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PPO activity of two varieties of pears. Control of enzymatic browning for temperature effect, presence of inhibitors and complexation with β-cyclodextrin

Actividad de PPO de dos variedades de pera. Control del pardeamiento enzimático por efecto de la temperatura, la presencia de inhibidores y la complejación con β-ciclodextrina

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Abstract

Polyphenoloxidase (PPO) is an enzyme that produces enzymatic browning, which causes deterioration of quality, both in fresh fruits and vegetables and on their products, such as juices, jams, syrup fruits, among others. This results in numerous losses in the industry. For this reason, the characterization and inhibition of this enzyme are very important. In this work, PPO from two types of pears (*Pyrus communis* L): Aranjuez White ($_A$PPO) and Williams ($_W$PPO) were characterized. The optimum pH and temperature for enzyme activity were determined ($_A$PPO: 7 and 30ºC; $_W$PPO: 7.5 and 24.5ºC). Both enzymes are heat-labile enzymes and exhibited similar behavior against temperature. The enzymes analyzed showed a higher affinity for 4-methylcatechol than for catechol. The inhibition of PPO through ascorbic acid, 4-hexylresorcinol, sodium isoascorbate and citric acid was determined. Additionally, the effect of β-Cyclodextrin was evaluated as preventive agent of fruit oxidation. This compound forms an inclusion complex with the substrates. The binding constant value of the complex β-Cyclodextrin with 4-tert-butylcatechol was 16888 M^{-1}.

Keywords: polyphenoloxidase, chemical inhibition, β-Cyclodextrin, inclusion complex.

Resumen

La Polifenoloxidasa (PPO) es una enzima que provoca el pardeamiento enzimático, el cual causa el deterioro de la calidad en frutas y vegetales frescos y en sus productos, tales como jugos, mermeladas, jarabes y otros. Esto da como resultado numerosas pérdidas en la industria. Por esta razón, la caracterización e inhibición de esta enzima son muy importantes. En este trabajo se caracterizaron las PPO de dos tipos de peras (*Pyrus communis* L): Blanca de Aranjuez ($_A$PPO) y Williams ($_W$PPO). Se determinaron el pH y la temperatura óptimos de la actividad enzimática ($_A$PPO: 7 y 30ºC; $_W$PPO: 7.5 y 24.5ºC). Ambas enzimas son termolábiles y presentan un comportamiento similar frente a la temperatura. Las enzimas analizadas mostraron una mayor afinidad por el 4-metilcatecol que por el catecol. Se determinó la inhibición de las PPO a través de ácido ascórbico, 4-hexilresorcinol, isoascorbato de sodio y ácido cítrico. Asimismo, se evaluó el efecto de β-Ciclodextrina como agente preventivo de la oxidación de la fruta. Este compuesto forma un complejo de inclusión con los sustratos. El valor de la constante de formación del complejo β-Ciclodextrina con 4-terbutil catecol fue 16888 M^{-1}.

Palabras clave: polifenoloxidasa, inhibición química, β-Ciclodextrina, complejo de inclusión.

1 Introduction

The loss of quality in fruits and vegetables has numerous well-known causes. One of them is enzymatic browning. Huge economic losses in fruit and vegetable industries are attributed to this. The browning is catalyzed by enzymes with polyphenol oxidase activity (PPO, E.C.: 1.14.18.1), which has been widely studied (Liu *et al*., 2013; Gómez-López 2002; Sulaiman and Silva, 2013).

PPO is a bifunctional copper-containing enzyme that catalyzes the oxidation of phenolic compounds in the presence of molecular oxygen. The oxidation of phenolics develops the browning which appears in the postharvest, processing and storage of foods (Ayaz *et al*., 2008; Brandelli and Lopes, 2005; Robles-Ozuna *et al*,. 2007).

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The characterization of PPO has been studied in numerous fruits and vegetables: taros, potatoes, avocados, apples, pears, artichokes, lychees, medlars, peaches, sunflowers, bananas, mangos, among others (Aydemir, 2004; Cheema and Sommerhalter, 2015; Carbonaro and Mattera, 2001; Duangmal and Owusu Apenten, 1999; Gómez-López, 2002; Rocha and Morais, 2001; Singh *et al*., 1999; Sun *et al*., 2008; Singh *et al*., 2018). The location of PPO in the plant cell and its physicochemical properties depend on species, cultivar, maturity and the specific phenological stage of the plant. For this reason, there are variations in the enzymatic activity, even among similar fruits (Gómez-López, 2002). Thus, the characterization becomes necessary in order to avoid its undesirable effects (enzymatic browning).

Previous research has assayed different compounds as inhibitors of PPO activity: sodium metabisulphite, sodium azide, ascorbic acid, EDTA, tartaric acid, citric acid, oxalacetic acid, glutathione, cysteine, kojic acid, substituted benzaldehydes, among others (Arias *et al*., 2011; Ayaz *et al*., 2008; Brandelli and Lopes, 2005; Jiménez *et al*., 2001; Son *et al*., 2000). Furthermore, some technologies, such as pulse electric fields, temperature, microwave, and ultraviolet irradiation, have also been used to control and inhibit PPO activity in food (Castorena-García *et al*., 2013, Falguera *et al*., 2012; Matsui *et al*., 2008; Riener *et al*., 2008).

Other type of compounds that have inhibitory effects on PPO activity are the cyclodextrins (CDs) which are natural cyclic oligomers built up from 6, 7 or 8 glucopyranose units (named α-, β- and γ-Cyclodextrin, respectively), linked by α-(1-4)-glycosidic bonds. In recent years, the use of CDs in food processing and food additives has notably increased, especially to: a) solubilize colorants, vitamins and flavorings; b) be a protecting agent for lipid components susceptible to degradation; c) suppress unpleasant odors and flavors; d) stabilize fragrances, flavorings and essential oils against unwanted changes; and e) release certain constituents of the foods (Cravotto *et al*. 2006; Szente and Szejtli 2004). Another beneficial effect of CDs on food is the reduction of enzymatic browning in juices or in fresh fruits and vegetables (López-Nicolás *et al*., 2007; López-Nicolás and García-Carmona, 2007; Fuentes Campo *et al*., 2019). These beneficial effects of CDs on foods are achieved through the formation of inclusion complexes with food components. The internal cavity of the CDs is hydrophobic, so they can encapsulate molecules of appropriate size and shape that are poorly soluble in water by means of non-covalent type driving forces. These polysaccharides are harmless, and the World Health Organization has recommended a maximum level of βCD in foods of 5 mg/kg per day (Astray *et al*., 2009).

Williams and Aranjuez White pear varieties are easily grown in Argentina. According to official data, about 550,000 to 680,000 tons of Williams pear are grown per year, out of which 35-40% is exported (Benitez, 2001; Toranzo, 2016). The Aranjuez White variety is grown in smaller quantities and is mainly intended for the internal market. The study of both pear varieties is important because of the relevance of this crop for the food industry. In addition, although the PPO has been widely studied in a considerable variety of fruits, the properties of this enzyme have not been thoroughly studied for Aranjuez White and Williams pears. In this work, the characterization of PPO from Aranjuez White and Williams pears was carried out. Several physicochemical and kinetic characteristics were evaluated to understand the behavior of the enzyme in pears. Moreover, the effect of βCD on PPO activity was assessed, and the numerical value of the formation constant of βCD-substrate complex was determined from the decrease in enzymatic activity by the presence of βCD. In the same way, the value of this constant was determined from a phase solubility diagram.

2 Materials and methods

2.1 Materials

The whole fruits of two pear cultivars (*Pyrus communis* L.: Aranjuez White (A) and Williams (W) were obtained at commercial maturity from a local market (San Luis, Argentina) and were stored at 4ºC for 24 h until used. The substrates catechol (C), 4-methylcatechol (MC) and, 4-tert-butylcatechol (TBC), like the inhibitors 4-hexylresorcinol (HR), ascorbic acid (AA), citric acid (CA), and sodium isoascorbate (IA) were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Anhydrous β-Cyclodextrin (βCD) was purchased from MP Biomedicals. The non-ionic tensoactive Triton X-100 was purchased from Fluka Chemicals Co. (Buchs, Switzerland). All other chemicals were of analytical grade.

2.2 Methods

The PPO extracts from Aranjuez White ($_A$PPO) and Williams ($_W$PPO) pears were obtained using the procedure described by Gauillard and Richard-Forget with some modifications (Garro and Gasull, 2010). Peeled crude samples (100 g) were homogenized for 2 min in an Ultracomb commercial blender (Buenos Aires, Argentina) in 100 mL of 50 mM sodium phosphate buffer at pH 6.5, containing 20 mM AA, 2% (v/v), ethylenglycol, and 1% (v/v) Triton X-100. Polyethylenglycol was used to bind phenols which could inactivate PPO activity during the extraction (Erat *et al*., 2006), while Triton X-100, a non-ionic surfactant, was used to achieve the full extraction of the enzyme (Sikora *et al*., 2019). After filtration of the homogenate through gauze, the filtrate was centrifuged at 30100 xg for 30 min in a Beckman Coulter J2-HS ultracentrifuge (Fullerton, California, USA). All steps were carried out at 4ºC. The supernatants were recovered and kept in tubes at -20ºC and were used as crude enzyme extracts which retained PPO activity for about 6 months.

2.3 PPO activity assay

PPO activity was assayed by a spectrophotometric procedure using a Cary 50 UV-Vis (Varian Inc., USA) double beam spectrophotometer equipped with a quartz cell of 10 mm path length, with Single Cell Peltier accessory for temperature control. Enzymatic activity was assayed by measuring the rate of increase in absorbance at 25ºC. The reaction mixture contained 3 mL of sodium phosphate buffer solution (50 mM, pH 7), 100 μL of enzyme extract and the substrate. The reference cuvette contained only buffer solution and the substrate. The straight-line section of the absorbance curve as a function of time was used to determine enzymatic activity. One unit of PPO activity (UE) was defined as the amount of enzyme that causes an increase in absorbance of 0.001/min at 420 nm per mL of enzyme assay solution mixture (Lim and Wong, 2018). All determinations were performed in triplicate.

2.4 pH effect on PPO activity

The enzymatic activity was tested using different buffer solutions in the pH range of 4 to 9, with intervals of 0.5 pH units. PPO activity was assayed using 50 μL of C and MC (1000 mM) as substrate and 100 μL of crude enzyme extract in 3 mL buffer solutions. These were tested in the pH range 4-5.5 in 50 mM acetate buffer, 6-7.5 in 50 mM phosphate buffer and 8-9.5 in 50 mM THAM buffer (tris(hydroxymethyl)aminomethane-HCl). PPO activity was determined by measuring absorbance at the maximum wavelength of the product (420 nm). All determinations were performed in triplicate.

2.5 Enzyme kinetics and substrate specificity

$_A$PPO activity was assayed using C (125.5 mM), MC (1.601 mM) and TBC (1.039 mM) in buffer at optimum pH values, while $_W$PPO was assayed using substrates C (1.504 mM) and MC (1.484 mM). The reaction rate was measured in terms of the increase in absorbance at the wavelength of maximum absorption for the corresponding products (λ = 420 nm). The assays were carried out in cuvettes (3 mL) containing the buffer solution at optimum pH, 100 μL of the crude enzyme extract and different amounts (10-150 μL) of the substrate solution. For each substrate, the kinetic data were plotted as 1/activity versus 1/substrate concentration, according to the Lineweaver-Burk method (Leskovac, 2003). Michaelis-Menten constant (K_M) and maximum velocity ($V_{\max}$) were determined from the linear regression curve. Substrate specificity ($V_{\max}/K_M$) was calculated using the previously obtained data on a Lineweaver-Burk plot. All the determinations were performed in triplicate.

2.6 Evaluation of thermal activity

PPO activity (at optimum pH values) was measured at different temperatures in a range of 10-50ºC using a circulation water bath. Before the addition of 100 μL of the enzyme solution, buffer solutions (3 mL) and substrate MC ($_A$PPO, 15.4 mM; $_W$PPO, 37.8 mM) and C ($_A$PPO, 16 mM; $_W$PPO, 35.2 mM) were kept between 10-50ºC for 15 min. All assays were performed in triplicate. The data obtained from the thermal activity have been used to analyze some thermodynamic parameters related to $_A$PPO and $_W$PPO activities in the crude enzyme extracts.

2.7 Thermal stability

Thermal stability of PPO extract was measured at optimum pH values. The solution was kept in a circulation water bath at constant temperatures: 25, 30, 35, 40, 45, 50 and 55ºC for different times lapses (5-30 min). After the mixture was cooled in an ice bath and brought to 25ºC, 3 mL of the

treated enzyme was mixed with approx. 0.1 mL of C 1M (32.9 mM) and approx. 0.05 mL of MC 1 M (15.8 mM), and residual PPO activity was determined spectrophotometrically. All solutions were prepared by weighing. The percentage of residual PPO activity was calculated by comparison with the unheated enzyme (Simas-Dias *et al*., 2018).

2.8 Inhibition of PPO activity

HR (0.015-1.10 mM), CA (3.10-21.5 mM), AA (0.620-6.400 mM) and IA (1.60-6.40 mM) were examined for their effectiveness as inhibitors of $_A$PPO and $_W$PPO using MC 1500 mM as substrate. Experiments were carried out using 3 mL of reaction mixture containing the buffer solution at optimum pH, crude enzyme extract (100 μL), different amounts (10-50 μL) of MC and variable volumes (μL) of the inhibitor at the concentrations detailed above (Ali *et al*., 2015).

2.9 Complexation of TBC with β-Cyclodextrin

The oxidation of TBC by $_A$PPO was followed spectrophotometrically at 420 nm (ε = 1150 M^{-1} cm^{-1}) in the presence of increasing concentrations (0-10 mM) of βCD at 25ºC. Experiments were performed for three concentrations of TBC (6.30, 9.54 and 12.58 mM) with 100 μL of crude enzyme extract (Orenes-Piñero *et al*., 2007).

The experimental determination of complex formation constant using the other substrates analyzed (C and MC) was not possible because the inclusion complexes with these substrates were not detected.

3 Results and discussion

3.1 Effects of pH: Stability and optimum pH

Figure 1 shows the pH versus activity profile for the enzymatic extract of the samples analyzed using MC as substrate. The curves obtained show that the $_A$PPO activity is the highest at pH 7, whereas $_W$PPO presents its maximum activity at pH 7.5. This is in agreement with the values reported by the literature, since the most usual pH range for the optimal PPO activity of pears is between 5 and 7 (Singh *et al*., 2018).

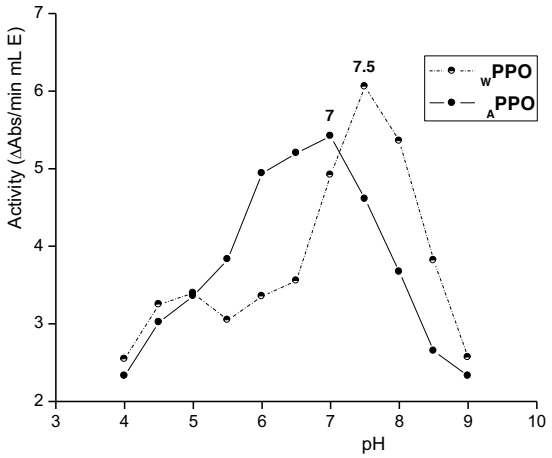

Fig. 1. pH activity profiles for Polyphenoloxidase from Aranjuez White ($_A$PPO) and Williams ($_W$PPO) pears (Substrate: 4-methylcatechol; T= 298 K). Buffer solutions: Acetate Buffer (pH 4 to pH 5.5); Phosphate Buffer (pH 6 to pH 7.5); THAM Buffer (pH 8 to pH 9).

It can also be observed that the PPO from both sources were inactive at pH lower than 4 and at alkaline pH greater than 9. The optimum pH may vary depending on some factors such as the enzyme source, maturity of the fruit, extraction method, temperature and substrate (Ziyan and Pekyardimic, 2004). In this case, we have used the same extraction procedure and the same substrates for both enzymes. Thus, the optimum pH values depend only on pear variety. The activity profile with respect to the incubation pH of the enzyme was assayed and the results were expressed as residual activity to the enzymatic activity at the optimum pH. According to the data obtained, the pH of highest enzyme stability is 6.5 for $_A$PPO and the range 6.5-7.5 for $_W$PPO. If the enzyme is at pH far from the optimum, its secondary and tertiary structure will be altered as a consequence of the protonation or deprotonation of the residues of the different amino acids that compose it. The consequence will be the unfolding or permanent (or irreversible) denaturation of the protein. If the environment in which the enzyme is found is not at an extreme pH, it can retract and return to its original conformation and activity, i.e. it can be renatured.

3.2 PPO activity and substrate specificity

V_{max} and K_M values for the substrates analyzed are listed in Table 1. The best substrate for each enzyme depends on two factors: strong substrate binding or high affinity (low K_M value) and high

Table 1. Kinetics and Thermodynamics Parameters of PPO from pears.

	Aranjuez White ($_A$PPO)		Williams ($_W$PPO)	
Substrate	C	MC	C	MC
pH_{Op}	7.0	7.0	7.5	7.5
K_M (mM)	24.34±2.23	17.04±0.05	41.37±4.17	29.69±1.29
V_{max} (UE)	13061±552	10889±12.0	9300±554	7116±104
SE (UE mM^{-1})	539.11±26.8	639.18±1.02	226.04±8.85	240.0±6.90
E_a (kJ mol^{-1})	11.8±0.7	7.1±0.5	18.1±0.1	19.1±1.1
A (UE)	593.7±185	78.62±14.3	16.29±6.85	16.94±9.01
T_{Op} (°C)	30.0	30.0	24.5	24.5
$\Delta H^{\neq}$ (kJ mol^{-1})	9.4±1.7	4.6±0.5	16.1±0.4	16.5±1.2
$\Delta S^{\neq}$(J K^{-1} mol^{-1})	-195±6	-217±2	-190±1	-172±4
$\Delta G^{\neq}_{323}$ (kJ mol^{-1})	72.3±0.2	74.7[a]	77.5[a]	72.2[a]

pH_{op}: Optimum pH; T_{op}: Optimum Temperature; K_M: Michaelis constant; V_{max}: Maximum Velocity; SE: Substrate Specificity; E_a: Activation Energy; $\Delta H^{\neq}$: Activation Enthalpy change; $\Delta S^{\neq}$: Activation Entropy change; $\Delta G^{\neq}_{323}$: Free Energy change. The error was calculated from the 95% confidence interval. [a] Values calculated with error <10%.

catalytic efficiency (high $V_{\max}$ value) for a fixed enzyme concentration. Thus, the criterion for the best substrate is the $V_{\max}/K_M$ ratio, known as substrate specificity (SE) that measures the enzyme efficiency against a given substrate (Rocha and Morais, 2001; Tuncay, 2011). According to the results obtained, kinetic parameters indicate that the two enzymes analyzed have higher affinity for MC than for C. This difference is more important for $_W$PPO than for $_A$PPO. The SE values listed in Table 1 coincide with those reported for "Jonagored" apples (Rocha and Morais, 2001), for taros and potatoes (Duangmal and Owusu Apenten, 1999), and for medlars (Dincer *et al*., 2002). The same behavior has been observed in our previous work (Garro and Gasull, 2010) for summerset peaches, while for September peaches the enzyme showed greater affinity for C. The same trend was observed in artichokes (Aydemir, 2004).

3.3 Evaluation of thermal activity

It is known that the temperature has a two-sided influence on enzymatic activity: the increase in temperature improves the enzymatic reaction rate, but it also causes denaturation of the enzyme.

The dependence of $V_{\max}$ with the temperature can be evaluated by the Arrhenius equation, presented in Eq. (1) (Martinez-Monteagudo, 2018):

$$\ln V_{\max} = \ln A - \frac{E_a}{RT} \qquad (1)$$

where: A is Arrhenius constant; E_a is activation energy (J/mol); R is the universal gas constant (8.314 J/K mol) and T is the absolute temperature in Kelvin (K).

Plotting $\ln V_{\max}$ versus $1/T$, the values of the Activation Energy (E_a) and the Frequency Factor (A) can be obtained from the slope and the intercept, respectively. For $_A$PPO and/or $_W$PPO, the plot showed no obvious deviation from linearity. The E_a values obtained for $_A$PPO are different (11.8 kJ mol^{-1} for C and 7.1 kJ mol^{-1} for MC), whereas for $_W$PPO they are very similar (18.1 kJ mol^{-1} for C and 19.1 kJ mol^{-1} for MC). The optimum temperature is 30ºC ($_A$PPO, Fig. 2) and 24.5ºC ($_W$PPO).

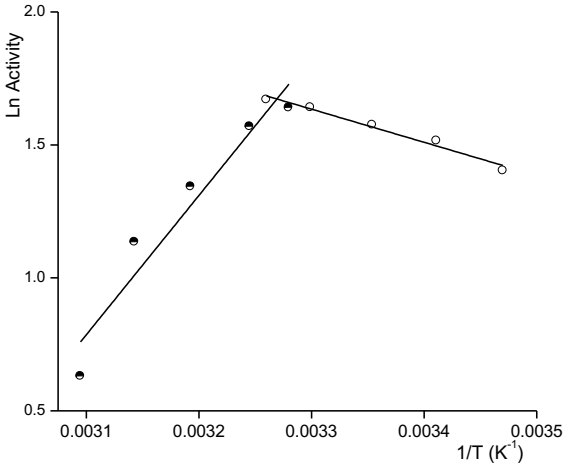

Fig. 2. Plot of ln Activity vs $1/T$ for Aranjuez White (APPO, Substrate: catechol).

Below and above these temperatures, enzymatic activity decreases gradually. This is consistent with the reported temperatures for PPO activities in lychees 35ºC (Sun *et al*., 2008) and medlars 30ºC (Ayaz *et al*., 2008), among others.

The activation parameters $\Delta S^{\neq}$ (entropy change) and $\Delta H^{\neq}$ (enthalpy change) were calculated using the Eyring equation, as shown in Eq. (2):

$$\ln\left(\frac{V_{\max}}{T}\right) = \ln\frac{k}{h}\exp\left(\frac{\Delta S^{\neq}}{R}\right) - \frac{\Delta H^{\neq}}{RT} \qquad (2)$$

where k (1.3806×10^{-23} J/K) is the Boltzmann constant, h (6.6261×10^{-34} J s) is known as the Planck constant, and R (8.314 J/K mol) is the universal gas constant. Results for these analyses, as well as E_a, A and free energy change $\Delta G^{\neq}$ for crude $_A$PPO and $_W$PPO are reported in Table 1.

It can be observed that, as expected, both enzymes behave in the same way against the two substrates analyzed. The values obtained from E_a and $\Delta H^{\neq}$ for $_W$PPO are significantly higher than those for $_A$PPO. Thus, we can conclude that $_W$PPO is more resistant to thermal inactivation. $\Delta H^{\neq}$ is considered as a measure of the number of noncovalent bonds broken in forming a transition state for enzyme inactivation. The positive values of $\Delta H^{\neq}$ indicate that PPO denaturation of both pears is an endothermic reaction. $\Delta S^{\neq}$ is a measure of the net enzyme and solvent disorder change accompanying transition state formation. The possible reason for negative $\Delta S^{\neq}$ is an increase in the order of the system through an aggregation process in which a few inter or intramolecular bonds are formed, achieving a certain ordering of the protein molecule. Taking into account the $\Delta S^{\neq}$ values, $_A$PPO is more heat-resistant than $_W$PPO. However, it can be observed that the differences are not significant. For this reason, we can conclude that $_W$PPO is more heat resistant than $_A$PPO. $\Delta G^{\neq}$ values (at 30ºC) are very similar for the two enzymes acting on the two substrates and are consistent with the characteristic value (100 kJ / mol) of protein denaturation reaction (Gouzi *et al*., 2012). In general, the activation parameter values are in agreement with those obtained in previous research (Dincer *et al*., 2002; Duangmal and Owusu Apenten, 1999).

3.4 Thermal stability

For $_W$PPO using MC and C as substrate, it was observed that the enzyme which is incubated in pH 7.5 buffer at 25ºC between 5 and 30 min retains its activity; whereas at 30ºC, the residual activity (RA%) decreases gradually as the temperature and the time of exposure to heat increase. The enzyme loses between 50 and 80% of its activity (Figure 3) when heated for 30 min. $_A$PPO behaves similarly under heating for the two substrates tested. Its residual activity was typically linear for all temperatures higher than 25°C when heated for up to 5 minutes. In the longest heating times, there is an apparent deviation from linearity. This deviation could indicate the presence of a second enzyme with greater thermal resistance. This is compatible with the fact that PPO is an isoenzyme. The values obtained for these two pears are within the ranges reported by other authors (Rapeanu *et al*., 2005; Dincer *et al*., 2002).

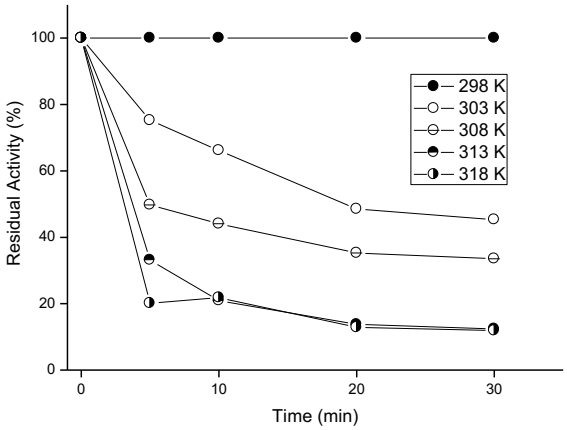

Fig. 3. Thermal stability of Polyphenoloxidase from Williams pear ($_W$PPO) (Substrate: 4-methylcatechol).

3.5 Inhibition of PPO activity

Various compounds were examined to determine their potential for inhibition of crude $_A$PPO and $_W$PPO activity, using MC as substrate. These inhibitors included analogous substrates and reducing agents. The type of inhibition produced by the compounds was identified applying linear regression analysis for the Lineweaver-Burk plots at different concentrations of substrates in the absence and presence of inhibitors. Subsequently, the representation of the slope and/or intercept against the inhibitor concentration used in each case allowed to obtain the numerical value of KI (dissociation constant of the enzyme-inhibitor complex). The KI values reported in Table 2 show that HR is more effective as an inhibitor of $_A$PPO and $_W$PPO (lower KI value) and presents uncompetitive inhibition. In addition, AC, AA and IA act as non-competitive inhibitors of $_A$PPO and as competitive inhibitors of $_W$PPO.

Table 2. Chemistry Inhibition of PPO of Aranjuez White and Williams pears.

	Aranjuez White ($_A$PPO)		Williams ($_W$PPO)	
Inhibitor	Type	K_I (mM)	Type	K_I (mM)
HR	UC	0.044±0.0014	UC	0.0104±0.011
AC	NC	77.26±2.490	C	0.1328±0.002
IA	NC	2.554±0.109	C	7.641±0.130
AA	NC	0.245±0.0043	C	0.044±0.0051

C: Competitive; UC: Uncompetitive; NC: No Competitive (Type Inhibition); HR: 4-hexylresorcinol; AA: ascorbic acid; CA: citric acid; IA: sodium isoascorbate. Substrate: 4-methylcatechol. The error was calculated from the 95% confidence interval.

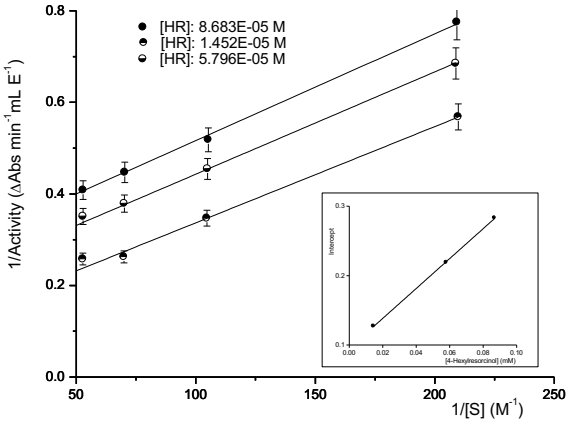

Fig. 4. Representation of Lineweaver-Burk (L-B) equation in the presence of HR as inhibitor; Substrate: 4-methylcatechol (Uncompetitive Inhibition). Inset: Intercept (L-B) vs HR concentration.

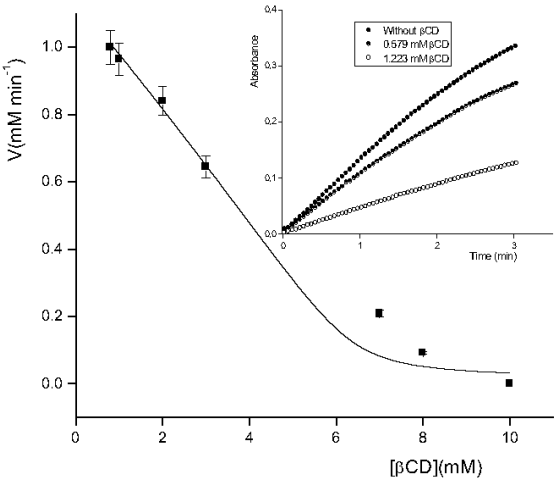

Fig. 5. Plot of Equation (8) from different concentrations of βCD for APPO. [TBC]: 0.989 mM. Inset: Absorbance vs. time in the presence of different concentrations of βCD.

The relative KI values were KI(AC) > KI(IA) > KI(AA) for $_A$PPO and KI(IA) > KI(AC) > KAI(AA) for $_W$PPO. In Figure 4, the graphical representation of the Lineweaver-Burk equation for $_A$PPO and the intercept of the previous line versus the concentration of HR (inset) are shown. The parallel straight lines obtained indicate that this inhibitor acts as a uncompetitive inhibitor.

3.6 Complexation of TBC with β-Cyclodextrin

When βCD was included in the reaction medium, the phenolic compounds in the fruit were oxidized by PPO to the same quinone produced as in the absence of βCD. However, as can be observed in Fig. 5 (inset), the oxidation rate (Abs/min) decreased in the presence of βCD. To understand this decrease in the oxidation rate, the effect of increasing amount of βCD in the reaction medium at fixed substrate concentrations was studied. The enzymatic browning decreased as βCD concentrations increased. This result is produced by the complexation of the substrate with this starch derivative, which acts as a host to include poorly soluble substrates as TBC. Its hydrophobic side chain enables it to form inclusion complexes with CDs, producing a decrease in color at low CDs concentrations. The sparingly soluble substrate (TBC) and βCD form an inclusion complex, characterized by a formation constant. This constant can be determined directly through phase solubility diagrams or some other analytical method (Filippa *et al*., 2013).

In order to determine the complexation constant (K_c) between TBC and βCD, the inhibition curves similar to those shown in Figure 5 were studied. Depending on the degree of enzyme saturation, that is, on total TBC concentration, the inhibition curve obtained was more or less sigmoidal.

Assuming that this inhibitory effect was due to the formation of the inclusion complexes and that free TBC is the only form of substrate to which PPO can react, the Michaelis-Menten rate equation could be expressed as equation (3):

$$v = \frac{V_{\max}[TBC]_f}{K_M + [TBC]_f} \tag{3}$$

where $[TBC]_f$ refers to the concentration of free TBC (substrate). This expression can be obtained as a function of the total concentrations of βCD and TBC. When a complex of stoichiometry 1-1 is formed between $[TBC]_f$ and $[\beta CD]_f$, the following equilibrium is presented in equation 4 (Sojo *et al.*, 1999):

$$[TBC]_f + [\beta CD]_f \xrightarrow{K_C} [TBC - \beta CD] \tag{4}$$

K_C is the complex formation constant, Eq. (5):

$$K_C = \frac{[TBC - \beta CD]}{[TBC]_f[\beta CD]_f} \tag{5}$$

Taking into account that both TBC and βCD can be found freely or forming complexes, the following mass balances can be established, Eq. (6) and (7):

$$[TBC]_t = [TBC]_f + [TBC - \beta CD] \tag{6}$$

$$[\beta CD]_t = [\beta CD]_f + [TBC - \beta CD] \tag{7}$$

Operating with equations (5), (6) and (7) gives an expression for $[TBC]_f$, which is replaced in Eq. (3) to obtain Eq. (8):

$$V = \frac{V_{\max}[(-([\beta CD]_t K_C - [TBC]_t K_C + 1) + \sqrt{([\beta CD]_t K_c - [TBC]_t K_C + 1)^2 + 4K_C[TBC]_t}/2K_C])}{K_M + [(-([\beta CD]_t K_C - [TBC]_t K_C + 1) + \sqrt{([\beta CD]_t K_c - [TBC]_t K_C + 1)^2 + 4K_C[TBC]_t}/2K_C]} \tag{8}$$

There is a nonlinear relationship between the enzymatic reaction rate and the concentration of βCD present in the reaction medium. Fitting the data by nonlinear regression using Origin v 8.0, a value of 16888 M^{-1} was obtained for K_c between TBC and βCD. In the previous expression, the values of V_{max} and K_M (9.36 mM min^{-1} and 13.19 mM) from the Lineweaver-Burk equation in the kinetic experiences without βCD were used. In agreement with previous research, the value for K_c is similar to those calculated by other authors. Sojo *et al*. reported 12010 M^{-1} (1999) and Orenes-Piñero *et al*. observed 12903 M^{-1} (2007) for the complex TBC-βCD in an environment where banana PPO and Streptomyces antibioticus were present.

Conclusions

The PPO enzyme was obtained from two varieties of pears: Aranjuez White and Williams, and they were characterized in physicochemical terms. If we compare results obtained for the two enzymes, we can conclude that as far as pH is concerned, their behaviors do not differ significantly. There is an important difference in the optimum temperature value. However, when the enzymes were incubated at different temperatures, the behaviors were very similar. The determination of the thermodynamic parameters of denaturation provides information on the enzyme thermal stability of the heat-induced denaturation process. For the two enzymes analyzed, values reported for this process were consistent with previous research.

In relation to the inhibitor effects, HR, which was shown to be the most efficient one, had the same type of inhibition in both PPO, and the KI values obtained were in the same order. Moreover, the compounds AC, IA and AA showed a non-competitive inhibition for $_A$PPO and a competitive inhibition for $_W$PPO.

For the Aranjuez White pear, we found that the decrease in browning due to βCD occurs through the formation of an inclusion complex between βCD and the substrate. It was possible to verify the complex formation and to determine the magnitude of the inclusion complex constant with TBC, which is a very insoluble substrate.

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References

Ali H.M., El-Gizawy, A.M., El-Bassiouny, R.E.I. and Saleh, M.A. (2015). Browning inhibition

mechanisms by cysteine, ascorbic acid and citric acid, and identifying PPO-catechol-cysteine reaction products. *Journal of Food Science and Technology 52*, 3651- 3659. https://doi.org10.1007/s13197-014-1437-0

Arias, E., Oria, R. and Lópes-Buesa, P. (2011). Spectroscopic and kinetic studies of pear polyphenoloxidase inactivation by 4-hexylresorcinol. *Journal of Food Biochemistry 35*, 1710-1714. https://doi.org/10.1111/j.1745-4514.2010.00509.x

Astray, G., Gonzalez-Barreiro, C., Mejuto, J.C., Rial-Otero, R. and Simal-Gándara, R.J. (2009). A review on the uses of cyclodextrins in foods. *Food Hydrocolloid 23*, 1631-1640. https://doi.org/10.1016/j.foodhyd.2009.01.001

Ayaz, F.A., Demir, O., Torun, H., Kolcuoglu, Y. and Colak, A. (2008). Characterization of polyphenoloxidase (PPO) and total phenolic contents in medlar (*Mespilus germanica* L.) fruit during ripening and over ripening. *Food Chemistry 106*, 291-298. https://doi.org/10.1016/j.foodchem.2007.05.096

Aydemir, T. (2004). Partial purification and characterization of polyphenol oxidase from artichoke (*Cynara scolymus* L.) heads. *Food Chemistry 87*, 59-67. https://doi.org/10.1016/j.foodchem.2003.10.017

Brandelli, A. and Lopes, C. (2005). Polyhenoloxidase activity, browning potential and phenolic content of peaches during postharvest ripening. *Journal of Food Biochemistry 29*, 624-637. https://doi.org/10.1111/j.1745-4514.2005.00026.x

Benitez, C. E. (2001). *Cosecha y Postcosecha de Peras y Manzanas en los Valles Irrigados de la Patagonia*. Ediciones INTA- Alto Valle, Argentina. ISBN: 987-521-033-1.

Carbonaro, M. and Mattera, M. (2001). Polyphenoloxidase activity and polyphenol levels in organically and conventionally grown peach (*Prunus persica* L., *cv. regina bianca*) and pear (*Pyrus communis* L., *cv. williams*). *Food Chemistry 72*, 419-424. https://doi.org/10.1016/S0308-8146(00)00248-X

Castorena-García, J.H., Martínez-Montes, Robles-López, M.R., Welti-Chanes, J.S., Hernandez-Sanchez, H. and. Robles-de-la-Torre, R.R. (2013). Effect of electric fields on the activity of polyphenol oxidases. *Revista Mexicana de Ingeniería Química 12*, 391-400.

Cheema, S. and Sommerhalter, M. (2015). Characterization of polyphenol oxidase activity in *Ataulfo* mango. *Food Chemistry 171*, 382-387. https://doi.org/10.1016/j.foodchem.2014.09.011

Cravotto, G., Binello, A., Baranelli, E., Carraro, P. and Trotta, F. (2006). Cyclodextrins as food additives and in food processing. *Journal of Current Nutrition and Food Science 2*, 343-350. https://doi.org/10.2174/157340106778699485

Dincer, B., Colak, A., Aydin, N., Kadioglu, A. and Guner, S. (2002). Characterization of polyphenoloxidase from medlar fruits (*Mespilus germánica L. rosaceae*). *Food Chemistry 77*, 1-7. https://doi.org/10.1016/S0308-8146(01)00359-4

Duangmal, K. and Owusu $_A$Penten, R.K. (1999). A comparative study of polyphenoloxidase from taro (*Colocasia esculenta*) and potato (*Solanum tuberosum var. romano*). *Food Chemistry 64*, 351-359. https://doi.org/10.1016/S0308-8146(98)00127-7

Erat, M., Sakiroglu, H. and Kufrevioglu, O.I. (2006). Purification and characterization of polyphenol oxidase from *Ferula sp. Food Chemistry 95*, 503-508. https://doi.org/10.1080/14756360320000141890

Falguera, V., Pagan, J., Garza, S., Gavin, A. and Ibarz, A. (2012). Inactivation of polyphenol oxidase by ultraviolet irradiation: Protective effect of melanins. *Journal of Food Engineering 110*, 305-309. https://doi.org/10.1016/j.jfoodeng.2011.04.005

Filippa, M.A., Sancho, M.I. and Gasull, E.I. (2013). Determination of apparent binding constants by NSAIDs-βcyclodextrin complexes: HPLC, phase solubility diagrams and theoretical studies. *Journal of Inclusion Phenomena and Macrocyclic Chemistry 77*, 223-230. https://doi.org/10.1007/s10847-012-0236-3

Fuentes Campo, A., Sancho, M., Melo, G., Dávila, Y. and Gasull, E. (2019). *In vitro* and *in vivo* inhibition of *Hass* avocado polyphenol oxidase enzymatic browning by paeonol, β-cyclodextrin, and paeonol: β-cyclodextrin inclusion complex. *Journal of Bioscience and Bioengineering 127*, 703-709. https://doi.org/10.1016/j.jbiosc.2018.11.009

Garro, A. and Gasull, E. (2010). Characterization of polyphenoloxidase from 2 peach (*Prunus persica* L.) varieties grown in Argentina. *Food Science and Biotechnology 19*, 627-632. https://doi.org/10.1007/s10068-010-0088-9

Gómez-López, V.M. (2002). Some biochemical properties of polyphenol oxidase from two varieties of avocado. *Food Chemistry 77*, 163-169. https://doi.org/10.1016/S0308-8146(01)00331-4

Jiménez, M., Chazarra, S., Escribano, J., Cabanes, J. and Garcia-Carmona, F. (2001). Competitive inhibition of mushroom tyrosinase by 4-substituted benzaldehydes. *Journal of Agricultural and Food Chemistry 49*, 4060-4063. https://doi.org/10.1021/jf010194h

Leskovac, V. (2003). *Comprehensive Enzyme Kinetics*. New York: Kluwer Academic Publishers. ISBN: 0-306-46712-7. https://doi.org/10.1007/0-306-48390-4_5

Lim, W. Y. and Wong, C.W. (2018). Inhibitory effect of chemical and natural anti-browning agents on polyphenol oxidase from ginger (*Zingiber officinale roscoe*). *Journal of Food Science and Technology 55*, 3001-3007. https://dx.doi.org/10.1007%2Fs13197-018-3218-7

Liu, W., Zou, L., Liu, J., Zhang, Z., Liu, C. and Liang, R. (2013). The effect of citric acid on the activity, thermodynamics and conformation of mushroom polyphenoloxidase. *Food Chemistry 140*, 289-295. http://dx.doi.org/10.1016/j.foodchem.2013.02.028

López-Nicolás, J.M. and García-Carmona, F. (2007). Use of cyclodextrins as secondary antioxidants to improve the color of fresh pear juice. *Journal of Agricultural and Food Chemistry 55*, 6330-6338. https://doi.org/10.1021/jf070819x

López-Nicolas, J.M., Nuñez-Delicado, E., Sanchez-Ferrer, A. and Garcia-Carmona, F. (2007). Kinetic model of apple juice enzymatic browning in the presence of cyclodextrins: The use of maltosyl-β-cyclodextrin as secondary antioxidant. *Food Chemistry 101*, 1164-1171. https://doi.org/10.1016/j.foodchem.2006.03.018

Martinez-Monteagudo, S.I. (2018). Analysis of thermoxidation kinetics of milk fat. *Revista Mexicana de Ingeniería Química 17*, 587-602. http://doi.org/10.24275/10.24275/uam/izt/dcbi/revmexingquim/2018v17n2/MartinezS

Matsui, K.N., Gut, J.A.W., de Oliveira, P.V. and Tadini, C.C. (2008). Inactivation kinetics of polyphenol oxidase and peroxidase in green coconut water by microwave processing. *Journal of Food Engineering 88*, 169-176. https://doi.org/10.1016/j.jfoodeng.2008.02.003

Orenes-Piñero, E., García-Carmona, F. and Sánchez-Ferrer, A. (2007). Kinetic characterization of diphenolase activity from *Streptomyces antibioticus* tyrosinase in the presence and absence of cyclodextrins. *Journal of Molecular Catalysis B: Enzymatic 47*, 143-148. https://doi.org/10.1016/j.molcatb.2007.04.009

R$_A$Peanu, G., Van Loey, A., Smout, C. and Hendrickx, M. (2005). Thermal and high-pressure inactivation kinetics of polyphenol oxidase in *Victoria* grape must. *Journal of Agricultural and Food Chemistry 53*, 2988-2994. https://doi.org/10.1021/jf0482515

Riener, J., Noci, F., Cronin, D., Morgan, D., and Lyng, J. (2008). Combined effect of temperature and pulse electric fields on apple juice peroxidase and polyphenoloxidase inactivation. *Food Chemistry 109*, 402-407. https://doi.org/10.1016/j.foodchem.2007.12.059

Robles-Ozuna, L.E., Goycoolea, F.M., Silveira, M. I. and Montoya B., L.C. (2007). Use of chitosan during blanching of nopal (*Opuntia ficus indica*) and its effect on quality. *Revista Mexicana de Ingeniería Química 6*, 193-201. https://www.redalyc.org/articulo.oa?id=62060208

Rocha, A.M.C.N. and Morais, A.M.M.B. (2001). Characterization of polyphenoloxidase (PPO) extracted from "Jonagored" apple. *Food Control 12*, 85-90. https://doi.org/10.1016/S0956-7135(00)00026-8

Sikora, M., Swieca, M., Franczyk, M., Jakubczyk, A., Bochnak, J. and Zlotek, U. (2019). Biochemical properties of polyphenol oxidases from ready-to-eat lentil (*Lens culinaris medik*.) sprouts and factors affecting their activities: a search for potent tools limiting enzymatic browning. *Foods 8*, 154. https://doi.org/10.3390/foods8050154

Simas-Dias, D., Acevedo-Jaramillo, L.Y., U. Vasconcelos, U. and N. Pereira, N. (2018) Characterization of β-glucosidases produced by *Aspergillus niger* at cc 1004 in submerged fermentation from sugarcane bagassed. *Revista Mexicana de Ingeniería Química 17*, 365-377. http://doi.org/10.24275/uam/izt/dcbi/revmexingquim/2018v17n1/Simas

Singh, N., Singh, R., Kaur, K. and Singh, H. (1999). Studies of the physic-chemical properties and polyphenoloxidase activity in seeds from hybrid sunflower (*Helianthus annuus*) varieties grown in India. *Food Chemistry 66*, 241-247. https://doi.org/10.1016/S0308-8146(99)00057

Singh, B., Suri, K., Shevkani, K., Kaur, A., Kaur, A. and Singh, N. (2018). Enzymatic browning of fruit and vegetables: a review. In: *Enzymes in Food Technology* (M. Kuddus ed.), P.p, 63-78. Springer Nature, Singapore. https://doi.org/10.1007/978-981-13-1933-4_4

Sojo, M.M., Nuñez-Delicado, E., García-Carmona, F. and Sánchez-Ferrer, A. (1999). Cyclodextrins as activator and inhibitor of latent banana pulp polyphenol oxidase. *Journal of Agricultural and Food Chemistry 47*, 518-523. https://doi.org/10.1021/jf9808978

Son, S.M., Moon, K.D. and Lee, C.Y. (2000). Kinetic study of oxalic acid inhibition on enzymatic browning. *Journal of Agricultural and Food Chemistry 48*, 2071-2074. https://doi.org/10.1021/jf991397x

Sulaiman, A. and Silva, F.V.M. (2013). High pressure processing, thermal processing and freezing of '*Camarosa*' strawberry for the inactivation of polyphenoloxidase and control of browning. *Food Control 33*, 424-428. https://doi.org/10.1016/j.foodcont.2013.03.008

Sun, J., Shi, J., Zhao, M., Xue, S.J., Ren, J. and Jiang, Y. (2008). A comparative analysis of property of lychee polyphenoloxidase using endogenous and exogenous substrates. *Food Chemistry 108*, 818-823. https://doi.org/10.1016/j.foodchem.2007.11.036

Szente, L. and Szejtli, J. (2004). Cyclodextrins as food ingredients. *Trends in Food Science and Technology 15*, 137-142. https://doi.org/10.1016/j.tifs.2003.09.019

Toranzo, J. (2016). *Producción Mundial de Manzana y Peras. Programa Nacional Frutales*. Ediciones INTA-Alto Valle, Argentina. ISBN 978-987-521-696-9.

Tuncay, D. and Yagar, H. (2011). Comparison of polyphenol oxidases prepared from different parts of artichoke (*Cynara scolymus* L.). *International Journal of Food Properties 14*, 809-821. https://doi.org/10.1080/10942910903453363

Ziyan, E. and Pekyardimci, S. (2004). Purification and characterization of pear (*Pyrus communis*) polyphenol oxidase. *Turkish Journal Chemistry 28*, 547-557.