Food Sci. Biotechnol. 19(3): 627-632 (2010)
DOI 10.1007/s10068-010-0088-9

RESEARCH ARTICLE

Characterization of Polyphenoloxidase from 2 Peach (*Prunus persica* L.) Varieties Grown in Argentina

Adriana Garro and Estela Gasull

Received: 21 August 2009 / Revised: 26 February 2010 / Accepted: 10 March 2010 / Published Online: 30 June 2010

Abstract Polyphenoloxidase was extracted from September peach ($_{SE}$PPO) and Summerset peach ($_{SU}$PPO) and its physicochemical characteristics were analyzed. The optimum pH was 6.5 for $_{SE}$PPO and 5.5 for $_{SU}$PPO. The optimum temperature was 35ºC for $_{SE}$PPO and 39.4ºC for $_{SU}$PPO. Activation energy (Ea) from thermal activation was 41.5 kJ/mol for $_{SE}$PPO and 37.5 kJ/mol for $_{SU}$PPO. Heating at 60ºC by 5 min, $_{SU}$PPO was denatured whereas $_{SE}$PPO retained 2.6% of activity. Activation enthalpy ($\Delta H^{\#}$) and activation entropy ($\Delta S^{\#}$) for $_{SE}$PPO heat-inactivation were 69.9 J/mol and −83.5 kJ/mol·K for $_{SU}$PPO, $\Delta H^{\#}$ was 91.8 J/mol while $\Delta S^{\#}$ was −21.0 kJ/mol·K. Substrate specificity (V_{max}/K_M) was 4-methylcatechol>catechol> pyrogallol for $_{SE}$PPO and 4-mehtylcatechol>pyrogallol> catechol for $_{SU}$PPO. For both enzymes, the order of inhibition effectiveness using reductor agents was metabisulphite> ascorbic acid. Benzaldehyde, 4-hydroxybenzaldehyde, and DL-dopa were competitive inhibitors, and their K_I values were 38.86, 8.43, and 2.08 mM, respectively.

Keywords: polyphenoloxidase, peach, activity, stability, inhibition

Introduction

Browning of raw fruits, vegetables, and beverages is a major problem in the food industry and it is believed to be the main cause of quality loss during post-harvest handling and processing. The browning mechanism in food is well characterized and is mainly enzymatic in origin (1). Polyphenoloxidase (PPO), also known as tyrosinase (EC 1.14.18.1), is a copper-containing oxidoreductase which catalyzes two different reactions using molecular oxygen: the hydroxylation of monophenols to *O*-diphenols (monophenolase activity) and the oxidation of the *O*-diphenols to *O*-quinones (diphenolase activity), which are later polymerized to dark-colored pigments (2,3). The degree of browning depends on the nature and amount of endogenous phenolic compounds, on the presence of oxygen, reducing substances, and metallic ions, on pH and temperature, and on the activity of PPO, the main enzyme involved in the reaction (4,5).

Browning can be prevented by heat inactivation, inhibition of the enzyme, removal or transformation of the substrates (oxygen and phenols), decrease of pH below the range of activity, addition of antioxidants which inhibit PPO, or prevent pigments formation (6-8).

In this work, the characterization of PPO from September ($_{SE}$PPO) and Summerset peach ($_{SU}$PPO) fruits was performed in terms of pH optimum, thermal activation and stability, substrate specificities, and degrees and types of inhibition by general PPO inhibitors, in order to help to predict the behavior of these enzymes.

Materials and Methods

Materials Whole fruits of peach (*Prunus persica* L., cv. Summerset, and cv. September) were obtained at commercial maturity from a local market (San Luis, Argentina) and were stored at 4ºC for 24 hr until used in the experiments. Catechol, 4-methylcatechol, pyrogallol, 3,4-dihydroxy-DL-phenylalanine (DL-dopa), *p*-cresol,3-[4-hydroxyphenyl-DL-alanine] (DL-tyrosine), and 3,3',4',5,7-pentahydroxyflavone (quercetin) were obtained from Sigma-Aldrich (St. Louis,

Adriana Garro, Estela Gasull (✉)
Área de Química Física, Departamento de Química, IMIBIO SL. Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, 5700, San Luis, Argentina
Tel: +54-26-5242-4689 Int 122; Fax: +54-26-5243-1301
E-mail: esgasu@unsl.edu.ar, egasull7@gmail.com

MO, USA). Triton X-100 was purchased from Fluka Chemicals Co. (Buchs, Switzerland). Benzaldehyde and 4-hydroxybenzaldehyde were from Merck Schuchardt (Hohenbrunn, Germany). All other chemicals were of analytical grade.

Methods The extraction of PPO from September peaches ($_{SE}$PPO) and from Summerset peaches ($_{SU}$PPO) was performed using the procedure of Gauillard and Richard-Forget (9) with some modifications. Peeled crude samples (100 g) were homogenized for 2 min in a Ultracomb commercial blender (Buenos Aires, Argentina) in 100 mL of 50 mM sodium phosphate buffer at pH 6.5, containing 20 mM ascorbic acid, 2%(v/v) polyethylenglycol, and 1%(v/v) Triton X-100. Polyethylenglycol was used to bind phenols which could inactivate PPO activity during the extraction (10), while Triton X-100, a non-ionic surfactant, was required to achieve a full extraction of enzyme (11). After filtration of the homogenate through gauze, the filtrate was centrifuged at 33,586×g for 30 min in a Beckman Coulter J2-HS ultracentrifuge (Fullerton, CA, USA). All steps were carried out at 4ºC. The supernatants were recovered and kept in tubes at –20ºC and were used as crude enzyme extract which retained PPO activity for about 4 months.

PPO activity assay PPO activity was assayed by a spectrophotometric procedure using a Shimadzu UV-160A (Kyoto, Japan) double beam UV-vis spectrophotometer equipped with a quartz cell of 10-mm path length, with temperature control. Enzymatic activity was assayed by measuring the rate of increase in absorbance at a given wavelength (variable for different substrates and PPOs) at 25ºC. The reaction mixture contained 3 mL of buffer solution and different quantities of enzyme ($_{SE}$PPO, 200 µL; $_{SU}$PPO, 100 µL) and substrate. The reference cuvette contained only buffer solution and the substrate (12). The straight-line section of the absorbance curve as a function of time was used to determine enzyme activity. One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001/min (13). When a lag phase occurred, the reaction rate was measured after the lag phase. All determinations were performed in triplicate.

Properties of crude $_{SE}$PPO and $_{SU}$PPO ***Effects of pH*****:** PPO activity was assayed in the pH range 4-5.5 in 50 mM acetate buffer, 6.5-7.5 in 50 mM phosphate buffer and 7.5-9.5 in 50 mM Tris buffer [Tris (hydroxymethyl) aminomethane)-HCl using catechol (50 mM)] as substrate and 200 ($_{SE}$PPO) or 100 µL ($_{SU}$PPO) of crude enzyme extract in 3 mL buffer solutions. PPO activity was determined by measurement of absorbance at the maximum wavelength of the product (408 nm for $_{SE}$PPO and 395 nm for $_{SU}$PPO).

Thermal activity**:** PPO activity (at optimum pH values) was measured at different temperatures in a range of 15-50ºC using a circulation water bath. Buffer solutions (3 mL) and substrate catechol ($_{SE}$PPO, 13 mM; $_{SU}$PPO, 2.5 mM) were kept for 15 min at temperatures as indicated above, prior to the addition of the enzyme solution ($_{SE}$PPO, 200 µL; $_{SU}$PPO, 100 µL). All assays were performed in triplicate. The data obtained from the thermal activity have been used to analyze some thermodynamic parameters related to peach PPO activity in the crude enzyme extracts.

Thermal stability**:** Thermal stability of extract PPO was investigated at optimum pH values at various constant temperatures from 20 to 60ºC. The enzyme solution in buffer was incubated in a circulation water bath at temperatures of 20, 30, 40, 50, and 60ºC for different times (5-40 min). After the mixture was cooled in an ice bath and brought to 25ºC, 3 mL heated enzyme solution was mixed with catechol ($_{SE}$PPO, 12 mM; $_{SU}$PPO, 4 mM), and residual PPO activity was determined spectrophotometrically. The percentage residual PPO activity was calculated by comparison with unheated enzyme.

Enzyme kinetics and substrate specificity**:** $_{SE}$PPO and $_{SU}$PPO activity was assayed using monohydroxyphenols (*p*-cresol and DL-tyrosine), dihydroxyphenols (catechol and 4-methilcatechol), trihydroxyphenols (pyrogallol), and a polyhydroxyphenol (quercetin) in buffers at optimum pH values. The rate of the reaction was measured in terms of the increase in absorbance at the wavelength of maximum absorption for the corresponding product. The assays were carried out in cuvettes (3 mL) containing the buffer solution at pH optimum, the extract crude of enzyme ($_{SE}$PPO, 200 µL; $_{SU}$PPO, 100 µL) and different amounts (5-100 µL) of the substrate solution (*p*-cresol, 200 mM; DL-tyrosine, 150 mM; catechol, 322 mM; 4-methylcatechol, 175 mM; pyrogallol, 160 mM; quercetin, 3.7 mM).

For each substrate, the kinetic data were plotted as 1/activity versus 1/substrate concentration, according to the method of Lineweaver-Burk (14). 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 by using the previously obtained data on a Lineweaver-Burk plot.

Effect of inhibitors**:** Benzaldehyde (0.8-25 mM), 4-hydroxybenzaldehyde (7.4-24 mM) and DL-dopa (0.6-2.25 mM) were examined for their effectiveness as inhibitors of $_{SE}$PPO and $_{SU}$PPO using catechol as substrate. To perform the experiments were used 3 mL of reaction mixture contained the buffer solution at pH optimum, the extract crude of enzyme ($_{SE}$PPO, 200 µL; $_{SU}$PPO, 100 µL) and different amounts of 330 mM catechol solution as substrate (25-100 µL).

In each case, the type of inhibition was deduced from Lineweaver-Burk double reciprocal plots. The inhibitory constants K_I were calculated from a replot of the slope of each reciprocal plot versus the corresponding inhibitor concentration (15).

In addition, the effect of increasing amounts of reducing compounds, as ascorbic acid (0.03-2.76 mM) and sodium metabisulphite (0.011-0.075 mM), was studied. The assays were carried out using catechol as substrate ($_{SE}$PPO, 12 mM; $_{SU}$PPO, 5 mM), and their $I_{50\%}$ were evaluated.

Results and Discussion

Effects of pH $_{SE}$PPO had a maximum activity around pH 6.5, and $_{SU}$PPO had a maximum activity at pH 5.5. The common pH range for optimum fruit PPOs activity is known to be 5.0-7.0 (16). The PPOs from both sources were inactive at pH near 4. Enzyme activity decreased rapidly at alkaline pHs (7-9). The optimum pH depends on genetic properties (variety), nature of phenolic substrates, and extraction methods. In this case, we have used the same procedure for extraction, and the same substrate. Thus, the optimum pH values depend only on peach variety. No reports were found for pH effects on the 2 peach varieties studied.

Thermal activity The temperature had a 2-sided influence on enzymatic activity: increasing temperature enhanced enzymatic reaction velocity but simultaneously led to denaturation of enzyme. Then, from 15ºC to the optimum temperature activity increased as temperature increased, and then started to decrease. The optimum temperature for $_{SE}$PPO activity was 35.0ºC and for $_{SU}$PPO it was 39.4ºC. Below and above these temperatures, enzyme activity decreased gradually. This was consistent with reported temperatures for PPO activities in grapes: 40 (17) and 45ºC (18); in medlar: 35ºC (19), and in plum: 37ºC (20).

Activation energy (Ea) was determined according to the Arrhenius equation by measuring the maximal initial rate at different temperatures and plotting the logarithmic value of V_{max} versus 1/T (15).

$$\ln V_{max} = \ln A - \frac{Ea}{RT} \quad (1)$$

For $_{SE}$PPO and/or $_{SU}$PPO the plot showed no obvious deviation from linearity, with an activation energy (Ea) of 41.55 kJ/mol for $_{SE}$PPO (R^2=0.998), and 37.50 kJ/mol for $_{SU}$PPO (R^2=0.995). As can be seen, the Ea values are very similar.

Thermal stability For crude $_{SU}$PPO the enzyme was stable after incubation for 30 min at 30ºC; then, its residual activity percentage decreased to 89%. For 40 and 50ºC, PPO activity gradually decreased. After heating for 5 min at 60ºC, the relative PPO activity was 32.6%.

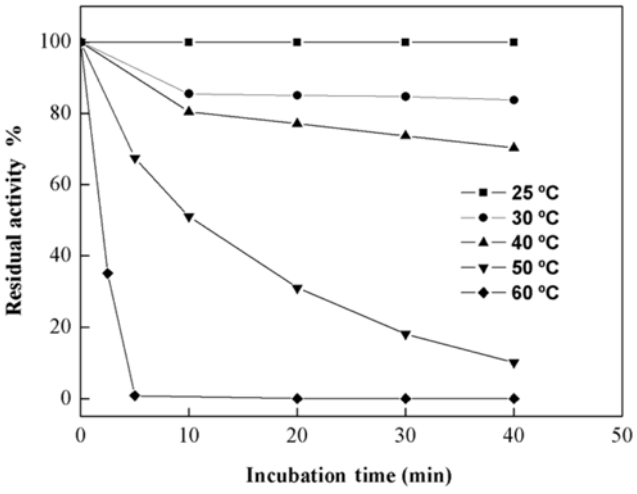

Fig. 1. Thermal stability of PPO from September peach. Assay condition: pH 6.5; 13.2 mM catechol; 200 µL enzyme

The thermal stability profile for $_{SE}$PPO presented in the form of residual activity percentage is shown in Fig. 1. The enzyme was stable at 25ºC for 40 min. At 30 and 40ºC, 14.5 and 20% respectively of activity in $_{SE}$PPO were lost after 10 min. When heated at 50ºC the $_{SE}$PPO activity was lost progressively. At 60ºC, heat denaturation of the enzyme occurred after 5 min of incubation. Very short exposures (1-2 min) to temperatures of 70-90ºC are sufficient for total irreversible destruction of its catalytic activity (21). The drop in percentage residual activity at high temperatures is actually due to the unfolding of the tertiary structure of the enzyme to form the secondary structure.

Often, thermal inactivation of enzymes can be described by a first-order reaction, and the rate constant for heat inactivation (k) was determined from the following relation (22):

$$k = -\frac{1}{600}\ln\frac{A_t}{A_o} \quad (2)$$

where A_o is the activity of unheated enzyme and A_t is the residual enzyme activity remaining after heating for 600 sec at different temperatures. The temperature-dependence of k (1/sec) was evaluated using the Arrhenius equation, and the Ea for PPOs using catechol as substrate was estimated as 72.6 kJ/mol ($_{SE}$PPO) and 94.5 kJ/mol ($_{SU}$PPO). The Ea values obtained are in agreement with those reported by Weemaes *et al.* (23) for apples, avocados, grapes, and plums.

The activation parameters enthalpy $\Delta H^{\#}$ and entropy $\Delta S^{\#}$ were determined from:

Table 1. Transition state parameters for the heat inactivation of September and Summerset PPOs extract

Thermodynamic parameter	$_{SE}$PPO	$_{SU}$PPO
Ea (kJ/mol)	72.6	94.5
$\Delta H^{\#}$ (kJ/mol)	69.9	91.8
$\Delta S^{\#}$(J/mol K)	-83.5	-21.0
$\Delta G^{\#}_{323}$ (kJ/mol)	96.9	98.6

$$\ln\left(\frac{k}{T}\right)=\ln\left(\frac{k}{h}e^{\frac{\Delta S^{\#}}{R}}\right)-\frac{\Delta H^{\#}}{RT} \quad (3)$$

where k ($1.3806.10^{-23}$ J/K) is the Boltzman constant, *h* ($6.6261.10^{-34}$ J sec) is the Planck constant, and R (8.314 J/K mol) is the universal gas constant. Results for these analyses, as well as free energy change $\Delta G^{\#}$ for crude $_{SE}$PPO and $_{SU}$PPO are reported in Table 1.

The activation parameters are consistent with the view that PPOs are relatively heat-labile enzymes. Results from this table suggest that $_{SU}$PPO is more heat-resistant than $_{SE}$PPO, apparently as a result of the larger $\Delta H^{\#}$ value for inactivation. If only $\Delta S^{\#}$ values are taken into account, $_{SE}$PPO is more heat-resistant than $_{SU}$PPO. $\Delta G^{\#}$ values (estimated in Table 1 for 323 K) are very similar. In general, $\Delta H^{\#}$ is seen as a measure of the number of noncovalent bonds broken in forming a transition state for enzyme inactivation, whereas $\Delta S^{\#}$ is a measure of the net enzyme and solvent disorder change accompanying transition state formation (22).

Enzyme kinetics and substrate specificity Monohydroxyphenols are not oxidized by the peach extracts. *O*-Diphenols and triphenols were oxidized significantly for $_{SE}$PPO and $_{SU}$PPO, displaying Michaelis-Menten kinetics. Linear regression analysis of 1/activity versus 1/substrate concentration determined for $_{SU}$PPO using pyrogallol as substrate is shown in Fig. 2. The best substrate for each enzyme depends on 2 factors: strong substrate binding or high affinity (low K_M value) and high 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 (24).

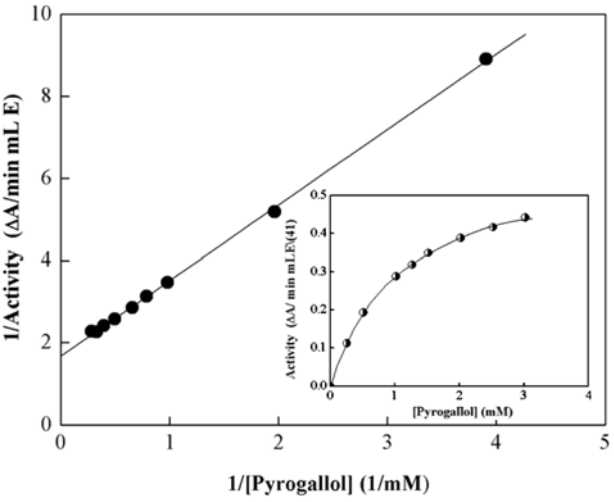

Fig. 2. Lineweaver-Burk plot and activity as a function of substrate concentration for PPO from Summerset peach. Assay condition: pH 5.5; substrate, pyrogallol; 100 μL enzyme

V_{max} and K_M for each substrate are presented in Table 2. For $_{SE}$PPO, catalytic efficiency was the lowest but substrate binding was the highest with pyrogallol; it was found to be the most efficient phenolic substrate, followed by catechol and 4-methylcatechol.

For $_{SU}$PPO, however, 4-metylcatechol was the best substrate, followed by pyrogallol and catechol. This result is consistent with a previous report (19) indicating that 4-metylcatechol is usually the best substrate for PPOs.

Quercetin (a polyhydroxyphenol) was oxidized by $_{SE}$PPO and $_{SU}$PPO but K_M and V_{max} cannot be calculated from a plot of 1/activity versus 1/substrate concentration by the method of Lineweaver-Burk.

Effects of inhibitors Various inhibitors were examined to determine their potential for inhibition of catechol by crude $_{SE}$PPO and $_{SU}$PPO activity. These inhibitors included analogous substrates and reducing agents. Table 3 shows the inhibition results with catechol as substrate. For analogous substrates, Lineweaver-Burk plots of 1/activity versus 1/substrate concentration by various inhibitor concentrations determined the type of inhibition. Good

Table 2. Substrate specificity of September and Summerset PPOs extract

Substrate	$_{SE}$PPO[1)]				$_{SU}$PPO[1)]			
	λ	V_{max}	K_M	V_{max}/K_M	λ	V_{max}	K_M	V_{max}/K_M
p-Cresol	-	-	-	-	-	-	-	-
DL-Tyrosine	-	-	-	-	-	-	-	-
Catechol	408	416	2.51	165.7	395	1321.6	5.33	247.5
4-Methylcatechol	450	506	3.63	139.4	400	33.6	0.03	1120
Pyrogallol	420	224.9	0.214	1,050.9	410	597.7	1.10	543.4
Quercetin	435	*	*	*	430	*	*	*

[1)]λ, wavelength (nm); V_{max}, UE/mL E; K_M, mM; *Inappropriate substrate, cannot be calculated from Lineweaver-Burk plot.

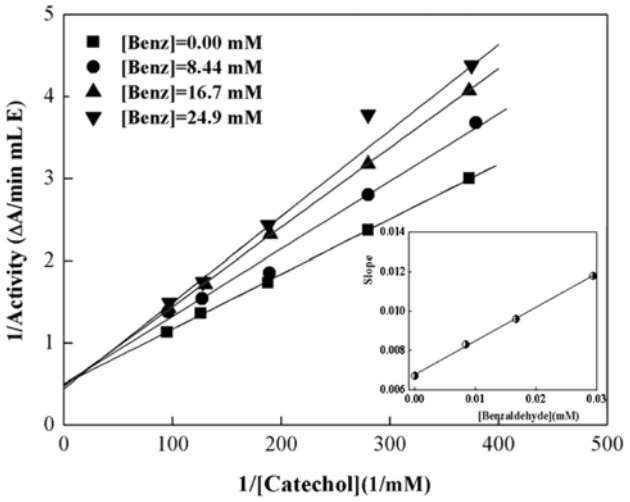

Fig. 3. Inhibition of PPO from Summerset peach by benzaldehyde [Benz]. Assay condition: pH 5.5; substrate, catechol (2.75-11 mM); 100 μL enzyme

straight lines were obtained. For $_{SE}$PPO and $_{SU}$PPO, benzaldehyde and 4-hydroxybenzaldehyde were competitive inhibitors, and their K_I constants are reported in Table 3. Jiménez *et al*. (25) have reported that benzaldehydes behave as classical competitive inhibitors by mushroom tyrosinase.

As can see been in Table 3, for $_{SE}$PPO and $_{SU}$PPO benzaldehyde is a worse inhibitor than 4-hydroxy-benzaldehyde. This difference in their inhibitory effects can be attributed to the substituent at the *para*-position. This fact was observed by Janovitz-Klapp *et al*. (26) who determined that *p*-hydroxy substitution enhanced the inhibitory properties of carboxylic acids.

A typical example for competitive inhibition and replots of slope versus inhibitor concentrations is shown in Fig. 3 for $_{SU}$PPO using catechol as substrate and benzaldehyde as inhibitor. For $_{SU}$PPO, DL-dopa was a competitive inhibitor, but we cannot ensure the type of inhibition for $_{SE}$PPO.

For reducing agents, $I_{50\%}$ values were determined and shown in Table 3. It can be seen that $_{SE}$PPO tends to be inhibited by ascorbic acid more easily than $_{SU}$PPO, whereas the latter is more strongly inhibited by sodium methabisulphite than $_{SE}$PPO. The inhibition reaction mechanism differs, depending on the reducing agent employed. Inhibitions by thiol compounds are attributed to either the stable colorless products formed by an addition reaction with *O*-quinones or binding to the active centre of PPO, like metabisulphite (27). In PPO assays, in which ascorbic acid was used as an inhibitor, a lag period was observed before any changes in absorbance were measured. The extent of the lag period increased with an increase in inhibitor concentration. The mechanism of ascorbic acid inhibition involves the reduction of quinones generated (2, 28). After the lag period, when nearly all ascorbic acid is converted to dehydroascorbic acid, the amount of *O*-quinones formed by PPO action increases.

Acknowledgments This work was supported by Secretaría de Ciencia y Técnica de la Universidad Nacional de San Luis (Argentina).

References

1. McEvily JA, Iyengar R, Otwell WS. Inhibition of enzymatic browning in foods and beverages. Crit. Rev. Int. J. Food Sci. 32: 253-273 (1992)
2. Martinez VM, Whitaker JR. The biochemistry and control of enzymatic browning. Trends Food Sci. Tech. 6: 195-200 (1995)
3. Sanchez-Ferrer A, Rodriguez-Lopez JN, Garcia Canovas F, Garcia-Carmona F. Tyrosinase: A comprehensive review of its mechanism. Biochim. Biophys. Acta 1247: 1-11 (1995)
4. Goupy P, Amiot MJ, Richard-Forget F, Duprat F, Aubert S, Nicolas J. Enzymatic browning of model solutions and apple phenolic extracts by apple polyphenoloxidase. J. Food Sci. 60: 497-501 (1995)
5. Carbonaro M, Matera M. Polyphenoloxidase activity and polyphenol levels in organically and conventionally grown peach (*Prunus persica* L., cv. Regina bianca) and pear (*Pyrus communis* L. cv. Wiliams). Food Chem. 72: 419-424 (2001)
6. Severini C, Baiano A, De Pilli T, Romaniello R, and Derossi A. Prevention of enzymatic browning in sliced potatoes by blanching in boiling saline solutions. Lebensm. -Wiss. Technol. 36: 657-665 (2003)
7. Guerrero-Beltran JA, Swanson BG, Barbosa-Canovas GV. Inhibition of polyphenoloxidase in mango puree with 4-hexylresorcinol, cysteine, and ascorbic acid. Lebensm. -Wiss. Technol. 38: 625-630 (2005)
8. Vamos-Vigyazo L. Enzymatic browning and its control. Chap. 4, pp.

Table 3. Effect of inhibitors on the activity of September and Summerset PPOs extract

Inhibitor	$_{SE}$PPO[1)]			$_{SU}$PPO[1)]		
Compound analogues	[Inhibitor]	Type	K_I	[Inhibitor]	Type	K_I
Benzaldehyde	6.16-17.90	competitive	20.15	0.84-24.70	competitive	38.86
4-OH-Benzaldehyde	7.40-21.8	competitive	6.47	7.81-23.22	competitive	8.43
DL-Dopa	*	*	*	0.59-2.25	competitive	2.08
Reducing agents	[Inhibitor]	Type	$I_{50\%}$	[Inhibitor]	Type	$I_{50\%}$
Ascorbic acid	0.03-0.200	-	0.29	0.41-2.76	-	4.48
Metabisulphite	0.025-0.074	-	0.23	0.011-0.006	-	0.07

[1)]Inhibitor and K_I, mM; *Cannot ensure

49-62. In: Enzymatic Browning and Its Prevention. Lee CY, Whitaker JR (eds). American Chemical Society, Washington, DC, USA (1995)
9. Gauillard F, Richard-Forget F. Polyphenoloxidase from Williams pear (*Pyrus communis* L., cv Williams): Activation, purification, and some properties. J. Food Sci. 74: 49-56 (1997)
10. Erat M, Sakiroglu H, Kufrevioglu OI. Purification and characterization of poyphenol oxidase from *Ferula* sp. Food Chem. 95: 503-508 (2006)
11. Rocha AMCN, Galeazzi, MAM, Cano P, Morais AMMB. Characterization of 'Starking' apple polyphenoloxidase. J. Sci. Food Agr. 77: 527-534 (1998)
12. Espin JC, Trujano MF, Tudela J, Garcia-Canovas F. Monophenolase activity of polyphenol oxidase from Haas Avocado. J. Agr. Food Chem. 45: 1091-1096 (1997)
13. Galeazzi MAM, Sgarbieri VCJ. Substrate specificity and inhibition of polyphenoloxidase from a dwarf variety of banana (*Musa cavendishii* L.). J. Food Sci. 46: 1404-1406 (1981)
14. Lineweaver H, Burk D. The determination of enzyme dissociation constant. J. Am. Chem. Soc. 56: 658-661 (1934)
15. Segel IH. Biochemical Calculations. How to Solve Mathematical Problems in General Biochemistry. Wiley, New York, NY, USA. pp. 278-280 (1975)
16. Rapenau G, Van Loey A, Smout C, Hendrickx M. Biochemical characterization and process stability of polyphenoloxidase extracted from Victoria grape (*Vitis vinifera* ssp. Sativa). Food Chem. 94: 253-261 (2004)
17. Sanchez-Ferrer A, Bru R, Cabanes J, Garcia-Carmona F. Characterization of catecholase and cresolase activities of monastrell grape polyphenol oxidase. Phytochemistry 27: 319-321 (1988)
18. Valero E, Varon R, Garcia-Carmona F. Characterization of polyphenol oxidase from Airen grapes. J. Food Sci. 53: 1482-1485 (1988)
19. Dincer B, Colak A, Aydin N, Kadioglu A, Güner, S. Characterization of polyphenoloxidase from medlar fruits (*Mespilus germanica* L., Rosaceae). Food Chem. 77: 1-7 (2002)
20. Siddiq M, Sinha K, Cash JN. Characterization of polyphenol oxidase from Stanley plums. J. Food Sci. 57: 1177-1179 (1992)
21. Vamos-Vigyazo L. Polyphenol oxidase and peroxidase in fruits and vegetables. Crit. Rev. Int. J. Food Sci. 15: 49-127 (1981)
22. Duangmal K, Owusu Apenten RK. A comparative study of polyphenoloxidases from taro (*Colocacia esculenta*) and potato (*Solanum tuberosum* var. Romano). Food Chem. 64: 351-359 (1999)
23. Weemaes CA, Ludikhuyze LR, Van den Broeck I, Hendrickx ME, Tobback PP. Activity, electrophoretic characteristics, and heat inactivation of polyphenoloxidases from apples, avocados, grapes, and plums. Lebensm. -Wiss. Technol. 31: 44-49 (1998)
24. Palmer T. Kinetics of single-substrate enzyme catalyzed reactions. pp. 107-127. In: Understanding Enzymes. Prentice Hall/Ellis Horwood, Hertfordshire, UK (1995)
25. Jiménez M, Chazarra S, Escribano J, Cabanes J, García-Carmona F. Competitive inhibition of mushhroom tyrosinase by 4-substituted benzaldehydes. J. Agr. Food Chem. 49: 4060-4063 (2001)
26. Janovitz-Klapp A, Richard F, Goupy P, Nicolas J. Inhibition studies on apple polyphenoloxidase. J. Agr. Food Chem. 38: 926-931 (1990)
27. Valero E, Garcia-Carmona F. Hysteresis and cooperative behaviors of a latent plant polyphenol oxidase. Plant Physiol. 98: 774-776 (1992)
28. Iyengar R, McEvily AJ. Anti-browning agents: Alternatives to the use of sulphites in foods. Trends Food Sci. Tech. 3: 60-64 (1992)