Polyphenoloxidase activity and browning in fresh-cut 'Rocha' pear as affected by pH, phenolic substrates, and antibrowning additives

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ABSTRACT

The effect of pH, phenolic substrates, and food additives on polyphenoloxidase (PPO) activity and on tissue browning was studied in fresh-cut 'Rocha' pear. Substrates 4-methylcatechol, caffeic acid, (+)-catechin hydrate, catechol, chlorogenic acid, dopamine hydrochloride, and pyrogallol, were prepared in citric acid-phosphate buffer at pHs ranging from 3.0 to 8.0. pH optima for PPO activity depended on the phenolic substrate. Activity was optimal at pH 5.0 for catechol and 4-methylcatechol; pH 6.0 for chlorogenic acid; pH 7.0 for dopamine, caffeic acid, and catechin; and pH 8.0 for pyrogallol. Discrepancies were observed between the pH dependency of PPO activity and browning, as assessed by objective color measurement. Significant correlations were obtained between enzyme activity and metric-hue difference (ΔH^*) over the pH range 3.0–8.0 for four of the eight phenolics. Chlorogenic acid, the main PPO substrate in 'Rocha' pear, induced high tissue browning but very low PPO activity at pH 3.0–4.0. Chemical inhibition of PPO was tested using catechol as substrate, and buffer solutions containing 250 mM Ca^{2+} in four salts (ascorbate, chloride, lactate and propionate), 57 mM ascorbic acid, 61 mM N-acetyl-L-cysteine and 3 mM 4-hexylresorcinol. PPO inhibition by additives was affected by the pH of the buffer, and was more effective with ascorbic acid, N-acetyl-L-cysteine and calcium ascorbate. It was concluded that inferences on tissue browning based on PPO activity can be misleading. Measurement of tissue color is proposed as a reliable means to assess the antibrowning effectiveness of additives and the pH of additives for cut pear should be corrected to reduce the browning potential.

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1. Introduction

Browning is a major limiting factor of shelf-life in susceptible fresh-cut fruit. Browning challenges the ability of the industry to meet the needs of the growing segment of consumers who seek convenience but will not lower their expectations about the intrinsic quality of fresh-cut fruit. Enzymic browning occurs when *o*-diphenol substrates react with oxygen to generate *o*-quinones, which subsequently polymerize, resulting in dark melanins (Yoruk and Marshall, 2003). The oxidative reaction is catalyzed by polyphenoloxidase (PPO; EC 1.14.18.1), although the co-involvement of peroxidase (POD; EC 1.11.1.7) in the presence of H_2O_2 has also been proposed in pear (Richard-Forget and Gaillard, 1997). Since PPO and phenolic substrates are localized in different cellular compartments, plastids and vacuoles, respectively, the breakdown of cellular compartmentation is required for browning to occur. Plant PPO have their pH optima in the range 4.0–8.0 and are nearly

inactive at pHs lower than 4.0 (Weemaes et al., 1998; Yoruk and Marshall, 2003). In pear, the reported pH optima for PPO activity depend on the cultivar and the phenolic substrate used to assay activity *in vitro*, with values ranging from 4.3 (Espín et al., 1998) to 7.2 (Ziyan and Pekayardimci, 2004). Acidifying additives (e.g., ascorbate, citrate) are frequently used in fresh-cut fruit to limit microbial development and to reduce PPO action (Martinez and Whitaker, 1995; Ahvenainen, 1996; Yoruk and Marshall, 2003). In addition to the chemical nature of the acidifying additives, pH, *per se*, affects *in vitro* activity of PPO and POD (Yoruk and Marshall, 2003; Fortea et al., 2009) and the surface color of pear slices (Gomes et al., 2010). Therefore, it is anticipated that conditions that, intentionally or unintentionally, alter the surface pH of fresh-cut fruit will affect enzymic browning.

Relationships between the rate of browning and PPO activity, type of phenolic substrate, and its concentration, have been documented (Coseteng and Lee, 1987; Amiot et al., 1992, 1995; Galvis-Sánchez et al., 2004). However, correlations between browning and enzyme activity or substrate availability are not always observed. For example, browning of fresh-cut potato is not correlated with the activity of PPO and POD or with the

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concentrations of phenolics, or even the antioxidant ascorbic acid (Cantos et al., 2002). Instead, membrane integrity can be the rate-limiting factor for browning (Cantos et al., 2002).

The objective of this study was to assess the effect of pH on *in vitro* PPO activity against various phenolic substrates and its relation to *in vivo* color development in fresh-cut 'Rocha' pear. The effect of pH on the inhibitory action of food additives (calcium ascorbate, calcium chloride, calcium lactate, calcium propionate, ascorbic acid, N-acetyl-L-cysteine, and 4-hexylresorcinol) on PPO was also evaluated. This information highlights the importance of pH on browning and can be considered when food additives are developed or used to reduce quality losses in fresh-cut pear.

2. Materials and methods

2.1. Plant material and processing

Pear (*Pyrus communis* L. 'Rocha') fruit were harvested at commercial maturity from orchards located in the Western Region of Portugal. Fruit raw material was stored in commercial cold rooms for 8 months at -0.5°C , 90–92% relative humidity, 2.5 kPa O_2 , and 0.7 kPa CO_2 (balance N_2). Fruit with a fresh weight of 152.7 ± 11.9 g and $12.2 \pm 0.7\%$ soluble solids, were processed with an average flesh firmness of 54 ± 3 N, measured with a penetrometer mounted on a stand drill and equipped with an 8 mm diameter probe. Whole fruit were sanitized with $150 \mu\text{L L}^{-1}$ NaOCl and rinsed with tap water before processing. Pears were cut by hand with a sharp knife previously sanitized with 70% ethanol, and one half of each fruit was used to measure tissue browning *in vivo* and the other half was frozen in liquid nitrogen and stored at -20°C (Siddiq et al., 1994; Siddiq and Cash, 2000) for 6 months, before extraction of PPO for *in vitro* activity assays.

2.2. Color measurement in pear slices

In vivo assays of color changes were conducted in pear slices 5–10 mm thick. Each one of seven slices, with a surface area of ca. 14 cm^2 , was covered with 1 mL of each treatment solution, consisting of all combinations of six pH values and eight phenolic substrates (Section 2.3). After 30 s the treated surface was blotted dry with paper towels and the color was immediately measured. The slices were then kept for 120 min at 24°C , protected from light on a tray covered with aluminum foil. Tissue browning was evaluated by reflectance colorimetry, immediately after the treatment and at 30 min intervals for a total period of 120 min. Color of the treated surface was measured in the CIE $L^*a^*b^*$ color space with a Konica-Minolta CR-400 chromameter (Osaka, Japan) equipped with a D₆₅ illuminant and the observer at 2° . Color coordinates lightness (L^*), a^* , b^* , chroma ($C^* = [(a^*)^2 + (b^*)^2]^{1/2}$), hue angle [$h^{\circ} = \tan^{-1}(b^*)/(a^*)$], metric-hue ($\Delta H^* = [(\Delta a^*)^2 + (\Delta b^*)^2 - (\Delta C^*)^2]^{1/2}$), and total color ($\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$) differences between the initial and final readings were computed and analyzed.

2.3. Buffer solution and phenolic substrates

The effect of pH was evaluated in a single buffer solution prepared with 100 mM citric acid–200 mM sodium phosphate and pH adjusted to 3.0–8.0 with one pH unit intervals. Phenolic compounds (Sigma–Aldrich, Madrid, Spain) used in *in vivo* color measurements and in *in vitro* PPO activity assays were the five *o*-dihydroxyphenols [4-methyl catechol, pyrocatechol (catechol), chlorogenic acid, caffeic acid, dopamine hydrochloride], one *o*-dihydroxy (bi)phenol [(+)-catechin hydrate], one trihydroxyphenol (pyrogallol), and one monohydroxyphenol (L-tyrosine). In the *in vivo* experiment the concentration of phenolic compounds was 20 mM, while 8.3 mM

were used in the reaction mixture of the *in vitro* experiment, except for tyrosine, caffeic acid and catechin that were used at 4.1 mM due to low solubility. The concentrations of phenolic substrates in the *in vivo* and in the *in vitro* experiments were chosen to maximize the measurement accuracy and discriminatory ability across the experimental conditions. Buffer solutions at pH 3.0–8.0 without phenolics were used as controls for *in vivo* color development. In the *in vitro* experiment an aqueous pear extract was used as the control.

2.4. Native substrate extraction and characterization

The effect of pH on PPO activity was also assayed *in vitro* against an extract containing the water soluble phenolic compounds present in pear. Triplicate frozen fruit samples (20 g) were peeled and homogenized for 2 min (Ultra-Turrax T25, IKA, Staufen, Germany) in 100 mL of absolute ethanol at -20°C . The homogenate was boiled under reflux for 20 min to inactivate enzymes, and maintained overnight at -20°C to assure complete precipitation of polymers. The suspension was filtered through Miracloth (Calbiochem Corporation, La Jolla, CA, USA), the supernatant collected and completely evaporated (Laborota 4000 HB/G1, Heidolph, Kelheim, Germany) at 35°C for 40 min. The residue was suspended in 20 mL of ultra-pure water.

Phenolic compounds in the extract were identified and quantified by HPLC with diode array detection at 320 nm as described by Andrade et al. (1998). The PPO substrates detected were 3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, catechin, epicatechin, and *p*-hydroxybenzoic acid. Chlorogenic acid was the main substrate in the extract, with a concentration of 0.080 mg kg^{-1} pear.

2.5. PPO extraction

Crude extracts of pear PPO were obtained as described by Coseteng and Lee (1987), with modifications. Frozen pear tissue (10 g) was peeled and homogenized for 2 min with 20 mL of cold 200 mM phosphate buffer containing 5 mM cysteine hydrochloride (pH 7.2). The samples were centrifuged at $600 \times g$ for 20 min, the supernatant was collected and the pellet suspended in 20 mL of cold 134 mM KCl and stirred for 30 min. Following centrifugation, the supernatants were combined and the final volume of the crude extract adjusted to 45 mL. Total protein in the extract, as determined by the dye-binding method of Bradford (1976) using bovine serum albumin as the standard, was 0.316 g L^{-1} .

2.6. In vitro PPO activity

PPO activity was determined using the method described by Coseteng and Lee (1987), adapted to use 100 mM citric acid–200 mM sodium phosphate buffer at pH values ranging from 3.0 to 8.0. Enzyme extract (0.5 mL) was added to a quartz cuvette containing the buffer solution with the phenolic substrate (2.5 mL) and the initial absorbance measured at 420 nm. PPO activity was determined by measuring the rate of increase in absorbance at 420 nm during an incubation period of 30 min at 25°C . One unit of enzyme activity was defined as a change in absorbance of 0.001 per min per milligram of protein in the enzyme extract. All measurements were performed in triplicate extracts from independent biological samples.

2.7. Chemical inactivation of PPO

An additional experiment was designed to study the effect of pH on the inactivation of pear PPO by additives suitable for application in fresh-cut fruit. Four calcium salts, ascorbate, chloride, lactate,

Table 1

Metric-hue difference (ΔH^*) after 30 min at 24 °C of the surface of pear slices treated with different phenolic compounds at pH ranging from 3 to 8. Mean values ($n=7$) within lines followed by the same letter are not significantly different at $\alpha=0.05$ by the Duncan's multiple range test.

Phenolic substrate	Buffer pH					
	3.0	4.0	5.0	6.0	7.0	8.0
Caffeic acid	4.34 ^b	3.42 ^c	2.97 ^c	5.06 ^b	7.33 ^a	7.72 ^a
Catechin	4.60 ^{d,e}	3.82 ^e	6.35 ^{c,d}	8.04 ^c	14.94 ^a	10.62 ^b
Catechol	15.22 ^a	15.93 ^a	16.32 ^a	6.01 ^b	4.29 ^b	5.26 ^b
Chlorogenic acid	5.35 ^{a,b}	5.71 ^a	4.59 ^{b,c}	3.56 ^d	3.96 ^{c,d}	3.35 ^d
Dopamine	2.89 ^c	10.04 ^a	10.23 ^a	7.26 ^b	3.41 ^c	2.73 ^c
4-Methylcatechol	18.00 ^a	18.57 ^a	16.87 ^a	18.36 ^a	16.27 ^a	12.28 ^b
Pyrogallol	0.71 ^e	1.59 ^{c,d}	1.70 ^c	0.93 ^{d,e}	2.92 ^b	3.89 ^a
Tyrosine	0.45 ^a	0.74 ^a	0.94 ^a	0.54 ^a	0.70 ^a	0.65 ^a
Control ^x	0.70 ^a	0.57 ^a	0.51 ^{a,b}	0.29 ^{b,c}	0.31 ^{b,c}	0.25 ^c

^x Controls were buffer solutions at different pH values without phenolics.

and propionate, all from Sigma–Aldrich (Madrid, Spain), were separately dissolved in the buffer solution at a final concentration of 250 mM of Ca²⁺ (1%). Ascorbic acid (57 mM or 1%), N-acetyl-L-cysteine (61 mM or 1%) and 4-hexylresorcinol (3 mM or 0.05%) were also dissolved in the buffer solution. PPO was assayed *in vitro* as described, using catechol as the phenolic substrate. Buffered catechol solutions without inhibitors were used as controls.

2.8. Data analyses

Data from the *in vitro* PPO activity assays were analyzed by a two-factor ANOVA with phenolic substrate and pH as fixed factors. Mean separation was performed with the Duncan's multiple range test at $\alpha=0.05$. The associations between color measurement (*in vivo*) and PPO activity (*in vitro*) were quantified using Pearson's correlation coefficient. All ANOVA and correlation analyses were performed with the statistical software SPSS 17.0 for Windows (SPSS, Chicago, USA).

3. Results and discussion

3.1. Effect of substrate and pH on browning intensity

Endogenous enzymes were able to oxidize the phenolic compounds applied to the pear slices, and surface color changed accordingly (Fig. 1). The kinetics of color changes revealed that most browning occurred within the first 30 min and color remained relatively constant thereafter (data not shown). Similarly, up to 75% of the browning occurring in buffer extracts of peach and nectarine skin tissue takes place in the initial 30 min, and is almost complete within the first 60 min (Cheng and Crisosto, 1995). Therefore, *in vivo* color changes are reported and discussed taking into account the measurement performed at 30 min. Tissue browning during 30 min at room temperature (24 °C) significantly affected all color coordinates, L^* , a^* , b^* , C^* , and h° (data not shown). L^* and h° decreased after the treatments, indicating darkening and increased redness, respectively. The color coordinates a^* , b^* , and C^* increased, indicating more saturated colors (C^*) with higher red (a^*) and yellow (b^*) components (data not shown). The metric-hue difference, ΔH^* (Table 1), adequately described the color changes occurring in the tissue. Since the other color coordinates were redundant for our analysis, ΔH^* will be used hereafter as a surrogate for browning.

Phenolic substrates significantly affected the surface color of pear slices. ΔH^* (Table 1) was higher when the pear mesocarp was in contact with 4-methylcatechol, irrespective of pH. After 4-methylcatechol, the higher browning intensity was induced by the substrates catechol and catechin. Color change was minor when tyrosine was used as substrate or in buffer without phenolic

substrate (control). Browning (ΔH^*) of slices treated with catechol and, specially, with chlorogenic acid followed the same trend of color development in buffers without phenolics ($R^2 > 0.9$) in the pH range from 3.0 to 8.0 (Fig. 1).

Flesh color changes were also affected by pH in a substrate-dependent manner (Fig. 1). Acidic buffers (pH 3.0–5.0) in the absence of phenolic substrates (control) or containing catechol, chlorogenic acid, and even dopamine or 4-methylcatechol, generally induced higher browning intensity (Table 1 and Fig. 1) than neutral buffers. Caffeic acid, catechin and pyrogallol increased browning when dissolved in neutral to basic buffers (pH 6.0–8.0).

Clearly, browning development on the tissue surface is affected by pH and by the phenolic substrate. Since the reactions leading to browning are initiated by PPO, it is warranted to examine the effect of pH and phenolic substrates on PPO activity.

3.2. Effect of substrate and pH on PPO activity

'Rocha' pear PPO activity was significantly affected by the phenolic substrate ($P < 0.001$), pH ($P < 0.001$), and there was a significant ($P < 0.001$) interaction between both variables. PPO from 'Rocha' pear exhibited activity toward di- and tri-hydroxyphenols (Table 2), but no activity was observed with the monohydroxyphenol L-tyrosine, in agreement with reports from other pear cultivars (Siddiq et al., 1994; Espín et al., 1998; Ziyani and Pekayardimci, 2004). Monophenolase activity has been documented in pear PPO (Espín et al., 1998). It is not clear if monophenolase activity is absent from 'Rocha' pear or if this cultivar has a low catalytic constant for monophenolase activity.

On average, over the pH range 3.0–8.0, enzyme activity was higher when catechin was used as substrate (74 U min⁻¹ mg⁻¹), followed by catechol (58 U min⁻¹ mg⁻¹), dopamine (52 U min⁻¹ mg⁻¹), and 4-methylcatechol (50 U min⁻¹ mg⁻¹). Average activities with pyrogallol, chlorogenic acid, and caffeic acid were lower than 30 U min⁻¹ mg⁻¹ (Table 2). High PPO activity at every pH tested was observed when catechol was used as a substrate. These results are consistent with previous reports considering catechol and 4-methylcatechol as the most reactive substrates for pear PPO (Siddiq et al., 1994; Espín et al., 1998; Siddiq and Cash, 2000; Ziyani and Pekayardimci, 2004). In contrast, lower concentrations of chlorogenic acid than catechol are required for maximum activity of 'Conference' pear PPO (Arias et al., 2007), justifying the use of the former substance as a standard substrate for enzyme assay.

PPO activity increased with pH from 3.0 to 7.0 and decreased slightly as pH increased from 7.0 to 8.0, but this general pattern was affected by the substrate. The optimum pH was found to be 8.0 for pyrogallol, 7.0 for catechin, dopamine, and caffeic acid, 6.0 for chlorogenic acid, and 5.0 for catechol and 4-methylcatechol (Table 2). PPO activity against an extract containing the native soluble phenolics was very low, with optimum at pH 3.0–4.0 (Table 2). The pH optima for PPO activity also depends on the pear cultivar (Siddiq et al., 1994; Siddiq and Cash, 2000). When 4-methylcatechol is used as substrate, optimum pH was found to be 4.3 for PPO from 'Blanquilla' (Espín et al., 1998), 4.7 and 5.5 for 'd'Anjou' and 'Bartlett', respectively (Siddiq and Cash, 2000), 5.0 and 5.5 for 'Bosc' and 'Red' (Siddiq et al., 1994), and pH 7.2 for 'Ankara' pear PPO (Ziyani and Pekayardimci, 2004). Maximum activity of 'Conference' pear PPO against catechol or chlorogenic acid is pH 4.5 (Arias et al., 2007), a pH optimum lower than that obtained here for 'Rocha' pear PPO (Table 2). No PPO activity against 4-methylcatechol was detected at pH 7.0 for 'Bosc' and 'Red' (Siddiq et al., 1994), but the activity of PPO from 'Rocha' pear at pH 7.0 was 45% of the maximum activity against this substrate observed at pH 5.0 (Table 2). Differences in the reported pH optima for pear PPO may be due to the methodologies used to extract, purify, and assay enzyme activity

Fig. 1. Color development on pear slices, reported as metric-hue difference (ΔH^*) (left), and in cuvettes during *in vitro* PPO activity assay ($\text{U min}^{-1} \text{mg}^{-1}$ of protein) (right).

(Yoruk and Marshall, 2003). Relative activity of 'Rocha' PPO at pH 3.0 was very low (below 20% of maximum) for most phenolic substrates, except catechol and the native pear extract (Table 2). This is consistent with the results reported for PPO from 'Red' ($\approx 10\%$), but not for 'Bosc' ($\approx 60\%$) pear (Siddiq et al., 1994). PPO inhibition by pH can have several causes, including protonation of catalytic groups, conformational changes in the active site, denaturation of the protein, or substrate instability (Yoruk and Marshall, 2003), which help explain the variability in pH optima on plant PPO.

Since pH and phenolic substrate have a strong effect on browning and on PPO activity of 'Rocha' pear, we have examined whether the relationship between PPO activity and color changes was differentially affected by these factors.

3.3. Relationship between PPO activity and tissue browning

Pearson's correlation coefficients were calculated to quantify the degree of association between enzyme activity and color

Table 2

Effect of pH on PPO activity ($\text{U min}^{-1} \text{mg}^{-1}$ of protein) against various phenolic substrates. Mean values $\pm$ S.E. ($n = 3$) within lines followed by the same letter are not significantly different at $\alpha = 0.05$ by the Duncan's multiple range test.

Phenolic substrate	Buffer pH					
	3.0	4.0	5.0	6.0	7.0	8.0
Caffeic acid	5.7 ± 0.2^b	12.2 ± 0.3^b	12.2 ± 0.2^b	24.0 ± 0.8^a	27.8 ± 0.1^a	24.4 ± 6.2^a
Catechin	2.9 ± 0.1^f	8.7 ± 0.1^e	42.9 ± 0.6^d	97.1 ± 0.6^c	151.3 ± 1.4^a	143.7 ± 1.3^b
Catechol	53.0 ± 0.8^d	69.3 ± 1.0^b	76.6 ± 0.9^a	58.8 ± 0.9^c	47.2 ± 0.2^e	46.0 ± 1.0^e
Chlorogenic acid	3.4 ± 0.1^e	20.0 ± 0.2^c	33.3 ± 0.0^b	37.3 ± 0.5^a	32.7 ± 0.6^b	12.7 ± 0.1^d
Dopamine	5.6 ± 0.2^e	31.5 ± 0.1^d	54.5 ± 0.5^c	71.8 ± 0.2^b	79.0 ± 0.1^a	69.8 ± 2.0^b
4-Methylcatechol	6.6 ± 0.3^e	70.3 ± 1.0^c	92.2 ± 1.6^a	79.9 ± 0.7^b	41.4 ± 1.2^d	9.6 ± 0.8^e
Pyrogallol	6.1 ± 0.1^f	10.0 ± 0.1^e	11.2 ± 0.1^d	17.5 ± 0.3^c	52.2 ± 0.8^b	82.4 ± 0.2^a
Tyrosine	NA	NA	NA	NA	NA	NA
Pear extract	$2.4 \pm 0.1^{a,b}$	2.7 ± 0.1^a	1.0 ± 0.3^d	0.8 ± 0.0^d	$1.4 \pm 0.1^{c,d}$	$1.9 \pm 0.4^{b,c}$

NA, no activity.

Table 3Pearson's correlation coefficient between PPO activity and surface color coordinates of pear slices as affected by pH ($n=6$).

PPO substrate	L^*	a^*	b^*	C^*	h°	ΔH^*	ΔE^*
Caffeic acid	−0.846*	0.868*	0.880*	0.892**	−0.858*	0.794*	0.780*
Catechin	−0.793*	0.935**	0.832*	0.856*	−0.959**	0.938**	0.856*
Catechol	−0.733*	0.751*	0.652	0.702	−0.773*	0.753*	0.741*
Chlorogenic acid	−0.449	−0.516	0.340	0.320	0.552	−0.401	0.367
Dopamine	−0.904**	0.203	−0.262	−0.197	−0.307	−0.062	0.459
4-Methylcatechol	−0.525	0.515	0.469	0.502	−0.210	0.513	0.487
Pyrogallol	−0.818*	0.787*	−0.540	−0.526	−0.788*	0.945**	−0.573
Control ^x	−0.403	−0.026	0.470	0.466	−0.312	0.569	0.430

Significant correlation (1-tailed) at the 0.05 and 0.01 level are indicated by * and **, respectively.

^x Controls were buffer solutions at different pH values without phenolics for color and an aqueous pear extract for PPO activity.

parameters. PPO activity was negatively correlated with L^* and h° (Table 3), and positively correlated with a^* or ΔH^* (Table 3), over the pH range 3.0–8.0, for the substrates caffeic acid, catechin, catechol and pyrogallol. Similar results with catechol were reported by Soliva-Fortuny et al. (2002), who correlated L^* and ΔE^* values of pear cubes with PPO activity throughout storage, while Galvis-Sánchez et al. (2004) observed a poor relationship between PPO activity and the color parameters a^* and b^* in the flesh of whole 'Rocha' pear after long-term storage. Browning of extracts from peach and nectarine skin was not correlated with PPO activity (Cheng and Crisosto, 1995), but changes in L^* in a peach puree were directly related to PPO activity and total phenolics (Lee et al., 1990). In this experiment with 'Rocha' pear, no significant correlations between PPO activity and color changes were observed when chlorogenic acid, 4-methylcatechol, or control buffers without added phenolic were used (Table 3), while with dopamine a significant negative correlation existed between PPO activity and L^* (Table 3).

Discrepancies were observed between the pH dependency of browning and PPO activity (Tables 1 and 2). With chlorogenic acid, the main PPO substrate in 'Rocha' pear, tissue browning (ΔH^*) was higher at pH ≤ 4.0 , although PPO activity against the same substrate was low in this pH range. Similarly with the other phenolic substrates, browning at pH 3.0 was higher than the corresponding PPO activity, which may be due to the acid inactivation of pear PPO at pH < 3.5 –4.0 (Rivas and Whitaker, 1973; Weemaes et al., 1998), and to the ability of the citric acid used in the buffer solution to chelate the copper of the PPO prosthetic group. In contrast, an activation of PPO and POD enzymes by low pH (acid shock) has been observed (Yoruk and Marshall, 2003; Fortea et al., 2009). Discrepancies were also observed at higher pH values, which can be attributed to the increased instability of *o*-quinones at high pH (Valero and García-Carmona, 1992). Differences between browning patterns and PPO activity can also relate to the presence of PPO isoforms in the pear extract or even other enzymes with diverse pH optima. However, only one peak of activity was observed when activity was assayed in the pH range 3.0–8.0 suggesting that PPO activity is predominant.

Clearly, there is not a simple and direct relationship between PPO activity and color changes in fresh-cut pear, as pH and substrate differentially affected these two variables. Additive effects of substrates and endogenous enzymes in interaction with the pool of cellular substances that can interfere with the enzyme's binding affinity, quinone polymerization or reversible inhibition of catalysis, may explain this lack of correlation. Substrates that induce rapid color changes can be used to develop quick assays of food additives, but interactions between pH and substrate must be considered. Based on these results, a rapid assay is proposed to assess the effects of additive formulations in fresh-cut pear. The assay consists of the direct measurement of surface color differences after 30 min incubation at room temperature in the presence of 20 mM of chlorogenic acid. This allows a faster assessment of browning potential than following the naturally occurring color changes and

chlorogenic acid has the advantage of providing an adequate contrast while avoiding the disadvantages of over dyeing.

3.4. Effect of inhibitory additives on PPO activity

Food additives are used in susceptible fresh-cut fruit to prevent or minimize enzymic browning and extend storage life. Potential additives that can be used by the industry for this purpose (Martinez and Whitaker, 1995; Oms-Oliu et al., 2010) were assessed for their effect on PPO activity at various pH values.

The spectrophotometrical determination of PPO activity was not possible when calcium propionate was used at every pH value, or when the calcium salts of ascorbate, chloride or lactate were used at pH above 7.0 due to precipitation of calcium in the phosphate buffer. Ascorbic acid and N-acetyl-L-cysteine concentrations were in the range proposed by Oms-Oliu et al. (2006), but concentrations of 4-hexylresorcinol lower than commonly reported were used to avoid the darkish pigments on the surface of the pear wedges (Oms-Oliu et al., 2006) and the objectionable flavor (Dong et al., 2000). All chemicals tested were able to reduce the activity of 'Rocha' pear PPO (Table 4). Ascorbic acid, N-acetyl-L-cysteine, and calcium ascorbate were the most effective inhibitors (Table 4). L-Cysteine and ascorbic acid are both reported to be very good inhibitors of PPO from pear (Siddiq et al., 1994) and lychee (Sun et al., 2008). Ascorbate delays the polymerization of oxidized substrate molecules to brown pigments and can reduce them to their original state, but does not interact directly with pear PPO (Arias et al., 2007). Ascorbic acid alone, in the range of 43–170 mg L^{−1}, did not prevent browning reactions in fresh-cut pears (Gorny et al., 2002; Oms-Oliu et al., 2006). Ascorbic acid acts differently on PPO activity and on browning, depending on concentration (Oms-Oliu et al., 2006). Some PPO activity was observed at pH 3.0 but decreased to negligible values at pH 4.0–6.0 (Table 4). Ascorbate in combination with calcium chloride is more effective in reducing browning of fresh-cut pear under neutral (pH 7.7) than under acidic (pH 3.3) conditions (Sapers and Miller, 1998), but calcium ascorbate (250 mM ascorbate) was equally effective in reducing discolorations in pear slices at pH 3.0 and pH 7.0 (Gomes et al., 2010). Wedges of 'Flor de Invierno' pear treated with 92 mM N-acetyl-L-cysteine maintained the initial color for 28 days at 4 °C due to the inhibition of PPO (Oms-Oliu et al., 2006). Differences in the effectiveness of inhibitors to inactivate enzyme activity (*in vitro*) and reduce color changes (*in vivo*) have been noted. N-acetyl-L-cysteine at 0.3 mM was very effective in inactivating PPO of loquat fruit, but 1 mM was required to prevent browning in fresh loquat juice (Ding et al., 2002).

PPO from 'Conference' pear is inhibited by 4-hexylresorcinol in a pH-dependent manner (Arias et al., 2007). The stronger inhibition of PPO by 4-hexylresorcinol at low pH was also observed in 'Rocha' pear (Table 4). In fresh-cut pear, 4-hexylresorcinol did not inhibit browning at 0.5 mM (not shown) or at 1.6 mM (Dong et al., 2000), and concentrations of 103 mM promote dark pigmentation of the cut surface (Oms-Oliu et al., 2006).

Table 4

Effect of selected food additives on relative PPO activity (catechol at pH 5.0 taken as 100%). Mean values $\pm$ S.E. ($n=3$) within lines followed by the same letter are not significantly different at $\alpha=0.05$ by the Duncan's multiple range test.

Compound	Buffer pH					
	3.0	4.0	5.0	6.0	7.0	8.0
Catechol	48.4 $\pm$ 0.6 ^d (0)	90.9 $\pm$ 0.2 ^b (0)	100.0 $\pm$ 1.0 ^a (0)	93.2 $\pm$ 1.7 ^b (0)	54.7 $\pm$ 1.6 ^c (0)	36.3 $\pm$ 0.5 ^e (0)
Calcium ascorbate	7.7 $\pm$ 0.3 ^a (84)	1.4 $\pm$ 0.5 ^c (98)	2.8 $\pm$ 0.3 ^b (97)	3.7 $\pm$ 0.2 ^b (96)	ND	ND
Calcium chloride	0.0 $\pm$ 0.0 ^b (100)	1.1 $\pm$ 0.3 ^b (99)	8.5 $\pm$ 3.0 ^b (92)	32.6 $\pm$ 11.1 ^a (65)	ND	ND
Calcium lactate	40.4 $\pm$ 3.5 ^d (17)	68.7 $\pm$ 0.8 ^b (24)	76.1 $\pm$ 2.1 ^a (24)	50.5 $\pm$ 1.1 ^c (46)	ND	ND
Calcium propionate	ND	ND	ND	ND	ND	ND
Ascorbic acid	1.9 $\pm$ 0.1 ^a (96)	1.4 $\pm$ 0.2 ^b (98)	1.4 $\pm$ 0.1 ^b (99)	0.4 $\pm$ 0.1 ^c (100)	0.0 $\pm$ 0.0 ^d (100)	0.0 $\pm$ 0.0 ^d (100)
4-Hexylresorcinol	1.9 $\pm$ 0.3 ^b (96)	7.2 $\pm$ 0.3 ^b (92)	8.4 $\pm$ 0.4 ^b (92)	10.1 $\pm$ 0.9 ^b (89)	15.1 $\pm$ 0.3 ^a (72)	13.8 $\pm$ 1.8 ^{a,b} (62)
N-acetyl-L-cysteine	0.8 $\pm$ 0.1 ^b (98)	0.6 $\pm$ 0.3 ^b (99)	3.4 $\pm$ 1.0 ^a (97)	4.6 $\pm$ 0.4 ^a (95)	0.0 $\pm$ 0.0 ^b (100)	0.0 $\pm$ 0.0 ^b (100)

ND—PPO activity not determined due to precipitation.

Activity within parenthesis is indicated as percentage of inhibition in relation to the activity measured in catechol at each pH.

Chloride, as sodium or calcium salt (de Poix et al., 1980), and sodium chlorite (Lu et al., 2006) are reported to reduce PPO activity in apple. In apple, PPO inhibition increase with the acidification of a sodium chlorite solution from pH 5.5 to values below pH 4.0, probably because chloride dioxide generated under acidic conditions inhibits the enzyme (Lu et al., 2006). Similarly, the activity of 'Rocha' pear PPO can be suppressed in a solution of calcium chloride at pH 3.0–5.0, but not at pH 6.0 (Table 4). However, color changes in fresh-cut pear did not follow the same trend observed for PPO; the acidic or neutral dips of calcium chloride were equally effective in reducing browning (Gomes et al., 2010).

Among the calcium salts, calcium lactate had the least inhibitory effect on PPO (Table 4). The effect of pH on the inhibitory effect of calcium lactate had the same trend as the pH effect on the catechol substrate without inhibitor (Table 4), which confirms that calcium lactate alone has no antibrowning effect on cut pear (Dong et al., 2000; Gorny et al., 2002; Gomes et al., 2010).

4. Conclusions

Significant correlations between PPO activity *in vitro* and color changes *in vivo* were observed for caffeic acid, catechin, catechol, dopamine, and pyrogallol but browning at pH 3.0 was generally higher than the corresponding PPO activity. PPO activity against a water-soluble pear extract did not correlate with tissue color changes. In addition, lack of a clear correlation between PPO activity and browning, and differences among inhibitors in their effectiveness to inhibit browning and PPO make it difficult to predict the response of fruits based on *in vitro* outcomes. The results reported herein suggest that PPO activity is not the best predictor of tissue browning in fresh-cut 'Rocha' pear, either in the presence or in the absence of inhibitors. Direct assessment of color changes after application of catechol or chlorogenic acid to the cut surface is a rapid and accurate method to anticipate color changes in fresh-cut pear and test the efficacy of antibrowning additives. The pH of additive solutions should be corrected to 6 or higher to reduce the browning potential.

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