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Biochemical characterization and thermal inactivation of polyphenol oxidase from radish (*Raphanus sativus* var. *sativus*)

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ABSTRACT

Polyphenoloxidase (PPO) is the target for the development of several food antibrowning agents. Different substrates (pyrocatechol, gallic acid, chlorogenic acid, caffeic acid, 3,4 dihydroxybenzoic acid, p-cumaric acid, L-tyrosine, pyrogallic acid and phloroglucinol) were analyzed to determine their affinities with radish PPO. Pyrocatechol, gallic acid and pyrogallic acid were the substrates that showed high affinity based on V_{max}/K_m ratio. The optimum pH for the PPO using these three substrates were pH = 7 and the optimum temperatures were 20, 60 and 20–40 °C for pyrogallic acid, gallic acid and pyrocatechol, respectively. The kinetics of thermal inactivation was successfully modeled by a biphasic model ($r^2 > 0.888$), attributed to the presence of two enzyme fractions, a heat-labile easily inactivated even at low blanching temperatures, and a heat-resistant fraction that requires blanching temperatures above 80 °C to reach 70% of inactivation. The kinetics constants of this model for both heat-labile and heat-resistant increased with temperature in the range from 60 to 90 °C. The activation energy ratio of resistant to labile fraction was found to be 6 (E_{aL} = 142 kJ/mol).

1. Introduction

Radish (*Raphanus sativus* L.), which belongs to the Brassicaceae family, is a root crop pungent or sweet in taste with a lot of juice. Roots have variable shape and skin color, but the round, red-skinned variety is the best know (Herman-Lara et al., 2012). Radishes offer many health and nutritional benefits. They are rich in folic acid, Vitamin C and anthocyanins (Patil, Madhusudhan, Ravindra Babu, & Raghavarao, 2009). Epidemiologic evidence has suggested that diets rich in vegetables are associated with reduced risk of several diseases due to potent antioxidant properties of phytochemicals decreasing oxidative stress in consumers (Zhang et al., 2013). Although radishes are widely used in salad preparations, the rapid deterioration mainly due to slices browning decreases the marketability of these preparations. The marketing of fresh-cut salads is limited by a short shelf-life and rapid deterioration of their components due to tissue damage by slicing and similar methods of preparation (Spagna, Barbagallo, Chisari, & Branca, 2005). Gonzalez Aguilar (2001) reported for radish slices that the combination of 4-hexylresorcinol (0.001 g/L), potassium sorbate (0.05 g/L) and N-acetylcysteine (0.025 g/L) was most effective in preventing browning and deterioration for up to 18 days at 10 °C. Technological strategies for enzymatic browning inhibition should be focused on the enzyme responsible for plant tissue browning. The undesirable browning of damaged tissues in fruits and vegetables occurs by the enzymatic oxidation of polyphenols. Such oxidation is mainly caused by polyphenoloxidase (EC 1.10.3.1: 0-diphenol: oxygen oxidoreductase, PPO). Characterization of radish PPO is important to identify its biochemical properties and function and, in turn, to understand how to prevent its deteriorative action during storage and processing. Many studies have investigated PPO with the goal of preventing this discoloration (Quieroz, Mendes Lopes, Fialho & Valente-Mesquita, 2008; Yoruk & Marshall, 2003). Andi et al. (2011) reported the purification and characterization of polyphenols oxidase from Japanese radish root, which is a white radish; however this radish belongs to a Japanese variety namely var. L. cv. *Aokubi soufuto*-L. The most consumed variety of radish in Argentine is red radish, (*Raphanus sativus* var. *sativus*) and for this variety a characterization of the PPO has not been previously conducted.

The aims of this research were to (1) biochemical characterize the PPO of radish by determining several selected substrates specificity, (2) determine their enzyme kinetic parameters by

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mathematical modeling, (3) determine the effects of pH and temperature on the enzyme activity in order to find optimal ranges of work, (4) determine the thermal stability of the enzyme, and (5) determine the kinetics of thermal inactivation during blanching in the range of 60–90 °C by means of a biphasic model.

2. Materials and methods

2.1. Plant material and sample preparation

Radishes were purchased from a local market from Mar del Plata city. They were kept at 5 ± 1 °C in darkness prior to processing. Radish roots were separated from leaves and they were washed in tap water to eliminate any surface contamination, cut with a manual cutter into slices of 3–4 mm, and then washed again in tap water, using a ratio of sliced radish to water of 1:10 (g:g).

2.2. Measurement of the enzyme activity

The activity of PPO was measured by the colorimetric method. 10 g of radishes were homogenized at a 1:2 (g:mL) ratio with 0.5 mol/L phosphate buffer (pH = 7.0) in the presence of 50 g/L polyvinylpyrrolidone (ICN Biomedicals, Inc. OH) with a commercial mixer and centrifuged at 12,700 × g for 30 min. The supernatant, which contained PPO activity, was used as the experiment enzyme source (PPO crude vegetable extract). Crude extract was maintained at 0 °C until use. The reaction cuvette contained 2.9 mL of substrate (concentrations range from 2 to 40 mmol/L) mixture and 0.1 mL PPO crude vegetable extract. The enzyme activity was defined as a 0.001 change in absorbance between 0 and 60 s under the assay conditions, according to previous experiments. Each solution was tested in triplicate. The reference cuvette contained distillated water.

2.3. Kinetic data analysis and substrate specificity

The specificity of radish PPO extract was investigated for nine commercial grade substrates (pyrocatechol, gallic acid, chlorogenic acid, caffeic acid, 3,4 dihydroxybenzoic acid, p-cumaric acid, L-tyrosine, pyrogallic acid and phloroglucinol) at different concentrations. PPO activity was assayed in triplicate. The activity of PPO extract as a function of the substrates concentration was investigated in order to determine the enzyme kinetics. Michaelis–Menten constant (K_m) and maximum rate for the enzymatic reaction (V_{max}) were determined by means of Lineweaver–Burk method (Erat, Sakiroglu, & Kufrevioglu, 2006).

2.4. Effect of pH on enzyme activity

The activity of PPO was measured at room temperature in 0.1 mol/L acetic acid/0.1 mol/L sodium acetate in the pH range of 3.0–6.0, in 0.1 mol/L disodium hydrogen phosphate/0.1 mol/L hydrochloric acid in the pH range of 7.0–9.0 and also in 0.1 mol/L disodium hydrogen phosphate/0.1 mol/L sodium hydroxide in the pH range of 10.0–11.0. The optimum pH for the PPO was obtained using three substrates: 28 mmol/L pyrocatechol, 4 mmol/L gallic acid and 6 mmol/L pyrogallic acid. The pH value corresponding to the highest enzyme activity was taken as the optimal pH and the enzyme activity was expressed as the percentage of maximum activity speed at 25 °C.

2.5. Effect of temperature on enzyme activity

The temperature effect on the activity of radish PPO was investigated by equilibrating the substrate in a water bath (0–70 °C, at intervals of 10 °C) for 10 min before introducing the enzyme at a pH = 7. The optimum temperature for the substrates was obtained using three of them: 28 mmol/L pyrocatechol, 4 mmol/L gallic acid and 6 mmol/L pyrogallic acid. The enzyme activity was expressed as the percentage of maximum activity speed.

2.6. Thermal stability

The thermal stability of radish was investigated at optimal substrate pH, at intervals of 10 °C, from 0 to 80 °C using an incubation time of 10 min. The remaining activity of PPO was measured under the standard conditions ($T = 30$ °C). Relative PPO activity was measured using the K_m concentration of each substrate. The enzyme activity was expressed as the percentage of maximum activity speed.

2.7. Kinetics analysis of enzyme inactivation

The fist order biphasic model proposed by Fante and Zapata Noreña (2012) was used to describe the kinetics of the heat inactivation of the PPO. The mathematical expression of the model is:

$$RA = a_L \exp(-k_1 * t) + b_R \exp(-k_2 * t) \quad (1)$$

Where RA represents the value of the residual enzyme activity, k_1 and k_2 are the velocity constants of the heat labile and heat resistant components, respectively, a_L and b_R are the initial concentrations of the labile and resistant components, respectively, and t is the immersion time.

The dependence of the rate constants with temperature was assumed to follow the Arrhenius Law (Jakób et al., 2010):

$$k = k_0 \exp\left(\frac{-E_a}{RT}\right) \quad (2)$$

Where E_a is the activation energy, k_0 is the pre-exponential factor, and T is the absolute temperature.

2.8. Estimation of model parameters

Model parameters of biphasic model were estimated from the mean experimental values for each set of experimental conditions using nonlinear least-squares routines applying the function *lsqcurvefit* of the program Matlab 7.7.

2.9. Statistical analysis

Experiments were performed in triplicate. Values are expressed as means ± standard deviations. One way ANOVA (at the level of significance $P < 0.05$) was performed to ascertain the significance of the means. Statistical analysis was performed using SAS program (software version 8.0, SAS 1999).

3. Results and discussion

3.1. Substrate specificity

Phenolic compounds are the primary substrates of PPO (Yoruk & Marshall, 2003). Radish PPO showed activity with monophenolic substrate (L-tyrosine), diphenols (caffeic acid, pyrocatechol) and polyphenolics (chlorogenic acid, gallic acid, pyrogallic acid) (Table 1). p-cumaric acid (monophenol), 3,4-dihidroxibenzoic acid (diphenol) and phloroglucinol (triphenol substrate), showed no specificity for the enzyme. Probably with these last substrates the spatial orientation of the hydroxyl groups prevents enzyme and

Table 1
Values for K_m and V_{max} of radish PPO for different substrates.

Substrate	K_m (mmol/L)	V_{max} (UA/min ml)	V_{max}/K_m (UA/mmol/L min ml)	Wavelength (nm)
Gallic acid	4.2	233	55	350
Pyrogallic acid	6.3	4348	690	334
Chlorogenic acid	7.2	302	42	420
L-tyrosine	9.3	495	53	420
Pyrocatechol	28.3	1587	56	420
Caffeic acid	77.0	1695	22	400

substrate approximation. The cited literature indicates that the type and degree of inhibition of PPO activity depended on the structure of the substrate leading to varied interactions between the enzyme active site and the substrate (Kanade, Suhas, Chandra, & Gorda, 2007).

Similar results for phloroglucinol acid were reported when studying specificity of substrates in sour cherries (Jia et al., 2011). Andi et al. (2011) reported that purified Japanese radish root PPO oxidized phloroglucinol, pyrogallic acid and gallic acid, but the enzyme did not oxidize catechol or chlorogenic acid. Gawlik-Dziki, Szymanowska, and Baraniak (2007) analyzing the PPO of broccoli florets did not find activity towards monophenols (tyrosine) and low activity – towards trihydroxyphenol–phloroglucinol. In general, polyphenol oxidase isolated from fruits and vegetables is most active towards mono- and diphenols. 4-Methyl catechol and catechol are often chosen as substrates for determining the activity of polyphenol oxidase isolated from foods derived from plants.

3.2. Kinetics parameters

Enzyme kinetics parameters were calculated from the Lineweaver–Burk graphs (Fig. 1) for the six substrates that showed activity with the enzyme. The correlation coefficients were for pyrocatechol ($r^2 = 0.860$, $n = 9$), gallic acid ($r^2 = 0.639$, $n = 13$), chlorogenic acid ($r^2 = 0.888$, $n = 11$), caffeic acid ($r^2 = 0.994$, $n = 10$), pyrogallic acid ($r^2 = 0.903$, $n = 11$) and L-tyrosine ($r^2 = 0.893$, $n = 6$). K_m and V_{max} values for the mentioned substrates are presented in Table 1. Assuming a stable pH, temperature, and redox state, the K_m for a given enzyme is constant, and this parameter provides an indication of the binding strength of that enzyme to its substrate. Moreover, a low K_m indicates a higher affinity for the substrate. The V_{max} is the maximum velocity as the total amount of enzyme participates in the reaction. The measurement is theoretical because at given time, it would require all enzyme molecules to be tightly bound to their substrates (Bisswanger, 2002).

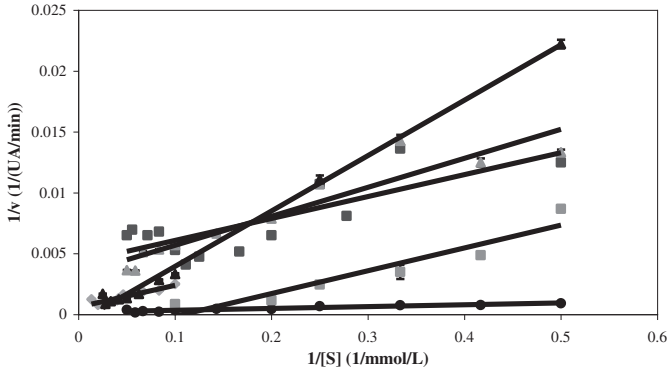

Fig. 1. Substrates specificities of radish PPO analyzed by a Lineweaver–Burke plot. Values are mean ± s.d., $n = 3$ ◆, Pyrocatechol; ■, Gallic acid; ▲, Chlorogenic acid;▲, Caffeic acid; ●, Pyrogallic acid; ■, L-tyrosine.

As seen in Table 1, the radish PPO had a great affinity for gallic acid (4.2 mmol/L), followed by pyrogallic, chlorogenic and L-tyrosine (6.3, 7.2 and 9.3 mmol/L, respectively). These phenols have also been shown to be the preferred substrate of PPO in a variety of foods such as grape (Rapeanu, Van Loey, Smout, & Hendrickx, 2006) and tomato (Spagna et al., 2005). Pyrocatechol and caffeic acid presented the highest K_m. However, pyrocatechol has been reported to act as substrate of PPO in potatoes and apples (Pereira Goulart, Donizeti Alves, Murad Magalhães, Luiz Carlos de Oliveira Lima, & Evangelista Meyer, 2003).

These results are comparable to the values of K_m reported by the available literature for the PPO of several vegetables. The K_m values obtained for PPO towards catechol from various plant sources were: 3.13 mmol/L from spinach, 10.5 mmol/L from beans, 4 mmol/L from artichoke and 18 mmol/L from thyme (Gawlik-Dziki, Złotek, & Swieca, 2008).

As seen in Table 1, the maximum reaction rate (V_{max}) value was 4348 UA/ml min for radish PPO with pyrogallic acid as substrate. Regarding pyrocatechol and caffeic acid, V_{max} within the same order were found, although they were 2.5–2.7 times lower respect pyrogallic acid V_{max}. This parameter depends on the structure of enzyme itself and the concentration of enzyme present, so for gallic acid, chlorogenic acid and L-tyrosine substrates, more enzyme was probably needed to achieve V_{max} values in the same order as the others substrates.

V_{max}/K_m ratio was taken as the criterion to evaluate substrates specificity (Altunkaya & Gokmen, 2008). Based in this criterion, the three substrates that shown the higher ratio were selected to analyze the effect of pH, temperature and thermal stability of the radish PPO activity: pyrocatechol (28 mmol/L), gallic acid (4 mmol/L) and pyrogallic acid (6 mmol/L). Moreover, gallic and pyrogallic acids are the substrates with the best PPO affinity and pyrocatechol is the more frequently substrate utilized for PPO measurement in different vegetables.

3.3. Effect of pH on PPO activity

The activity of radish PPO was measured at different pH, ranging from 3 to 11. Fig. 2 shows the influence of pH on radish PPO for the three tested substrates.

Differences in PPO pH optimum with various substrates were reported (Yoruk & Marshall, 2003) varying from 4.0 to 7.0, depending on the origin of the material, extraction method, and substrate. For the three substrates assayed the optimum pH for radish PPO was found to be 7.0 (Fig. 2). In general, most plants show maximum PPO activity at or near neutral pH values. However while using pyrocatechol and pyrogallic acid the maximal activity was obtained at pH 7.0, using gallic acid as substrate the PPO activity remains relatively high at pH in the range of 7–11. For longan (*Dimocarpus longan* Lour.) a subtropical fruit, the pH stability of PPO increased from pH 4.0 to 7.0, and then decreased from 7.0 to 8.0 (Yue-Ming, 1999). However, for broccoli florets, the optimal pH of phenol oxidase was found to be pH 5.72 for both catechol and methyl catechol substrates (Gawlik-Dziki et al., 2007). The common pH range for optimal grape PPO activity, as well as other fruits, is known to be pH 5.0–7.0 (Rapeanu et al., 2006). At acid pH (2.5–4), grape PPO still remained active (70% at pH 3.5).

Furthermore, below pH = 8, an important decreased of the enzyme activity was evidence when pyrocathecol and pyrogallic acid were the substrates. These results could be related to the residual proteins presented in the enzyme extract that might have formed insoluble complexes with the reaction products, namely, oxidized catechol (quinone polymers). Because increased exposures of hydrophobic groups are expected for proteins dissolved in alkaline solutions, an enhanced hydrophobic protein–polyphenol

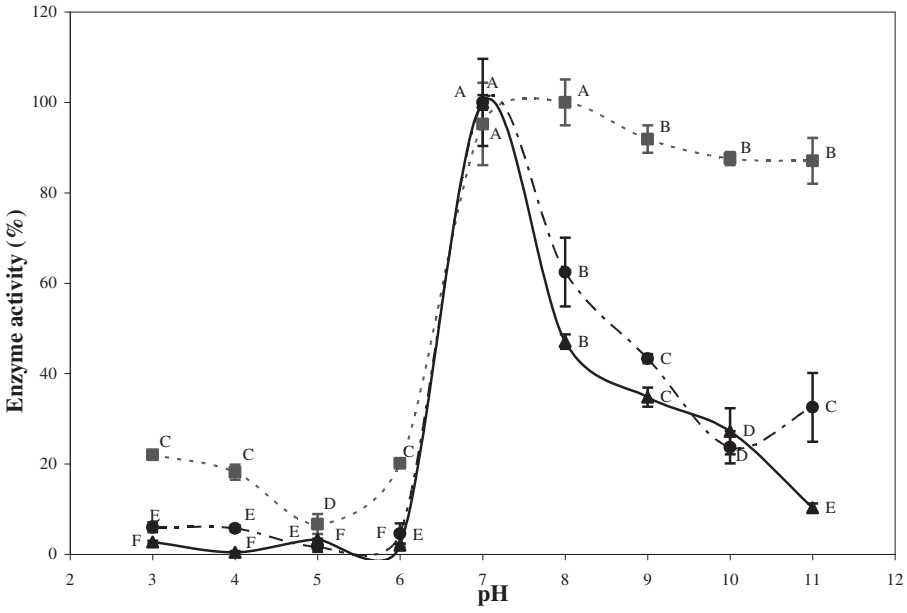

Fig. 2. Activity of radish PPO extract as a function of pH for: 6 mmol/L pyrocatechol (●), 4 mmol/L gallic acid (■) and 28 mmol/L pyrogallic acid (▲), 25 °C. Values are mean ± s.d. Values with the same letter are not significantly different ($P < 0.05$), $n = 3$.

association, could led to denaturation of the enzyme (Fang, Wang, Xiong, & Pomper, 2006). Moreover, the changes in ionization of prototropic groups in the active site of an enzyme at lower acid and higher alkali pH values may prevent proper conformation of the active site, binding of substrates, and/or catalysis of the reaction. Kinetic behavior of PPO was reported to alter depending on the pH of the assay due to pH-induced conformational changes in the enzyme (Yoruk & Marshall, 2003).

3.4. Effect of temperature on PPO activity

Thermal activity of radish PPO is presented in Fig. 3. The optimum temperature for radish PPO was dependent on substrate used. While for pyrogallic acid was 20 °C, for gallic acid was 60 °C and for pyrocatechol was in the range 20–40 °C. The optimum temperature for mulberry PPO activity has been found regarding to vary the substrate of the enzyme. Whereas the optimum temperature of enzyme for 4-methyl catechol and pyrogallol oxidation was 20 °C, for catechol it was 45 °C (Arslan, Erzengin, Sinan, & Ozensoy, 2004). Variations in optimal temperature for fruit PPO activity ranging from 18 °C to 37 °C have been reported by other authors (Ayaz, Demir, Torun, Kolcuoglu & Colak, 2008).

For pyrocatechol and pyrogallic acid, high temperatures (60–70 °C) lead to almost 80% loss of the enzyme activity, indicating that these temperatures provoke denaturation of the enzyme resulting in irreversible conformational changes that affect its functional activity. This was consistent with reported temperatures for PPO activities in Concord grapes (25–30 °C) (Rapeanu et al., 2006).

3.5. Thermal stability of PPO

The thermal stability profile of radish PPO, presented as residual activity after preincubation for 10 min at the specified temperature and the optimum pH, is shown in Fig. 4. The highest enzyme stability

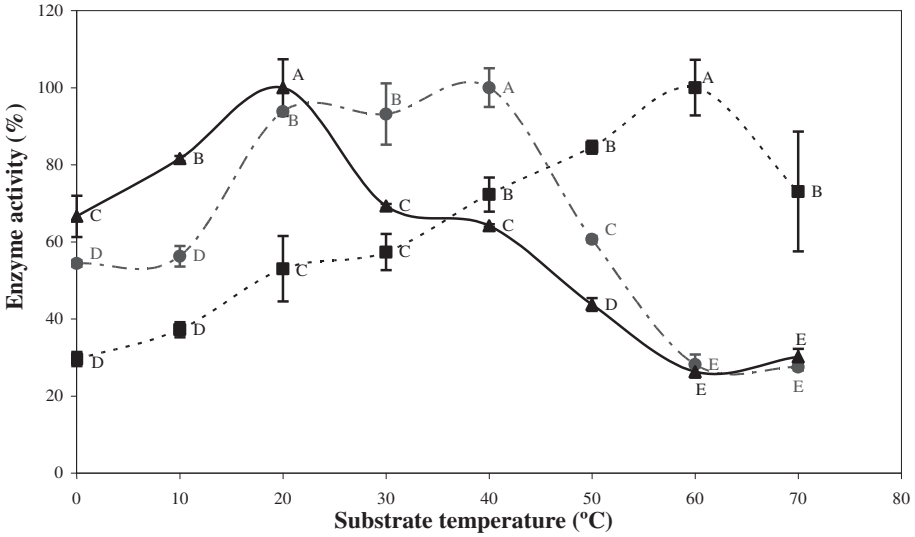

Fig. 3. Activity of radish PPO extract as a function of substrate temperature for: 28 mmol/L pyrocatechol (●), 4 mmol/L gallic acid (■) and 6 mmol/L pyrogallic acid (▲), pH = 7. Values are mean ± s.d. Values with the same letter are not significantly different ($P < 0.05$), $n = 3$.

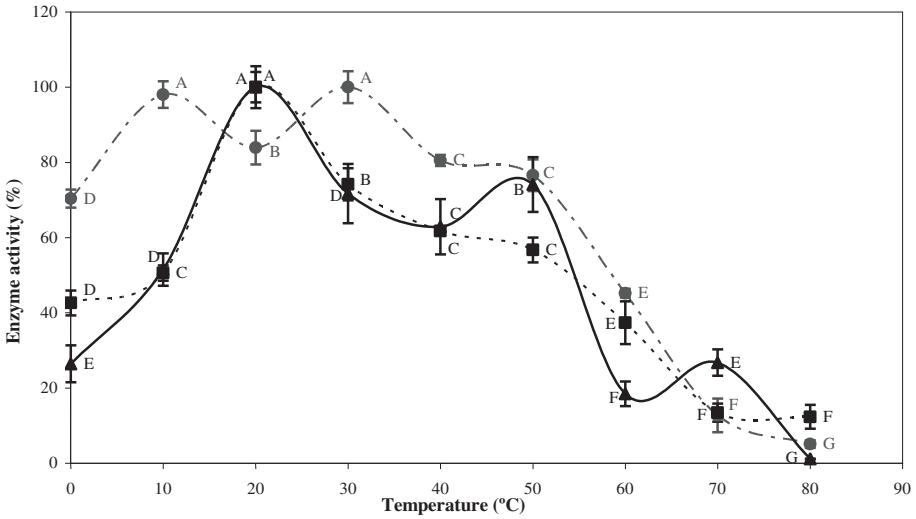

Fig. 4. Activity of radish PPO extract as a function of enzyme incubation temperature for: 28 mmol/L pyrocatechol (●), 4 mmol/L gallic acid (■), and 6 mmol/L pyrogallic acid (▲), pH = 7, substrate temperature = 30 °C; Values are mean ± s.d. Values with the same letter are not significantly different ($P < 0.05$), $n = 3$.

Table 2
Kinetics parameters for the inactivation of radish PPO.

Temperature (°C)	K_1 (1/min)	K_2 (1/min)	r^2
60	3.687e-13	0.4504	0.888
70	0.0402	1.5082	0.967
80	0.1351	14.0336	0.942
90	0.7630	24.3297	0.976

was found using pyrocatechol as substrate, with relatively high activities in the temperature ranges 10–50 °C, with retentions higher than 70%. The stability profiles obtained using gallic and pyrogallic acids were similar, with the higher activity retentions (60–100%) in the range 20–40 °C. With preincubations of 10 min at 50 °C the activity loss was 40%, while at 70 °C the losses reached 80% for all the substrates assayed. In general, exposure of PPO to temperatures of 70–90 °C destroys their catalytic activity, but the time required to inactivation depends on the product (Queiroz et al., 2008).

Looking for a mild heat shock as a physical technology to reduce the browning of radish slices, temperatures higher that 70 °C may be used to account a significantly PPO loss. However, these temperatures are highly enough to produce significant texture loss of the radish slices. Moreover, during blanching several thermolabile compounds, such as phenolics, may lose their activity due to oxidation or diffusion (or leaching) into water during blanching. Therefore, the retention of texture and phenolics compounds during blanching could be a reliable indicator for evaluation of radish quality (Lin et al., 2012). To solve this, hurdle technologies should be used, the sum of the enzyme inhibiting barriers could allow the use of thermal shock at lower temperatures without affecting product texture. Therefore, thermal inactivation of PPO as well as the addition of antibrowning agents is required to minimize nutritional and sensory qualities losses of product caused by browning.

3.6. Kinetics analysis of enzyme inactivation

The first order biphasic model to describe the kinetics of heat inactivation of enzymes consists of the separation into two different groups with respect to their heat stability, one component being heat labile and the other heat resistant (Fante and Zapata Noreña, 2012). The labile fraction and resistant fraction were 0.4691 and 0.5302, respectively. Fitting the data of inactivation of PPO achieved success by using a biphasic model. Table 2 shows the kinetics parameters of the enzyme at the different temperatures. It can be seen that the

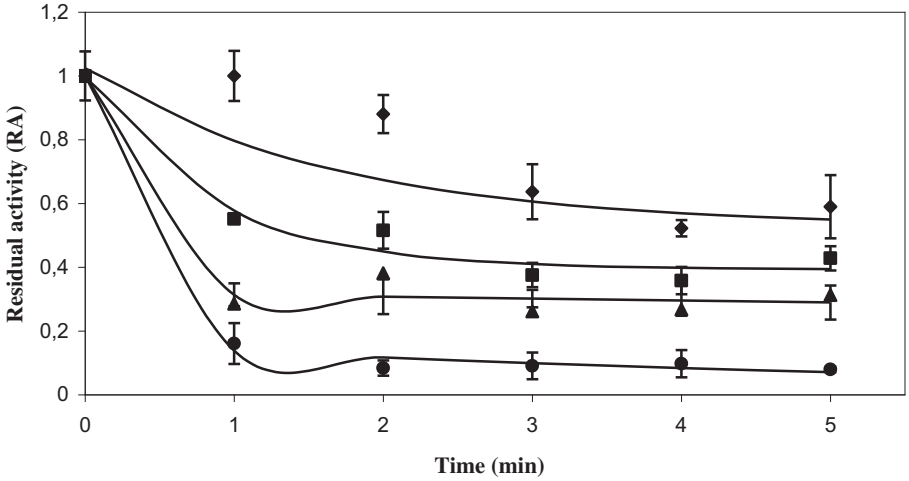

Fig. 5. Loss of activity for the radish PPO as function of blanching time and temperature ($n = 3$). Values are mean ± s.d., $n = 3$; ◆, 60 °C; ■, 70 °C; ▲, 80 °C; ●, 90 °C; –, Eq. (1).

reaction velocity constants increased with increased temperature for both the labile and the heat resistant components. Comparable results were reported in previous investigations of inactivation of PPO of apples (70–80 °C) and PPO of garlic (80–100 °C) (Fante and Zapata Noreña, 2012; Zhu, Pan, McHugh, & Barrett, 2010). The activation energy ratio of resistant to labile fraction was found to be 6 (E_{aL} = 142 kJ/mol). Fante and Zapata Noreña (2012) reported activation energies of 67.40 and 202.81 kJ/mol of garlic PPO for the resistant and labile form, respectively.

The residual PPO activities against blanching time at different processing temperatures are presented in Fig. 5. It can be appreciated that the rate at which PPO inactivates depends on temperature and that it increased with increasing temperatures. The residual activity of PPO decreased with time, decreasing rapidly in the first minutes, and then decreasing slowly up to 5 min of blanching. Working at 90 °C produces an enzyme inactivation of 90% after 2 min of thermal treatment. Although the residual activity presents a sharp initial decreased, it does not fall to zero and persists over time, even at 90 °C. The two clear zones of the curves of Fig. 5 for each temperature could be attribute to the presence of two isoenzymes, a heat-labile fraction and a heat-resistant fraction that requires more aggressive conditions to be inactivated (Agüero, Ansorena, Roura, & del Valle, 2008). Comparable results were reported for PPO from garlic (Fante and Zapata Noreña, 2012); butternut squash (Agüero et al., 2008) and pineapple puree (Chutintrasri & Noomhorm, 2006).

4. Conclusions

Different substrates specificities on the activity of PPO in sliced radish were analyzed. Based on enzyme kinetics which was performed by means of the model of Lineweaver–Burk, the kinetics parameters (V_{max}/K_m) were determined for the substrates under study. Pyrocatechol, gallic acid and pyrogallic acids showed the higher ratio (V_{max}/K_m). The optimum pH for the PPO using the mentioned three substrates was pH = 7 and the optimum temperatures were 20, 60 and 20–40 °C for pyrogallic acid, gallic acid and pyrocatechol, respectively. Regarding the thermal stability of PPO, temperatures higher that 70 °C may be used to account a significantly PPO loss. Although, blanching treatments at 90 °C for 2 min inactivated more than 90% of initial PPO, significant texture loss of the radish slices was observed. To solve this, hurdle technologies should be used to apply thermal shock at lower temperatures without affecting product firmness but reducing the PPO activity. Therefore, thermal inactivation of PPO as well as the addition of antibrowning agents is required to minimize nutritional and sensory qualities of sliced radishes caused by browning in order to increase the consumer's acceptability and therefore causes significant economic impact, both to food producers and to food processing industry.

Acknowledgments

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