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Browning Prevention by Ascorbic Acid and 4-Hexylresorcinol: Different Mechanisms of Action on Polyphenol Oxidase in the Presence and in the Absence of Substrates

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ABSTRACT: We have investigated the mechanism of action of 4-hexylresorcinol (4-HR) and ascorbic acid (AA) on the polyphenol oxidase (PPO) catalyzed oxidation of phenolic substrates. Incubation of PPO with 4-HR diminishes strongly PPO activity. This effect can be erroneously interpreted, due to the high affinity of 4-HR for PPO, as irreversible inactivation of PPO. However, PPO activity can be recovered by dialysis after incubation with 4-HR. 4-hexylresorcinol is a canonical enzyme inhibitor that binds preferentially to the *oxy* form of PPO. It is a mixed-type inhibitor, because it influences both apparent V_{max} (1.26 compared with 0.4 units in the absence and presence of 4-HR, respectively) and K_m values (0.28 mM compared with 0.97 mM in the absence and in the presence of 4-HR, respectively) of PPO. AA can prevent browning by 2 different mechanisms: in the absence of PPO substrates it inactivates PPO irreversibly, probably through binding to its active site, preferentially in its *oxy* form. In the presence of PPO substrates, AA reduces PPO oxidized reaction products, which results in a lag phase when measuring PPO activity by monitoring dark product formation but not when monitoring O_2 consumption. The simultaneous use of both 4-HR and AA on PPO results in additive prevention of browning.

Keywords: ascorbic acid, browning, inhibition, polyphenol oxidase, 4-hexylresorcinol

Introduction

Fruit browning is one of the most serious alteration processes in the fruit and vegetable industry. It is estimated that over 50% of fruit losses occur as a result of enzymatic browning (Whitaker and Lee 1995). The control of browning from harvest to consumer is therefore critical for minimizing losses and maintaining economic value for agriculture and the food processing industry alike. The most important browning reactions in fruits and vegetables, especially in fresh ones, are caused by enzymatically catalyzed reactions. These are catalyzed mainly by peroxidases and polyphenol oxidases (PPOs). The latter are generally considered as being mainly responsible for enzymatic darkening of foods of vegetable origin.

PPOs (E.C. 1.10.3.1) can catalyze 2 types of reactions: the hydroxylation of monophenols to give *o*-diphenols and the oxidation of *o*-diphenols to give *o*-quinones. Not all the PPOs catalyze the 1st reaction but all catalyze the 2nd. The product of the 2nd reaction, a benzoquinone, is very unstable and polymerizes promptly to give a colored compound (Taylor and Clydesdale 1987; Mc Evily and others 1992). The absorbance of this compound in the visible region of the spectrum is often used to measure PPO activity.

PPO activity is favored by mechanical wounding of the vegetable surface (Gorny and others 1998, 2000). Wounding promotes increased phenolics synthesis and allows direct contact between PPO and its 2 substrates: phenolics and molecular oxygen. This process is consubstantial to each vegetable processing operation that includes cutting or peeling. Indeed, PPO catalyzed browning is the main limiting factor of the shelf life of fresh cut produce.

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Several strategies are being used to minimize or reduce PPO catalyzed browning in fresh cut produce. They range from appropriate cultivar selection and ripeness degree based on low browning potential (a combination of low phenolics content and low PPO activity) (Buta and Abbott 2000; Gorny and others 2000; Soliva-Fortuny and others 2004) to the use of nonthermal technologies (Weemaes and others 1999; Gorny and others 2002; Fan and others 2005; Hajare and others 2006) or the addition of enzyme browning reaction inhibitors (Sapers and Miller 1998; González-Aguilar and others 2004; Pilizota and Sapers 2004). Cold processing as well as washing away phenolics are alternatives for reducing PPO catalyzed browning reactions. All these methods can be used alone but they are normally used in combination so as to reduce their potential negative impact on vegetable quality. This is a strategy similar to the "hurdle technology" devised by Leistner 1992) to stop microbial growth in food (Luo and Barbosa-Cánovas 1995).

The most important hurdles currently used to minimize browning are the use of low temperatures throughout the production chain and the addition of PPO inhibitors and/or antioxidants (Sapers and Miller 1998; Buta and Abbott 2000; Gorny and others 2002). The most frequently used antioxidants are ascorbic acid (AA) and sulfites. The latter have been banned in the United States in fresh fruit and vegetables and are going to be prohibited also in the European Union in the near future. There is therefore a growing interest in alternative compounds to prevent browning. Many inhibitors of PPO are known, but only a few have been considered as potential alternatives to sulfites. Several reports have appeared in recent years about the use of 4-hexylresorcinol (4-HR; 4-hexyl-1,3-benzenediol) to prevent enzymatic browning in vegetables, mainly in fruits (McEvily and others 1991, 1992; Monsalve-González and others 1993; Luo and Barbosa-Cánovas 1995; Whitaker and Lee 1995; Guerrero-Beltrán and others 2005). However, there is no clear picture Q1

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of the mechanism, or mechanisms, by which this compound retards browning. This is probably because of the kinetics of PPO, which can use many different substrates and for which complex phenomena such as suicide inactivation, slow inhibition, or slow tight inhibition have been described (Escribano and others 1989; Valero and others 1991, 1992; Jimenez and García-Carmona 1997; Pérez-Gilabert and García-Carmona 2001). We therefore explore in this work the mechanisms of action of 2 of the most antibrowning agents: 4-HR and AA.

Material and Methods

Materials

The 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. Mushroom tyrosinase (Sigma, T-3824), DL-DOPA, AA, citric acid, $CaCl_2$ (anhydrous) and 4-hexyresorcinol were purchased from Sigma-Aldrich Co. (Spain). All other chemicals were of analytical grade.

Methods

Preparation of pear slices and their antibrowning treatments. The pears were initially washed with chlorinated water (100 ppm of active chlorine for 5 min) to prevent surface contamination. Subsequently, the pears were rinsed and drained. A semiautomatic peeler Pelamatic (mod. Orange Peel) was used for peeling, and core removing and slicing was performed manually. Special knifes for fruit (Granston, mod. Messermeister, England) were used for slicing (10 to 12 slices/pear). The pear samples were immersed in antibrowning aqueous solutions (treatment 1: 2% AA + 1% citric acid + 1% $CaCl_2$[anhydrous], treatment 2: 0.01% 4-HR + 1% citric acid + 1% $CaCl_2$[anhydrous] and treatment 3: 2% AA + 0.01% 4-HR + 1% citric acid + 1% $CaCl_2$[anhydrous]) for 15 min at 4 °C. Control slices were simply dipped in water. Both control slices and treated ones were drained for 15 min at 4 °C. Pear slices were stored in the dark at 4 °C, during 12 d.

Spectrophotometrical enzyme assay. PPO activity was spectrophotometrically (UNICAM UV 500) determined as described by Lopez and others (1994), using DL-DOPA as substrate. Briefly, the assays were performed at 30 °C and were started by addition of PPO. The enzyme was dissolved in 50 mM sodium phosphate buffer, pH 7, at a concentration of 10 μg/mL. The sample cuvette contained 990 μL of substrate dissolved in the same buffer and 10 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/min at 475 nm.

Polarographic enzyme assay. O_2 consumption was measured using a Clark-type polarographic electrode from Strathkelvin Instruments Limited (1302 Microcathode Oxygen Electrode) connected to a computer by a 928 6-channel interface and was carried out following the method described by Golan-Goldhirsh and Whitaker (1984). To calibrate the oxygen electrodes we used a zero oxygen solution and at the high-end air-saturated water. Zero oxygen solution can be produced by adding a small amount of sodium sulfite to distilled water in a beaker and swirling to dissolve. To obtain air-saturated water, air was bubbled through the liquid 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 assays were performed at 30 °C and were started by addition of PPO. The sample glass tube contained 4950 μL of substrate dissolved in 50 mM sodium phosphate buffer, pH 7, and 50 μL of enzyme solution (the enzyme was dissolved in 50 mM sodium phosphate buffer, pH 7, at a concentration of 10 μg/mL). Enzyme activity was calculated from the linear portion of the curve (O_2 consumption compared with time).

Incubation of PPO. Incubation of PPO with AA and/or 4-HR in the absence of substrate was performed at 30 °C at different concentrations of inhibitor, in a final volume of 1 mL, using 50 mM sodium phosphate, pH 7, as buffer solution. Aliquots (10 μL) were taken at various times, and PPO activity was assayed immediately as described above. Activity at zero time was taken as 100%.

Kinetic parameters determination. V_{max} and K_m corresponding to DL-DOPA were determined using nonlinear regression, by fitting the experimental data obtained to the Michaelis–Menten equation using the Enzfitter software program (Leatherbarrow, 1987).

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.

Image analysis. The measurement of surface browning by image analysis was carried out using the method described by Mendoza (2004), with slight modifications. The vision system consisted of the following elements. (1) Lighting system: For image acquisition, the pear slices were illuminated using 2 halogen lamps (Philips) with a color temperature of 3000 K. The 2 lamps were arranged at 40 cm above the sample and at an angle of 45° with the sample. (2) Digital camera: A 6.3 Mp CMOS sensor color digital camera, model Canon EOS 10d, equipped with 50-mm lens, was placed in front of the sample at a distance of 40 cm. The angle between the camera lens and the lighting source axis was approximately 45°. Images from 1 side of the pear slices were taken on a matte black background using the following camera settings: manual mode with the lens aperture at $f = 2.8$ and speed 1/2000, no zoom, no flash, autofocus, maximum resolution, and storage in JPEG format. (3) Image processing: The digital images were preprocessed using a median filter. This low pass filter allowed to presmoothing of noisy images, improving their quality. The preprocessing of full images, color analysis, browning quantification, and image texture analysis were determined using the program ADImag, which was developed in the Image Processing Centre of the University of Zaragoza (Spain). All pear images were processed using the same procedure and measurements were done in 20 replications.

Results

The superficial browning, during the storage period, was determined by image analysis. The increase in the percentage of the brown area (from initial time to the last day of storage) is greater in the case of the control samples (rinsed in tap water) than the treated samples (1.8% compared with 1.6% [treatment 1], 1.5% [treatment 2], and 1.0% [treatment 3]). The greatest degree of browning inhibition occurred in the samples treated with both AA and 4-HR. The samples subjected to treatments 1 or 2 (where AA and 4-HR were separately applied) suffered more superficial browning than treatment 3 (1.6% and 1.5% compared with 1% ($P < 0.05$), applying treatment 1 and 2 or treatment 3, respectively) over the 12 storage days. Similar results have been reported by other authors for several fruits (Monsalve-Gonzalez and others 1993; Luo and Barbosa-Canovas 1997; Sapers and Miller 1998; Dong and others 2000). We decided to investigate the mechanisms involved in each case in order to gain an insight into the process and to discover how it could best be influenced. As a model system we used commercial fungal tyrosinase preparations.

We first tested in vitro the apparent inhibition of mushroom tyrosinase by AA (Figure 1A). The main effect of increasing the AA concentration on PPO activity was to increase the lag time of the browning reaction (Figure 1B), but the maximum activity, measured exactly when the absorbance began to increase, did not change (see the parallel lines in Figure 1C). The most plausible explanation for this behavior is to attribute the lag phase to the antioxidant activity of AA; once AA is consumed, the activity of the enzyme is the same independently of how much AA was present at the beginning of the test. Indeed, if enzyme activity is measured by controlling O_2 consumption, there is no difference in PPO activity when AA is present than in the control experiments (Figure 1D). We consider this as a direct proof that, under these experimental conditions, AA does not act directly on PPO but on the product of the PPO catalyzed oxidation reaction.

The inhibition of PPO browning reactions with increasing 4-HR concentrations showed a different behavior: there is no lag phase but the maximum activity diminishes dramatically when the 4-HR concentration rises (Figure 2A). Inset in Figure 2 could suggest the presence of at least 2 PPO isoenzyms with different sensitivity to 4-HR. Several isoenzymes of the same fungal PPO source have been already described in the literature (Flurkey 1991; Kumar and Flurkey 1991). It could also reveal the presence of 2 forms of the same isoenzyme with different affinity for 4-HR. Exactly this is what we have found in experiments described in Figure 4, and it has been described for the inhibition of tyrosinase by tropolone, which binds preferentially (or exclusively) to the *oxy* form of mushroom PPO (Espin and Wichers 1999). If O_2 consumption is followed (Figure 2B), no decrease in O_2 concentration can be observed, contrary to what is seen when AA is present in the assay tube. This means that 4-HR acts as a canonical enzyme inhibitor, interacting directly with the enzyme molecule.

Due to the structural similarity between 4-HR and PPO substrates and given the very well-described PPO suicide inactivation that occurs with some PPO substrates (Escribano and others 1989), we wondered whether 4-HR could inhibit browning reactions by modifying PPO irreversibly. Figure 3A shows the effect of incubating PPO with different amounts of 4-HR. Apparent inactivation ranges from approximately 50% at 0.1 mM 4-HR to almost 95% at 20 mM. At intermediate concentrations (2.5 mM) about 70% of the activity apparently disappears. Results like these have been interpreted (Weemaes and others 1999) as a covalent inactivating effect of 4-HR. However, if PPO is dialyzed after incubation with 4-HR, PPO activity recovers partially (see Figure 3B), and this is fully inconsistent with 4-HR being able to inactivate PPO covalently. This result also disagrees with that of Dawley and Flurkey (1993), who did not see any recovery of PPO after dialysis of PPO-4-HR complexes.

The apparent inactivation of PPO by 4-HR can be explained by the high affinity of 4-HR for PPO that makes the 4-HR-PPO complex stable even after a dilution step such as that performed in the experiment shown in Figure 3B. Moreover, 4-HR has been classified as a *slow inhibitor* (Jimenez and García-Carmona 1997), which binds preferentially intermediate forms of the enzyme in the catalytic cycle. The PPO catalytic cycle is composed of several steps in which the oxidation state of the active center changes between *oxy*, *deoxy*, and *met* forms (Whitaker 1995). 4-hexylresorcinol binds preferentially to the *oxy* form that is an intermediate state of the catalytic cycle, and this is the reason why it would take some time, after substrate addition, until the entire enzyme is in the *oxy* form. However, we do not see a biphasic time course for the PPO catalyzed reaction, probably because our PPO is already in the *oxy* form. According to this model, if the entire enzyme was in the *deoxy* form, the apparent inactivation (or rather, the reversible inhibition) described in Figure 3A should be of less intensity. In fact, if the enzyme is deprived of oxygen before it comes into contact with 4-HR, the apparent inactivation (or reversible inhibition) by the latter becomes less intense as is shown in Figure 4 for 2 different 4-HR concentrations. Our results, especially those shown in Figure 3A, B, are fully in agreement with the model

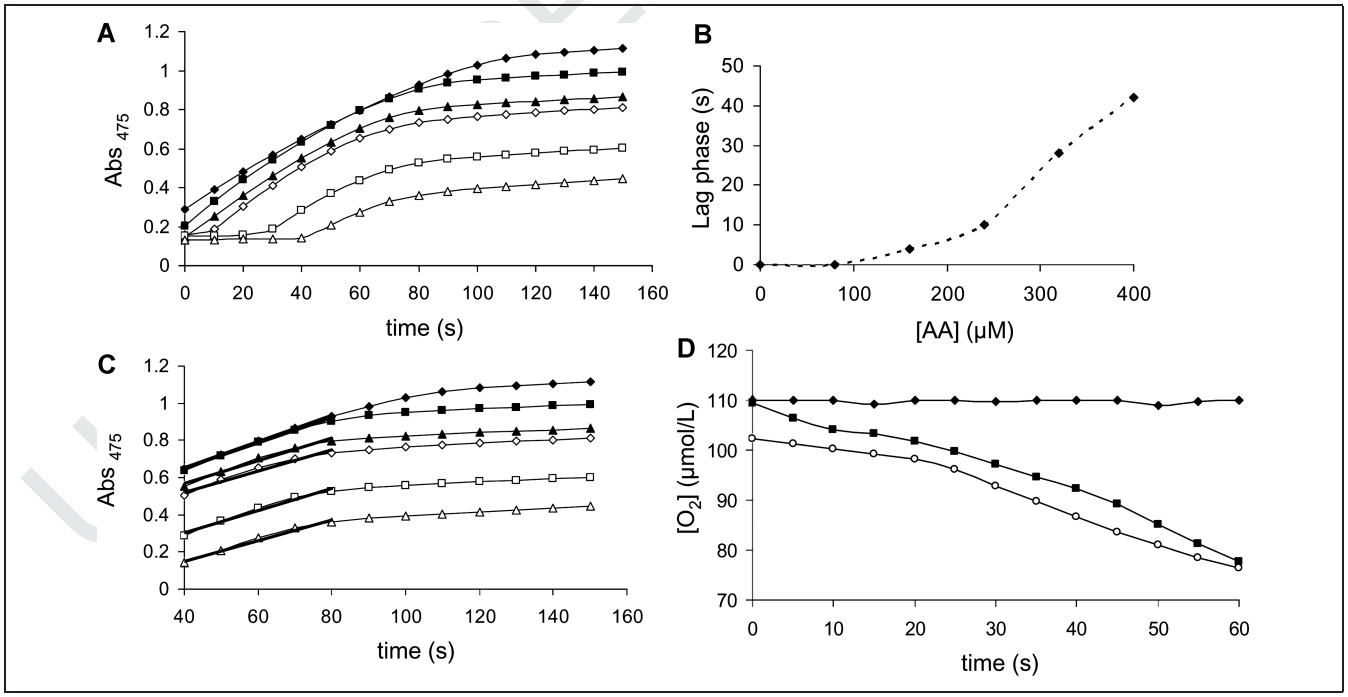

Figure 1 — Ascorbic acid (AA) effects on PPO activity. (A) PPO activity measured by monitoring absorbance at 475 nm in the presence of different AA concentrations:[•] 0, [■] 80, [▲] 160, [○] 240, [□] 320, and [△] 400 μM. (B) Effect of AA concentration ([AA]) on PPO lag phase. (C) PPO activity in the presence of AA measured after lag phase. (D) PPO activity measured following O_2 consumption, in the presence of AA.[○] Control without enzyme [♦], control without AA and 4-HR [■].

proposed by Jimenez and Garcia Carmona (1997). In fact, preferentially binding to an intermediate form of the enzyme catalytic cycle has been proposed as a proof for the existence of a slow inhibition phenomenon (Morrison 1982).

We then incubated PPO with different amounts of AA prior to allowing the enzyme to react with their substrates. Figure 5A shows that there is a decrease in activity which dependents both on incubation time and AA concentration. This points also to an apparent inactivation effect of AA on PPO. As in the case of 4-HR, PPO apparent inactivation by AA is dependent on the oxidation state of the active site of PPO, the *oxy* form being considerably more sensitive to AA than the *deoxy*: 40% compared with 65% residual activity, respectively, after incubation with AA (see Figure 5B). However, the apparent inactivation of PPO by AA is in fact different from the apparent inactivation of PPO by 4-HR because it is not reversed by dialysis (see Figure 5C, where analogue experiments with 4-HR are also presented for comparative purposes). These results point to AA as being an irreversible inhibitor of PPO, which acts by direct binding to the active site of the enzyme (Golan and Whitaker 1984)

Finally, if both AA and 4-HR are incubated simultaneously with PPO, a combined effect is observed (see Figure 6): the apparent inactivation effects before dialysis are stronger, but less than additive, whereas reactivation after dialysis is negligible.

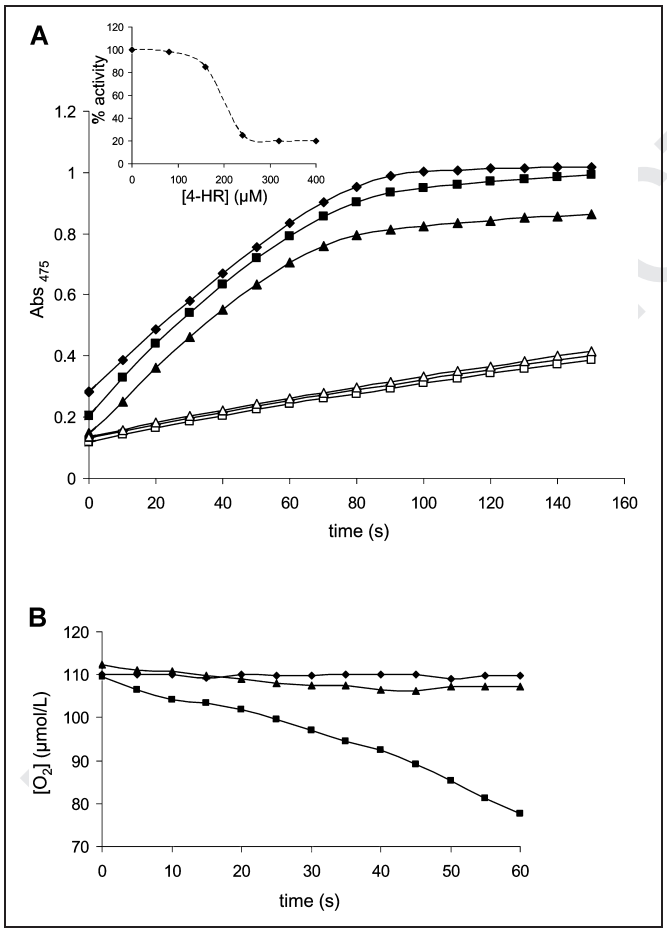

Figure 2—4-hexylresorcinol effect on PPO activity. (A) PPO activity measured by monitoring absorbance at 475 nm in the presence of different 4-HR concentrations: [•] 0, [■] 80, [▲] 160, [○] 240, [□] 320, and [△] 400μM. Inset: Degree of browning inhibition at different 4-HR concentrations. (B) PPO activity measured following O_2 consumption, in the presence of 4-HR. [▲] Control without enzyme, [♦] control without AA, [■] and 4-HR.

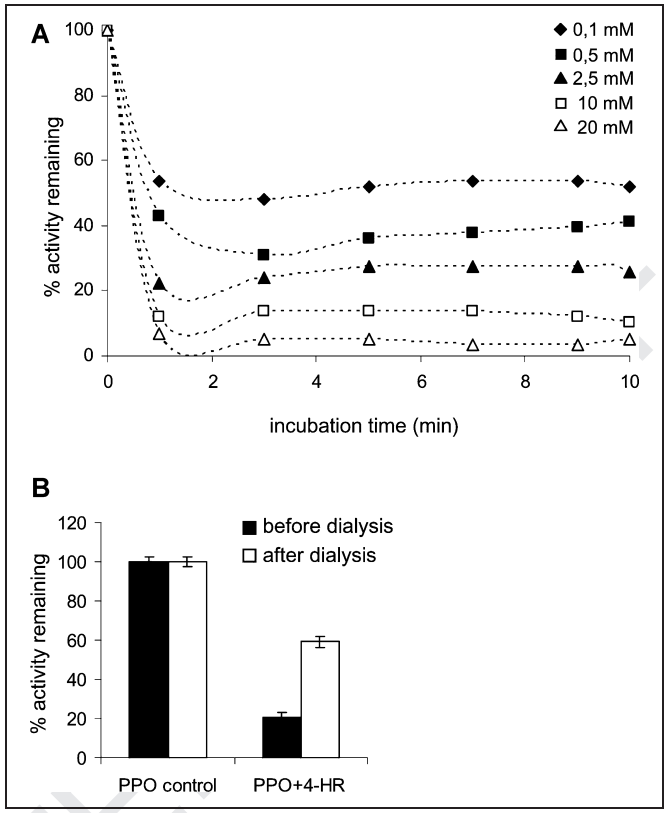

Figure 3—(A) PPO activity after its incubation with different 4-HR concentrations. (B) Activity recovery by dialysis after PPO incubation with 5 mM 4-HR.

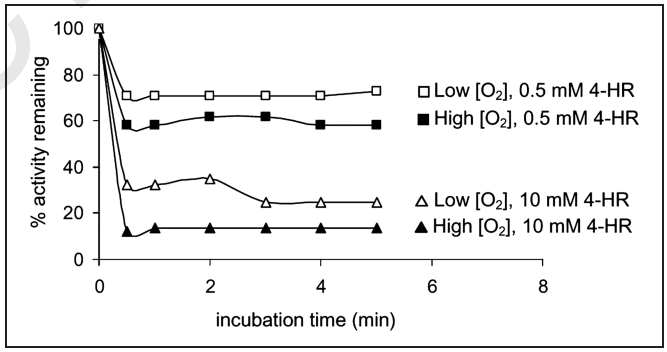

Figure 4—The effect of reducing O_2 concentration on the apparent PPO inactivation by 2 different 4-HR concentrations. Filled symbols, saturating O_2 concentration; open symbols, reduced O_2 concentration; squares, 0.5 mM 4-HR; triangles, 10 mM 4-HR.

Discussion

Our results show that the effects of AA and 4-HR on PPO activity are very dependent on environmental conditions. This is especially true in the case of AA, which can behave as an indirect inhibitor of PPO activity, reversing the oxidation of PPO reaction products, or irreversibly inactivating the enzyme. The latter effect is probably mediated by direct interaction of AA with the active site of the enzyme, as demonstrated by the differential sensitivity of 2 forms of the enzyme whose main difference lies in the oxidation state of the active site (see Figure 5B). Direct reaction of AA with the active site of the enzyme will be reduced first by the presence of competing molecules that act as substrates and, therefore, sterically hinder direct interaction of AA with the active site, and later by the very same oxidized substrate molecules with which AA would react

as an antioxidant in direct competition with the active site. Which reaction will be favored, AA with the active site or AA with the oxidized substrate molecules, will depend on the constant rate of the respective reactions and the relative abundance of the reactants involved in each of them. It is very difficult to predict the outcome of such a complex situation from a theoretical point of view, as these reaction constants are not known and the relative concentration of each reactant is constantly changing due to the many possible reactions involved. In any case, it is highly probable that as a consequence of the above-mentioned reactions, in all the assays at saturating substrate concentrations (of both O_2 and phenolics), AA will react preferentially with oxidized PPO reaction products reversing its oxidized state and being consumed in the process. This results in AA consumption, in the appearance of a lag phase, as shown in Figure 1B, and in no inhibition effects of the enzyme, as shown in Figure 1C. But if no substrate is present, AA will react directly with the active site, inactivating the enzyme, as has been previously reported by Golan-Goldhirsh and others (1992). These authors report a direct relation between enzyme inactivation by AA and the disappearance of hystidine residues to which Cu ions are bound in the active site of PPO. This reaction has been shown to require O_2 (Whitaker 1995). We do not see an absolute requirement for O_2 to observe PPO inactivation, but rather a higher percentage of inactivated enzyme in the presence of O_2.

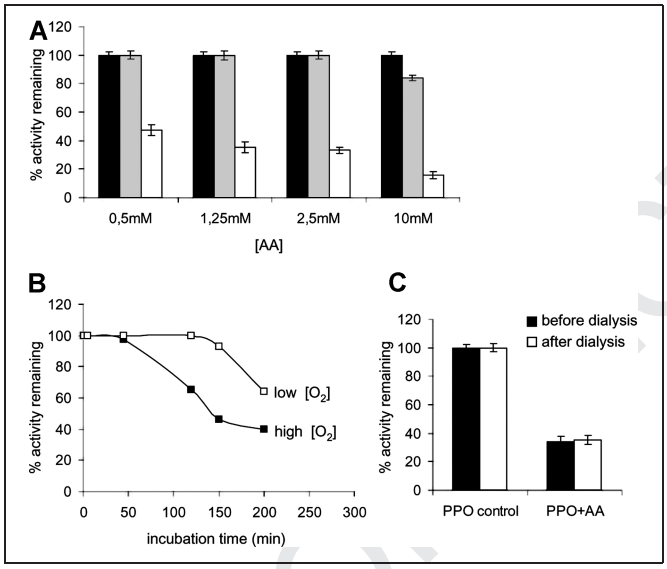

Figure 5—PPO activity after incubation with AA. (A) The effect of different AA concentrations at 3 different time points: [■] initial time; [□] after 10 min incubation; after 2 h incubation. (B) The effect of lowering O_2 concentration on PPO apparent inactivation by AA: [■] saturating O_2 concentration, [□] reduced O_2 concentration. (C) Lack of activity recovery by dialysis after PPO incubation with 5 mM AA.

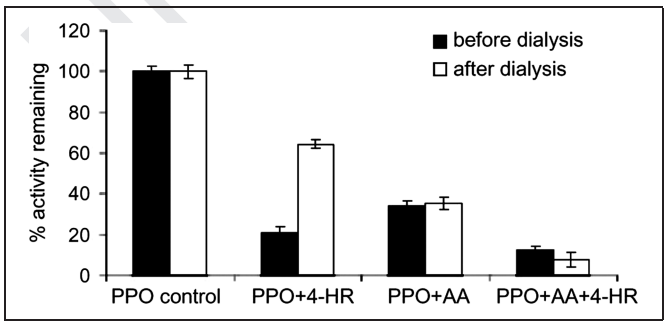

Figure 6—The effect of simultaneous incubation of PPO with 4-HR and AA.

The 4-HR effects on PPO are also dependent on environmental conditions, basically on the presence of PPO substrates, but for very different reasons. If no phenolic substrate is present, 4-HR seems to inactivate PPO (see Figure 3A). This kind of experiment has been interpreted as inactivation of PPO by 4-HR (Weemaes and others 1999), but the results shown in Figure 3B demonstrate that they are due to the enzyme inhibition because the apparent inactivation of PPO can, at least partially, be reversed by dialysis of the PPO-4-HR complex. Recovery of activity after dialysis is a proof of the absence of irreversible inhibition (Dixon and Webb 1979). The high affinity of 4-HR for PPO explains ($K_i = 0.1\ \mu$M, acting on DOPA at pH 6.5 and 25 °C; Osuga and others 1994) why it still has inhibitory effects after the dilution step that follows incubation of PPO with 4-HR and the subsequent analysis of PPO enzymatic activity. This high affinity is probably due to the fact that 4-HR is a substrate analogue of PPO: it contains an aromatic ring with hydroxyl groups, like PPO substrates, and an apolar aliphatic chain, which increases its affinity for PPO. Indeed, the increase in length of this aliphatic chain, results in an increase in PPO inhibition (Mc Evily and Iyengar 1992). The benzene ring and 1 hydroxyl group interact probably with the copper ions of the active site due to its structural similarity with PPO substrates, and the aliphatic chain interacts probably with apolar residues in the vicinity of the active site, increasing the stability of the enzyme-inhibitor complex.

The interaction of 4-HR with the active site is rather peculiar. It has a higher affinity for 1 of the 3 (*met*, *oxy*, *deoxy*) possible states of PPO active site. During the PPO catalytic cycle, the oxidation state of the active site changes due to substrate binding. PPO catalysis has an ordered bi-bi mechanism (Lerch and Ettlinger, 1972) in which O_2 is the 1st substrate to bind. It binds to the *deoxy* form and as a consequence of the O_2 binding, the active site changes to the *oxy* form. It is precisely this *oxy* form that exhibits the higher affinity for 4-HR. This leads to the *slow binding* character of 4-HR; it takes some time, until all the enzyme molecules bind the 1st substrate, O_2, and for this ES complex to transform itself in the *oxy* form. This model is supported by ample experimental evidence (Valero and others 1991, 1992; Pérez-Gilabert and García-Carmona 2001) and also by the result shown in Figure 4, where a larger apparent inactivation effect of PPO by 4-HR is shown when O_2 is eliminated and PPO is therefore predominantly in the *deoxy* form, the one with lower affinity for 4-HR.

Finally, if both AA and 4-HR are incubated with PPO (see Figure 6), more than 90% inhibition of activity can be observed. This effect is approximately additive which indicates that both molecules, AA and 4-HR, bind differently to the active site because if this was not the case, the simultaneous addition of AA and 4-HR would result in less than additive inhibition of PPO activity, due to the competition of both molecules for the active site. In this experiment, the enzyme is probably in *oxy* form; it has not been possible to reproduce the experiment with PPO in the deoxyform, because during the dialysis of PPO with AA and/or 4-HR, O_2 remixes easily with the PPO containing solutions (data not shown). This behavior of PPO toward the combination of both AA and 4-HR explains why enzymatic browning is delayed when these chemicals are used in fresh cut pears (Sapers and Miller 1998; Dong and others 2000; Abbott and Buta 2002) and has practical implications for the technological use of AA and 4-HR to prevent enzymatic browning, especially when they should be used in combination.

The results shown in this work can also be helpful for the understanding or analysis of experiments of PPO enzyme kinetics. First, it is clear that no accurate data of PPO kinetics can be obtained if

AA is present, because AA can probably act as a covalent inactivator of PPO, although apparently sound results can be obtained (see Figure 7A). Artifacts are more probably at low substrate concentrations, because AA competes with substrates for the PPO active site. If the PPO reaction in the presence of AA is followed measuring color development, the result will be very inaccurate because it is impossible to know the exact substrate concentration at which the reaction takes place since part of the substrate will have been transformed before any increase in color is detected (see the lag phase in Figure 1B). This problem will be even more obvious at low substrate concentrations. This cannot be avoided if the PPO reaction is followed measuring O_2 consumption (see Figure 7B). In this figure, the PPO appears not to follow Michaelis–Menten kinetics; however, the figure most probably reveals that AA is inactivating PPO at low O_2 concentrations.

On the other hand, inhibition of PPO by 4-HR can be analyzed by kinetic means using Michaelis–Menten kinetics. We do not observe, as Jimenez and García-Carmona (1997) do, biphasic curves when following the PPO reaction in the presence of 4-HR, but only a decrease in activity which is still linear. In our kinetic experiments, 4-HR concentration is much higher (approximately 1000 fold) than that of the PPO, which is a requisite for the use of Michaelis–Menten equations. Figure 8 shows the effect of 4-HR on PPO at different DL-DOPA concentrations. The apparent K_m for this substrate is approximately 3-fold higher in the presence of 4-HR (0.28 mM compared with 0.98 mM in the absence and in the presence of 4-HR, respectively) and the apparent V_{max} is reduced by about 3 fold (PPO, 1.27 units/mg enzyme; PPO + 4-HR, 0.43 units/mg enzyme). Therefore, 4-HR can be considered as a mixed type inhibitor of PPO.

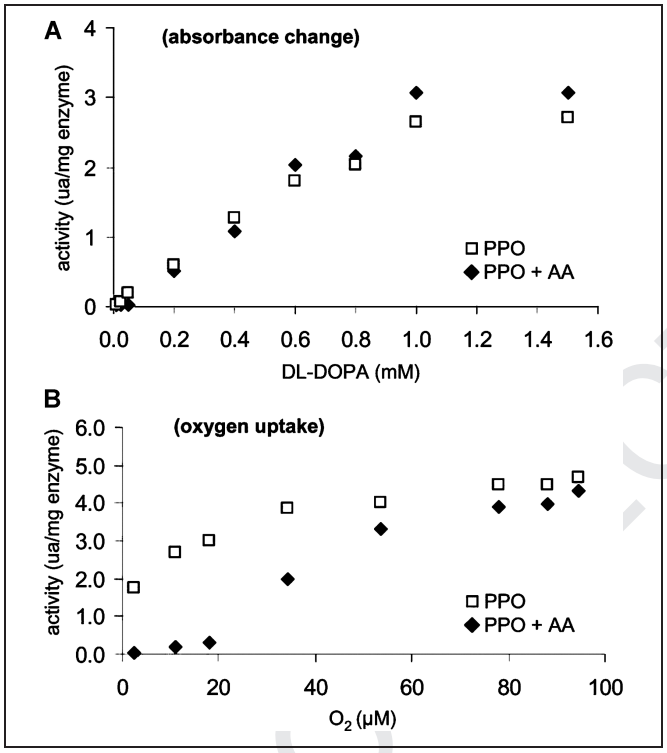

Figure 7—The effect of 0.2 mM AA on PPO activity at different (A) DL-DOPA and (B) O_2 concentrations.

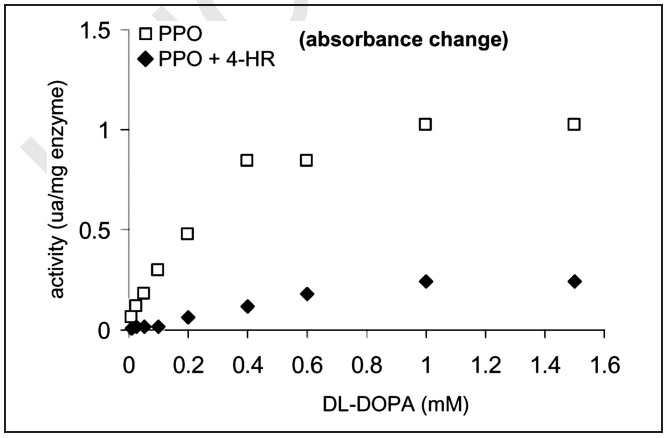

Figure 8—The effect of 0.2 mM 4-HR on PPO activity at different DL-DOPA concentrations.

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Queries

Q1 Author: The year for reference Guerrero-Beltrán and others has been changed from 2004 to 2005, as per the list. Please check.

Q2 Author: Please provide the name of the city for 'Sigma-Aldrich Co.'.

Q3 Author: Please provide the manufacturer location for 'UNICAM UV 500'.

Q4 Author: Please provide the manufacturer location for 'Strathkelvin Instruments Limited'.

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Q8 Author: Please provide the complete page range in reference Mc Evily et al. (1992).

Q9 Author: Please provide the name of the publisher in reference Osuga et al. (1994).

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