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Food Chemistry 87 (2004) 59–67
Food Chemistry
www.elsevier.com/locate/foodchem

Partial purification and characterization of polyphenol oxidase from artichoke (*Cynara scolymus* L.) heads

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Received 25 April 2003; received in revised form 20 October 2003; accepted 20 October 2003

Abstract

Partial characterization of polyphenol oxidase activity in artichoke heads is described. Stable and highly active PPO extracts were obtained using 1.0% (w/v) polyethylene glycol (PEG), 1.5% (w/v) Triton X-100 and 0.1% NaCl in 0.2 M potassium phosphate buffer, pH 6.0. Three isoenzymes of the artichoke PPO were detected by polyacrylamide gel electrophoresis. The pH optimum for artichoke PPO was found to be a very broad (5.0–7.0) and the enzyme activity was stable in the range 6.0–7.0 at 25 °C for 60 min. The optimum temperature was 25 °C. The enzyme was heat-stable between 20 and 30 °C and completely inactivated at 80 °C after 5 min. The activation energy (E_a) with catechol was 15.8 kJ/mol at pH 6.0. PPO showed activity to catechol, pyrogallol and 4-methylcatechol, DL-dopa, L-dopa and gallic acid. (K_m and V_{max} values were 10.2 mM and 19,662 U/ml min for catechol, 12.4 mM and 12,500 U/ml min for 4-methylcatechol, 14.3 mM and 8065 U/ml min for pyrogallol, 37.7 mM and 5865 U/ml min for L-dopa 36.3 mM and 6060 U/ml min for DL-dopa, 43.6 mM and 4620 U/ml min for gallic acid, respectively). L-tyrosine was also tested but was not oxidized by artichoke PPO. The I_{50} values of inhibitors studied on PPO were determined by means of activity percentage (I_{50}) diagrams. The values were 6.17×10^{-5} M, 6.32×10^{-5} M, 9.11×10^{-5} M, 1.76×10^{-5} M, 1.47×10^{-5} M, 8.33×10^{-5} M, 4.12×10^{-5} M, 1.94×10^{-4} M and 1.83×10^{-5} M for glutathione, thiourea, sodium azide, sodium metabisulfite, dithiothreitol, β-mercaptoethanol, sodium diethyl dithiocarbamate, oxalic acid and ascorbic acid, respectively. Therefore, the most effective inhibitor was dithiothreitol, followed in decreasing order by sodium metabisulphide and ascorbic acid. Metal ions (Zn^{++}, Ba^{++}, Cu^{++}) were poor inhibitors of the enzyme at 10 mM.

Keywords: Artichoke (*Cynara scolymus* L.); Polyphenol oxidase; Characterization; Inhibitors

1. Introduction

Polyphenol oxidase (PPO E.C.1.14.18.1) is a copper-containing enzyme which catalyzes the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-dihydroxyphenols to *o*-quinones, utilising molecular oxygen. These quinones are highly reactive, electrophilic molecules which covalently modify one crosslink to a variety of cellular constituents. The reactions produce undesirable blackening or browning in food processing and post-harvest physiology of plant products and are the main focus of interest in PPO in food technology.

The role of PPO in plants is not yet clear, but it has been proposed that it may be involved in necrosis development around damaged leaf surfaces and in defence mechanisms against insects and plant pathogen attack. Phenolic compounds may function by inhibiting bacterial growth or serve as precursors in the formation of physical polyphenolic barriers, limiting pathogen translocation. PPO-generated quinones modify plant proteins, decreasing the plant's nutritive availability to herbivores or invaders. Polymeric polyphenols seem to be more toxic to potential phytopathogens than are the phenolic monomers.

PPO has been studied in several plant tissues, such as apples (Murata, Kurokami, & Homma, 1992, 1993, 1995); bananas (Galeazzi, Scarbieri, & Constantinides, 1981; Kahn, 1985; Yang, Fujita, Ashrafuzzaman, Nakamura, & Hayashi, 2000), peaches (Flurkey & Jen, 1980), grapes (Lamikandra, Sharon, & Mitwe, 1992; Wissemann & Lee, 1985), plums (Siddig, Sinha, & Cash,

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doi:10.1016/j.foodchem.2003.10.017

1992), herbs (Arslan, Temur, & Tozlu, 1997), spinach (Golbeck Cammonata, 1995), broad beans (Flurkey, 1989; Huntcheson & Buchanan, 1980), field beans (Paul & Gowda, 2000), wild potatoes (Kowalski, Eannetta, Hirzei, & Steffens, 1992), Jerusalem artichoke (Zawistowski, Biliaderis, & Murray, 1988a, 1988b), cabbage (Fujita et al., 1995) and tea leaves (Halder, Tamuli, & Phaduri, 1998; Takeo & Baker, 1972). However, little research has been reported on the isolation and characterization of artichoke PPO. During the plant growing season, artichoke heads are stored at low temperature in polyethylene bags. When stored in a refrigerator, the plant develops unpleasant colours and flavours and loses nutrients. Therefore, it is necessary to characterize PPO, to develop more effective methods for controlling browning in artichoke.

In the present study, PPO was extracted and partially purified from artichoke heads and the characteristics of the enzyme were examined. In addition, the inhibition of enzyme activity was studied by using some ions and inhibitors.

2. Material and methods

2.1. Plant material

Artichoke heads of marketable quality were harvested from the region of Ege, in Turkey (February and March).

2.2. Reagents

Catechol, DL-dopa, L-dopa, Triton X-100, PEG (8000), glutathione, thiourea, dithiothreitol, β-mercaptoethanol, ascorbic acid and sodium metabisulfite were obtained from Sigma Chemical Co (St. Louis, USA). Potassium cyanide, ammonium sulfate and L-tyrosine were purchased from Merck, Germany. Other reagents were all of analytical grade.

2.3. Enzyme extraction and partial purification of artichoke PPO

Enzyme extracts were prepared so that PPO activity was at the highest level (Table 1). In all assays, 20 g of fresh artichoke heads were homogenized in 50 ml of extraction buffer. The homogenate was filtered through four layers of cheesecloth and centrifuged at 4000 *g* for 10 min at 4 °C. Solid $(NH_4)_2SO_4$ was added to the supernatant to obtain 50% saturation and then centrifuged at 14,000 *g* for 60 min at 4 °C. The precipitate was dissolved in 0.2 M phosphate buffer (pH 6.0). The enzyme extract was extensively dialyzed against the same buffer at 4 °C overnight. The dialyzed sample was used as the PPO enzyme source in the following experiments.

Table 1
Influence of extraction buffer composition on artichoke PPO activity

Buffer composition	PPO activity (U/ml min)
0.05 M Potassium phosphate (pH 6.0) + 1% (W/V) PEG	9292
0.05 M Potassium phosphate (pH 6.0) + 1.5% (W/V) Triton X-100	9856
0.05 M Potassium phosphate (pH 6.0) + 1.5% (W/V) Triton X-100 + 1% (W/V) PEG	10,116
0.05 M Potassium phosphate (pH 6.0) + 1.5% (W/V) Triton X-100 + 1% (W/V) PEG + 0.1% NaCl	10,469
0.2 M Potassium phosphate (pH 6.0) + 1% (W/V) PEG	9552
0.2 M Potassium phosphate (pH 6.0) + 1.5% (W/V) Triton X-100	10,954
0.2 M Potassium phosphate (pH 6.0) + 1.5% (W/V) Triton X-100 + 1% (W/V) PEG	12,050
0.2 M Potassium phosphate (pH 6.0) + 1.5% (W/V) Triton X-100 + 1% (W/V) PEG + 0.1% NaCl	13,484

2.4. PPO activity assay

PPO activity was determined by measuring the initial rate of quinone formation, as indicated by an increase in absorbance at 420 nm (Coseteng & Lee, 1978). A Jasco spectrophotometer was employed throughout the investigation. One unit of enzyme activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 per min. PPO activity was assayed in triplicate measurements. The sample cuvette contained 2.95 ml of 20 mM catechol solution in 0.1 M phosphate buffer (pH 6.0) and 0.05 ml of the enzyme solution. The blank sample contained only 3 ml of substrate solution. Molarity of the reaction buffer was selected as indicated in Table 2.

2.5. Electrophoresis and gel staining

Polyacrylamide slab gel electrophoresis was performed according to the method of Laemmli (1970) for separating PPO isoenzymes of artichoke in natural conditions. Crude extract sample was mixed with glycerol and bromophenol blue before being applied to 7.5% polyacrylamide gel. Electrophoresis was performed at a constant current of 25 mA at 4 °C for 3 h. The gel was

Table 2
Effect of reaction mixture buffer molarity on artichoke PPO activity

Buffer composition	PPO activity (U/ml min)
0.05 M Potassium phosphate (pH 6.0)[a]	10,400
0.1 M Potassium phosphate (pH 6.0)[a]	13,300
0.2 M Potassium phosphate (pH 6.0)[a]	12,250

[a] The pH 6.0 was selected by a previous pH study of optimal conditions for spectrophotometric PPO assay.

cut into two symmetrical parts and then stained for PPO activity by 2.5 mM L-dopa and DL-dopa in phosphate buffer pH 8.0. After 1 h of incubation of the gels, isoenzyme bands were developed. The gels were shaken in 1 mM ascorbic acid solution for 5 min and stored in 30% ethanol and then their photographs were taken.

2.6. Protein estimation

Protein content was determined according to the dye-binding method of Bradford (1976) using bovine serum albumin as standard.

2.7. Effect of pH on enzyme activity

PPO activity, as a function of pH, was determined under standard assay conditions using various buffers: acetate 0.1 M (pH 3.0–5.0), phosphate 0.1 M (pH 5.0–7.0) and Tris–HCl 0.1 M (pH 7.0–10.0). To determine pH stability, the enzyme was preincubated in acetate (0.1 M, pH 4.0 and 5.0), phosphate (0.1 M, pH 5.0 and 7.0) or Tris–HCl (0.1 M, pH 8.0 and 9.0) for 60 min at 25 °C. Residual PPO activity was measured under standard assay conditions.

2.8. Effect of temperature on enzyme activity and determination of activation energy

PPO activity, as a function of temperature, was determined under standard assay conditions, using temperatures from 5 to 80 °C. Thermal stability of PPO was determined by heating the enzyme solution at various temperatures between 20 and 80 °C for 60 min at pH 6.0. Residual PPO activity was measured under standard assay conditions. Activation energy (E_a) was determined, according to the Arrhenius equation, by measuring the maximal initial rate at different temperatures and plotting the logaritmic value of V_{max} vs. $1/T$ (Segel, 1976).

2.9. Substrate specificity and enzyme kinetics

Michaelis Menten constant (K_m) and maximum reaction velocity (V_{max}) were determined using seven substrates (catechol, 4-methylcatechol, pyrogallol, DL-dopa, L-dopa, gallic acid and L-tyrosine) in five different concentrations and under standard conditions. Data were plotted as $1/V$ and 1/[S] concentration according to the method of Lineweaver and Burk (1934).

2.10. Effect of various inhibitors on enzyme activity

To determine the effect of inhibitors, PPO activity was measured in the standard reaction medium in the presence or absence of the stated concentration of inhibitor.

Using five different concentrations of the substrates, PPO activities were measured at three constant inhibitor concentrations with the inhibitors. Values $1/V$ and 1/[S] were employed to draw Lineweaver–Burk graphs. Finally, K_i constant values were obtained from the graphs. To determine the inhibitor concentration that reduced the enzyme activity by 50% (I_{50}), regression analysis graphs were drawn by using percent inhibition values by a statistical package programme on a computer. I_{50} values were determined from the graphs.

2.11. Effects of different ions on the enzyme activity

PPO activity was measured in the presence (final concentration 1.0 or 10.0 mM) and absence of various ionic compounds under the standard conditions.

3. Results and discussion

3.1. Selection of conditions for enzyme assay

Several buffer compositions were employed to select the most suitable to extract PPO from artichoke heads (Table 1). The increase in molar concentration of potassium phosphate buffer increases the extraction of PPO activity to nearly 20% when PEG, Triton X-100 and NaCl were also employed. Addition of detergent Triton X-100 produced a slight increase of PPO activity of extracts made from low and high concentrated buffer. The increase of ionic strength by addition of sodium chloride only produced a slight increase in PPO activity when extracts were made from low and high concentration buffers. Therefore, a 0.2 M phosphate buffer, containing 1% (w/v) PEG, 1.5% (w/v) Triton X-100 and 0.1% NaCl was employed for all enzyme assays.

Tanning reactions during enzyme extraction can cause partial inactivation of the enzyme. Such reactions can be partially prevented by isolation under N_2, or in the presence of reducing agents or phenol-adsorbing agents, such as polyethylene glycol (PEG), polyamide or polyvinylpyrrolidone (PVP) (Mayer & Harel, 1979). In the present work, the use of PEG alone was not enough to absorb all phenolics and, consequently, extracted PPO activity was low. However, the use of a detergent (Triton X-100), together with PEG improved the extraction, combining polar and nonpolar binding capacities. An increase of enzyme activity by this detergent treatment has been reported (Cano, Lobo, Ancos, & Galeazzi, 1996; Zhou & Feng, 1991).

3.2. Electrophoresis study

Three isoenzymes were separated by native PAGE and detected using L-dopa and DL-dopa substrates. In both cases, the fast moving third bands were more active

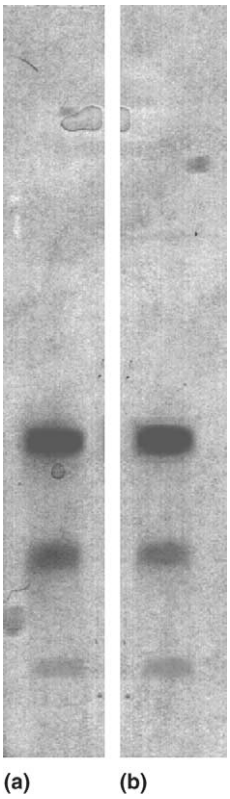

Fig. 1. Native PAGE of the artichoke PPO. (a) Activity staining by 2.5 mM L-dopa; (b) activity staining by 2.5 mM DL-dopa.

than the slow moving first and second bands (Fig. 1a and b).

Park and Luh (1985) reported four forms of PPO as isoenzymes in kiwis. Rivas and Whitaker (1973) reported the existence of two types of PPO in Bartlett pears. Our result differed from these but agreed with those of Flurkey and Jen (1980), who reported three types of PPO in peach. Possibly the number of PPO isoenzymes differs among species.

3.3. The effect of pH on PPO activity and stability

Different pH optima were determined for each substrate. The pH optima for catechol was found to have a very broad optimum, between 5.0 and 7.0, with more than 95% of the maximum activity occurring between these limits. These results was similar to the optimum for PPO in yali pear (between pH 5.8 and 7.8) (Zhou & Feng, 1991) and kiwi between pH (6.8 and 7.3) (Park & Luh, 1985) using catechol as substrate (Fig. 2).

PPO activity decreased below pH 5.0 and above pH 7.0 but the enzyme was still active at pH 3.0 with relative activity close to 40%. At pH 10.0, the retained activity of enzyme was 45%. Maximum PPO activities were obtained at pH 6.0 for 4-methylcatechol and 4-methylcatechol, pH 8.0 for DL-dopa and L-dopa and pH 6.5 for pyrogallol. An increase of ionic strength, from 0.05

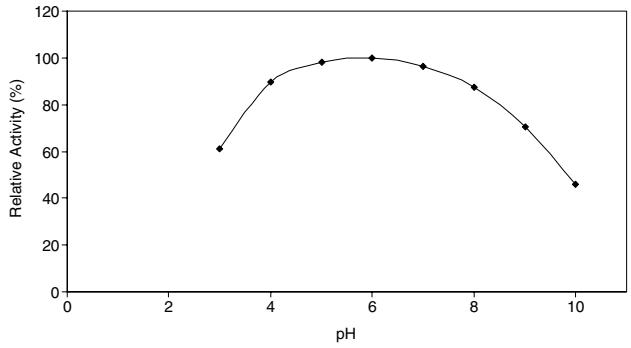

Fig. 2. Effect of pH on artichoke PPO activity with catechol as substrate.

to 0.1 M of potassium phosphate buffer, produced a nearly 22% increase in PPO enzyme activity (Table 2).

Optimum pH values for the enzyme from different sources have been reported: 4.5 for green olive (Ben Shamol, Kahn, Harel, & Mayer, 1977) 5.0 for potato (Balasingam & Ferdinand, 1970) and 7.0 for peppermint (Kavrayan & Aydemir, 2001) with catechol as substrate. Aylward and Haisman (1969) reported that the optimum pH for maximum PPO activity in plants varies from about 4.0–7.0, depending on the extraction methods, the substrate used for assay, and the localization of the enzyme in the plant cell.

The stability of the enzyme was examined by incubating the protein preparation at different pH values at 25 °C for 60 min; as shown in Fig. 3, the enzyme retained more than 95% of its original activity at pH 6.0 and 7.0 but lost its activity below pH 5.0 or above pH 7.0. The stability of the partially purified enzyme in basic media was lower than that in acidic media. Incubation of the enzyme for 60 min at pH 4.0, 8.0 and 9.0, caused 30%, 70% and 85% loss of activity, respectively. Kavrayan and Aydemir (2001) reported that *M. piperita* PPO was stable betwen pH 6.0 and 7.0 at 25 °C for 30 min. Rivas and Whitaker (1973) reported the instability of pear PPO below pH 3.5. Siddig et al. (1992) reported

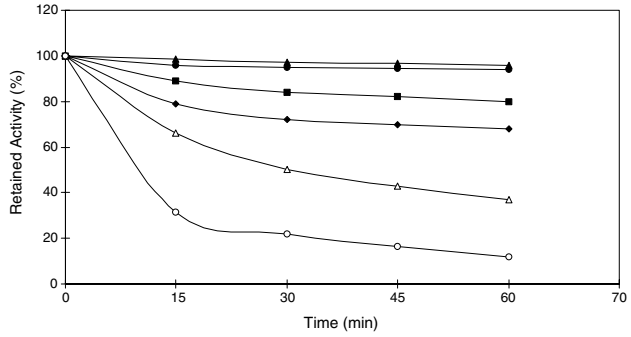

Fig. 3. pH stability of artichoke PPO with catechol as substrate. (-▲-) pH 6.0; (-●-) pH 7.0; (-■-) pH 5.0; (-◆-) pH 4.0; (-△-) pH 8.0; (-○-) pH 9.0.

that plum PPO was unstable below pH 4.5. These results indicate that plant PPO was stable near neutral pH.

3.4. The effect of temperature on PPO activity and stability

The effect of temperatures between 5 and 80 °C on PPO activity showed that optimum temperature for the PPO enzyme was 25 °C (Fig. 4). This value was similar to those of Amasya apple [18 °C (Oktay, Kühevioğlu, & Şakiroğlu, 1995)], grape [25 °C (Yokotsuka, Makino, & Singleton, 1988)] and stanley plums [20 °C (Siddig et al., 1992)]. The enzyme was incubated at different temperatures for 60 min at pH 6.0 and, after cooling, the residual enzyme activity was measured. Consequently, it was found that the enzyme was stable at 20 and 30 °C for 60 min but was unstable at temperatures above 30 °C. Fig. 5 shows that temperatures above 30 °C resulted in loss of enzyme activity. At high temperature, the enzyme activity was rapidly lost. For instance, when the temperature was increased from 40 to 60 °C, the activity of PPO decreased from 75% to 15%. This indicated that the enzyme was rapidly denatured at higher temperatures. The times required for 50% inactivation of activity at 60 and 70 °C were found to be 6 and 4 min, respectively.

It has been reported that *Allium* sp. PPO was stable at 40 °C for 30 min (Arslan et al., 1997). Stanley plum PPO at 70 °C (Siddig et al., 1992) and banana, at 70 °C, were stable for 30 min (Yang et al., 2000) and Jerusalem artichoke PPO at 60 °C for 30 min (Zawistowski et al., 1988a).

E_a value for PPO in artichoke with catechol substrate is estimated as 15.8 kJ/mol. The activation energy of artichoke PPO with caffeic acid substrate was 21.7 kJ/mol (Leoni, Palmeri, Lattanzio, & Van Sumere, 1990). E_a values were reported for purified PPO from kiwi fruit (17 kJ/mol) (Park & Luh, 1985), partially purified PPO from wild rice (20 kJ/mol) (Owusa-Ansak, 1989) and purified PPO from banana (18.6 kJ/mol) (Palmer, 1963).

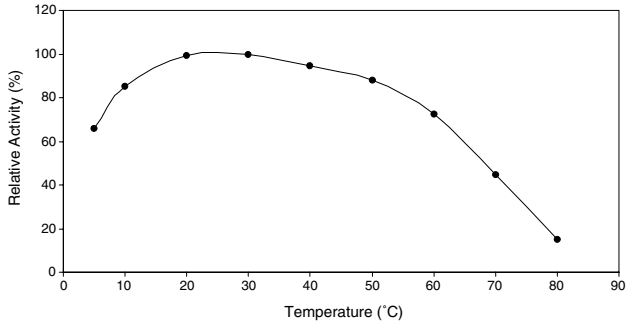

Fig. 4. Effect of temperature on artichoke PPO activity with catechol as substrate.

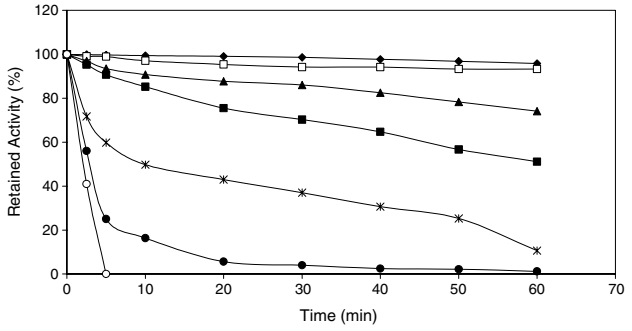

Fig. 5. Heat inactivation of artichoke PPO at various temperatures with catechol as substrate. (-◆-) 20 °C; (-□-) 30 °C; (-▲-) 40 °C; (-■-) 50 °C; (-∗-) 60 °C; (-●-) 70 °C; (-○-) 80 °C.

3.5. Substrate specificity and enzyme kinetics

Extracted PPO from different sources has been shown to have varying substrate specificity, as reported by Wong, Luh, and Whitaker (1971) and Jen and Kahler (1974).

The oxidizing ability of the partially purified PPO of artichoke heads was determined using monophenols, diphenols and triphenols as substrates. The substrate with highest activity was catechol, followed by 4-methylcatechol, pyrogallol, DL-dopa, L-dopa and gallic acid (Table 3). Artichoke PPO also showed no activity toward tyrosine (the monophenols), suggesting the absence of monophenolase (cresolase) activity. These results are similar to those reported by Halim and Montgomery (1978) and Zhou and Feng (1991) for d'Anjou and yali pear PPO, respectively.

Michaelis constants (K_m) and maximum reaction velocities (V_{max}) were determined using these substrates at various concentrations and under optimum conditions (pH and temperature). The Lineweaver–Burk plot analysis of this enzyme preparations showed K_m values of 10.2 mM (catechol), 12.4 mM (4-methylcatechol), 14.3 mM (pyrogallol), 36.3 mM (DL-Dopa), 37.7 mM (L-Dopa) and 43.6 mM (gallic acid) (Table 2). This value for catechol was similar to that of tea leaf (12.5 mM) (Halder et al., 1998), and field bean seed [10.5 mM] (Paul & Gowda, 2000). The artichoke K_m value is lower than the 34 mM for Amasya apple (Oktay et al., 1995), 20 mM for Stanley plum (Siddig et al., 1992) and compares with a K_m value of 67 mM for Concord grape (Cash, Sistrunk, & Stutte, 1976), with catechol as substrate. Artichoke PPO showed the lowest affinity for L-Dopa. On the other hand, K_m value for pyrogallol was similar to those of spinach, 15.7 mM (Golbeck & Cammarata, 1981) and tea leaf, 17.8 mM (Halder et al., 1998).

3.6. The effect of storage time on enzyme activity

Effects of different storage temperatures on partially purified artichoke PPO were studied over a 60 day period at pH 6.0 and the residual activity was measured. At 25 °C, the enzyme completely lost its activity after

Table 3
Optimum pH, temperature, K_m and V_{max} values of the polyphenol oxidase with different substrates

Substrate	Optimum pH	Optimum temperature (°C)	K_m (M)	V_{max} (U/ml min)
Catechol	6.0	25	10.2×10^{-3}	19,662
4-Methylcatechol	6.0	25	12.4×10^{-3}	12,500
DL-Dopa	8.0	30	36.3×10^{-3}	6060
L-Dopa	8.0	30	37.7×10^{-3}	5865
Pyrogallol	6.5	25	14.3×10^{-3}	8065
Gallic acid	6.0	25	43.6×10^{-3}	4620

three days. At 4 °C, 25% loss in PPO activity was observed during the first seven days; however, at the end of 21 days, more than 87% of its activity was lost. The enzyme was more stable at −15 °C; it lost only 10% of its enzyme activity on storage for two weeks and was completely inactivated after 2 months.

Nagai and Suzuki (2001) reported that cabbage PPO enzyme completely lost its activity after 14 days at 4 °C. Wissemann and Montgomery (1985) reported that 25% of the enzyme activity was retained during storage at 4 °C for 15 days. On the other hand, Kowalski et al. (1992) reported that wild potato PPO was stable at −70 °C for several weeks, but it was stable at 4 °C for only one day.

3.7. The effect of inhibitors

Various inhibitors were examined to determine their potential for inhibition of PPO activity. These inhibitors included a substrate analogue, which is also a metal chelator and reducing agents.

Lineweaver–Burk plots of $1/v$ versus 1/[S] at three inhibitor concentrations, determined the type of inhibition. Table 4 shows the inhibition results with catechol as the substrate. From the K_i constants, it is concluded that inhibition modes of the inhibitors are as follows: dithiothreitol, β-mercaptoethanol and ascorbic acid are competitive; and the others, noncompetitive. I_{50} values were also obtained with these inhibitors, using catechol

Table 4
K_i values and inhibition types of polyphenol oxidase with different inhibitors

Compounds	I (M)	K_i (M)	Type of inhibition	% Inhibition	I_{50}
Dithiothreitol	7.0×10^{-6}	5.1×10^{-6}	Competitive	34	1.47×10^{-5}
	1.0×10^{-5}	$2.6 \times .10^{-6}$		42	
	2.5×10^{-5}	2.5×10^{-6}		67	
Thiourea	3.0×10^{-5}	6.0×10^{-5}	Noncompetitive	31	6.54×10^{-5}
	5.0×10^{-5}	3.7×10^{-5}		39	
	8.0×10^{-5}	2.9×10^{-5}		59	
Sodium azide	5.0×10^{-5}	8.2×10^{-5}	Noncompetitive	36	9.11×10^{-5}
	8.0×10^{-5}	5.3×10^{-5}		55	
	1.1×10^{-4}	2.3×10^{-5}		64	
β-mercaptoethanol	3.3×10^{-5}	8.2×10^{-5}	Competitive	15	8.33×10^{-5}
	6.6×10^{-5}	5.3×10^{-5}		34	
	1.0×10^{-4}	2.3×10^{-5}		60	
Glutathione	3.0×10^{-5}	5.4×10^{-5}	Noncompetitive	23	7.80×10^{-5}
	5.0×10^{-5}	4.1×10^{-5}		31	
	9.0×10^{-5}	2.9×10^{-5}		60	
Sodium metabisulfite	7.0×10^{-6}	1.2×10^{-5}	Noncompetitive	25	1.76×10^{-5}
	1.5×10^{-5}	6.9×10^{-6}		42	
	2.5×10^{-5}	4.1×10^{-6}		61	
Sodiumdiethyldithiocarbamate	3.0×10^{-5}	4.9×10^{-5}	Noncompetitive	37	4.12×10^{-5}
	5.0×10^{-5}	3.6×10^{-5}		66	
	8.0×10^{-5}	2.6×10^{-5}		73	
Oxalic acid	2.3×10^{-4}	5.2×10^{-5}	Noncompetitive	36	1.94×10^{-4}
	1.7×10^{-4}	9.3×10^{-5}		44	
	1.0×10^{-4}	1.7×10^{-4}		73	
Ascorbic acid	1.1×10^{-5}	7.1×10^{-6}	Competitive	23	1.80×10^{-5}
	1.8×10^{-5}	6.2×10^{-6}		41	
	0.6×10^{-5}	8.3×10^{-6}		68	

as the substrate. The values were 6.17×10^{-5} M, 6.32×10^{-5} M, 9.11×10^{-5} M, 1.76×10^{-5} M, 1.47×10^{-5} M, 8.33×10^{-5} M, 4.12×10^{-5} M, 1.94×10^{-4} M and 1.83×10^{-5} M for glutathione, thiourea, sodium azide, sodium metabisulfite, dithiothreitol, β-mercaptoethanol, sodium diethyl dithiocarbamate, oxalic acid and ascorbic acid, respectively.

Of all inhibitors used in the study, dithiothreitol was most effective for inhibition of the observed artichoke PPO activity, followed by sodium metabisulfite and ascorbic acid.

The inhibitor reaction mechanism differs, depending on the reducing agent employed. Inhibition by thiol compounds is attributed to either the stable colourless products formed by an addition reaction with *o*-quinones (Ikediobi & Obasuyi, 1982) or binding to the active centre of PPO, like metabisulfite (Valero & Garcia-Carlmona, 1992). Ascorbate acts more as an antioxidant than as an enzyme inhibitor because it reduces the initial quinone formed by the enzyme to the original diphenol before it undergoes secondary reactions which lead to browning. Ascorbic acid has also been reported to cause irreversible inhibition (Golan, Goldhirsh, & Whitaker, 1984).

The effects of various compounds on the artichoke PPO activity are listed in Table 5. Thirty percent decrease in enzyme activity was observed at 10 mM concentration of $MnCl_2$, $CoCl_2$, $CaCl_2$ and $NiCl_2$. EDTA was a poor inhibitor of the enzyme, as reported for PPO in peach (Luh & Phithakpol, 1972) and avocado (Kahn, 1976). These compounds also inhibited PPO in Japanese pear (Tono, Fujita, Kawasaki, & Li, 1986).

Cu^{++} and Fe^{+++} ions activated the oxidation of PPO at 1 mM (Table 5). Similar PPO activations by Cu^{++} and Fe^{+++} ions were recorded, both by Simpson, Marshall, and Otwell (1987) and Leoni et al. in the same concentrations (1990). Leoni et al., reported that these PPO activations do not seem to be important enough to suggest that Cu^{2+} and Fe^{3+} ions have important roles in enzymic browning of artichoke heads during storage. The enzyme activity was markedly inhibited by sodium metabisulfite and potassium cyanide. However, metal ions (Zn^{++}, Ba^{++}, Cu^{++}) were poor inhibitors of the enzyme at 10 mM. Similar effects of these compounds were found on the purified PPO from Satsuma Mandarin (Fujita & Tono, 1979) and cabbage (Fujita et al., 1995). PPO was markedly inhibited by $Hg(CH_3COO)_2$ and NaF at 10 mM.

The possible PPO inhibitory effects of three nontoxic compounds, ascorbic acid, citric acid, and NaCl were studied. NaCl did not show away significant effect on enzyme activity. But Luh and Phithakpol (1972) reported 16% inhibition of peach PPO using 0.6% NaCl. Citric acid and acetic acid weakly inhibited the artichoke PPO activity at 10 mM under standard pH conditions (0.1 M phosphate buffer, pH 6.0). However, under low buffer capacity (using 0.01 M phosphate buffer, pH 6.0), PPO activity was markedly inhibited by citric acid and acetic acid at 10 mM, in which the pH values of the reaction mixtures were 3.0 and 3.5, respectively. Similar inhibitory effects of acetic acid and citric acid were found in the browning of head lettuce (Castner, Gil, Artes, & Tomas-Barberan, 1996). Of these, only ascorbic acid proved to be significantly inhibitory towards artichoke PPO. These results suggest that L-ascorbic acid, citric acid and acetic acid are good inhibitors of enzymatic browning of artichoke (Lattanzio, Linsalata, Sandro, & VanSumere, 1989). Research has shown that ascorbic acid and citric acid improve the shelf life of artichoke heads.

Table 5
Effects of various compounds on the activity of PPO from artichoke

Compounds	Relative activity (%)	
	1 mM[a]	10 mM[a]
None	100	100
$CuSO_4$	109	90
$CaCl_2$	87	70
$ZnSO_4$	106	91
$BaCl_2$	98	96
$MnCl_2$	84	72
$NiCl_2$	86	69
NaF	76	54
$CoCl_2$	95	73
$MgSO_4$	100	99
$FeCl_3$	105	102
NaCl	100	99
KCN	51	21
$Hg(CH_3COO)_2$	47	15
EDTA	97	90
Citric acid	98	89
Acetic acid	99	93
L-Ascorbic acid	0	0

[a] Final concentration of various compounds.

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