakikai Co.'.

Q4 Author: Reference Resigno and others (2002) is not listed. Please check.

Q5 Author: Reference Monsalve-Gonzalez and others (1995) is not listed. Please check.

Q6 Author: Reference Jaenicke and Decker (2004) is not listed. Please check.

Q7 Author: The year for reference Jackman and others has been changed from 1992 to 1998, as given in the list. Please check.

Q8 Author: Author name Esther Arias Álvarez has been changed to E. Arias (as given in the 'author field' below the article title).

Q9 Author: Please check the initials for author Lee and Watkins in reference Calderón-López et al. (2005).

Q10 Author: Please provide the last accessed date in reference IFPA (2000).

Q11 Author: Please provide the last accessed date in reference Zagory (1998).