Characterization and Purification of Polyphenol Oxidase from Artichoke (Cynara scolymus L.)

ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · MARCH 2005

Impact Factor: 2.91 · DOI: 10.1021/jf049053g · Source: PubMed

4 AUTHORS, INCLUDING:

Yusuf Turan
International BURCH University
32 PUBLICATIONS **191** CITATIONS
SEE PROFILE

Hatibe Ertürk Kara
Balikesir University
8 PUBLICATIONS **68** CITATIONS
SEE PROFILE

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization and Purification of Polyphenol Oxidase from Artichoke (*Cynara scolymus* L.)

SERAP DOĞAN,*,† YUSUF TURAN,† HATIBE ERTÜRK,† AND OKTAY ARSLAN§

Departments of Biology and Chemistry, Faculty of Science and Literature, University of Balikesir, 10100 Balikesir, Turkey

In this study, the polyphenol oxidase (PPO) of artichoke (*Cynara scolymus* L.) was first purified by a combination of $(NH_4)_2SO_4$ precipitation, dialysis, and a Sepharose 4B−L-tyrosine−*p*-aminobenzoic acid affinity column. At the end of purification, 43-fold purification was achieved. The purified enzyme migrated as a single band on sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis indicated that PPO had a 57 kDa molecular mass. Second, the contents of total phenolic and protein of artichoke head extracts were determined. The total phenolic content of artichoke head was determined spectrophotometrically according to the Folin−Ciocalteu procedure and was found to be 425 mg 100 g^{-1} on a fresh weight basis. Protein content was determined according to Bradford method. Third, the effects of substrate specificity, pH, temperature, and heat inactivation were investigated on the activity of PPO purified from artichoke. The enzyme showed activity to 4-methylcatechol, pyrogallol, catechol, and L-dopa. No activity was detected toward L-tyrosine, resorsinol, and *p*-cresol. According to V_{max}/K_m values, 4-methylcatechol (1393 EU min^{-1} mM^{-1}) was the best substrate, followed by pyrogallol (1220 EU min^{-1} mM^{-1}), catechol (697 EU min^{-1} mM^{-1}), and L-dopa (102 EU min^{-1} mM^{-1}). The optimum pH values for PPO were 5.0, 8.0, and 7.0 using 4-methylcatechol, pyrogallol, and catechol as substrate, respectively. It was found that optimum temperatures were dependent on the substrates studied. The enzyme activity decreased due to heat denaturation of the enzyme with increasing temperature and inactivation time for 4-methylcatechol and pyrogallol substrates. However, all inactivation experiments for catechol showed that the activity of artichoke PPO increased with mild heating, reached a maximum, and then decreased with time. Finally, inhibition of artichoke PPO was investigated with inhibitors such as L-cysteine, EDTA, ascorbic acid, gallic acid, D,L-dithiothreitol, tropolone, glutathione, sodium azide, benzoic acid, salicylic acid, and 4-aminobenzoic acid using 4-methylcatechol, pyrogallol, and catechol as substrate. The presence of EDTA, 4-aminobenzoic acid, salicylic acid, gallic acid, and benzoic acid did not cause the inhibition of artichoke PPO. A competitive-type inhibition was obtained with sodium azide, L-cysteine, and D,L-dithiothreitol inhibitors using 4-methylcatechol as substrate; with L-cysteine, tropolone, D,L-dithiothreitol, ascorbic acid, and sodium azide inhibitors using pyrogallol as substrate; and with L-cysteine, tropolone, D,L-dithiotreitol, and ascorbic acid inhibitors using catechol as a substrate. A mixed-type inhibition was obtained with glutathione inhibitor using 4-methylcatechol as a substrate. A noncompetitive inhibition was obtained with tropolone and ascorbic acid inhibitors using 4-methylcatechol as substrate, with glutathione inhibitor using pyrogallol as substrate, and with glutathione and sodium azide inhibitors using catechol as substrate. From these results, it can be said that the most effective inhibitor for artichoke PPO is tropolone. Furthermore, it was found that the type of inhibition depended on the origin of the PPO studied and also on the substrate used.

KEYWORDS: Artichoke; *Cynara scolymus* L.; polyphenol oxidase; substrate specificity; optimum pH and temperature; heat denaturation; purification; inhibition; inhibitors

1. INTRODUCTION

Artichoke (*Cynara scolymus* L.) is widely cultivated in Europe and America, and its head is eaten as a vegetable. Artichoke is of considerable economic importance for Turkey. The artichoke head, an immature flower, constitutes the edible part of this vegetable. The artichoke (*Cynara scolymus* L.) is not only a good food, known for its pleasant bitter taste, but also an interesting and widespread herbal drug. The chemical components of artichoke leaves have been studied extensively and have been found to be a rich source of polyphenolic

* Author to whom correspondence should be addressed (e-mail sdogan@balikesir.edu.tr).
† Department of Biology.
§ Department of Chemistry.

10.1021/jf049053g CCC: $30.25
Published on Web 01/14/2005

compounds, with mono- and dicaffeoylquinic acids and flavonoids as the major chemical components (*1*−*4*). The chemical components in the edible portion of the artichoke head remain unknown. The extracts of artichoke are used (i) in folk medicine against liver complaints, (ii) for the treatment of hepatitis and hyperlipidemia in European traditional medicine, (iii) to exert a hepatoprotective effect, (iv) to prepare herbal teas or herbal medicinal products, (v) in the treatment of hepatobiliary dysfunction and digestive complaints, such as loss of appetite, nausea, and abdominal pain, (vi) in various pharmacological test systems, antibacterial, antioxidative, anti-HIV, bile expelling, hepatoprotective, urinative, and choleretic activities and have the ability to inhibit cholesterol biosynthesis and low-density lipoprotein (LDL) oxidation, and (vii) to inhibit oxidative stress generated by reactive oxygen species in human leukocytes (*2*, *5*−*10*). Recently, research has focused on the antioxidant activity of artichoke leaf extracts. All of these properties make the artichoke very important in the food industry. One other important point is that this vegetable contains an enzyme called polyphenol oxidase (PPO). PPO (EC 1.14.18.1) is a copper-containing enzyme, widely distributed in nature, which is responsible for melanization in animals and browning in plants (*11*). Polyphenol oxidase also catalyzes the ortho-hydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones (*12*). *C. scolymus* L. is used as a material for pickled vegetables, and it is consumed in Turkey and other countries on a daily or weekly basis. When it is stored in a refrigerator, the plant develops unpleasant colors and flavors and loses nutrients when it browns. Therefore, it is necessary to chracterize the PPO to develop more effective methods for controlling browning in *C. scolymus* L.

Enzymatic browning of fruits and raw vegetables is related to oxidation of phenolic endogenous compounds into highly unstable quinones, which are later polymerized to brown, red, and black pigments. 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. Enzymatic browning is also an economic problem for processors and consumers (*11*). At least five causes of browning in processed and/or stored fruits and plants are known: enzymatic browning of the phenols, Maillard reaction, ascorbic acid oxidation, caramelization, and formation of browned polymers by oxidized lipids. The oxidation of the *o*-diphenols to *o*-quinones by PPO is the most important cause of the change in color as the *o*-quinones quickly polymerize and produce brown pigments (*12*, *13*). Enzymatic browning also causes a loss in the nutritional value through oxidation of ascorbic acid.

Enzymatic browning has been studied in several plant tissues such as aubergine (*14*), *Origanum* (*15*), apricot (*16*), *Thymus* species (*17*−*19*), *Salvia* (*20*), spinach (*21*), and tea leaves (*22*). There are a few investigations related to polyphenol oxidase activity obtained from *C. scolymus* L.: López-Molina et al. (*23*) investigated the enzymatic removal of phenols from an aqueous solution by artichoke extracts; Espin et al. (*24*) investigated the effect of pH and temperature on the monophenolase activity of PPO obtained from artichoke heads using 4-hydroxyanisole as substrate; Ziyan and Pekyardımdcı (*25*) investigated the characterization of PPO from Jerusalem artichoke; Aydemir (*26*) investigated the partial purification and characterization of PPO from artichoke; Lattanzio et al. (*27*) investigated the beneficial effect of citric and ascorbic acid on the phenolic browning reaction in stored artichoke heads and the browning phenomena in stored artichoke heads; and Leoni et al. (*28*) investigated PPO from artichoke. Enzymatic browning can be controlled in different ways. In addition to heat treatment and acidification, a wide range of chemicals inhibit PPO activity, but only a limited number of them are considered to be acceptable for the sake of consumer safety and/or cost and could act as potential alternatives to sulfites, which are very effective in controlling browning but subject to regulatory restrictions.

In the studies above, PPO was partially purified. Not investigated in detail were the enzyme and inhibiton kinetics; the contents of phenolic compounds and protein amount were not determined, and also not determined was the molecular mass of enzyme. Because little information is available on the characterization and purification of PPO from artichoke, this study has been aimed to assess some of its properties such as substrate specificity, optimum pH and temperature, heat inactivation, and molecular mass. PPO catalyzes the browning reaction occurring during fruit storage. The inhibitory potency and I_{50} values of various inhibitiors on PPO activity were determined in order to prevent or weaken browning of the artichoke PPO throughout the process. This information will be useful in devising effective methods for inhibiting browning during storage.

2. MATERIALS AND METHODS

2.1. Materials. *C. scolymus* L. used in this study was harvested fresh from a local garden in January (generative stage) in İzmir county (Turkey) and was kept frozen at −20 °C. All chemicals used in this study were of the best grade available and were used without further purification as they were purchased from Sigma Chemical Co. (Deisenhofen, Germany). Enzyme assays were carried out with the aid of a Cary |1E|g UV−visible spectrophotometer (Varian, Australia).

2.2. Extraction and Purification Procedure. *C. scolymus* L. (10 g) was placed in a Dewar flask under liquid nitrogen for 10 min to decompose cell membranes. A 10 g sample of *C. scolymus* L. was homogenized using a Waring blender for 2 min in 100 mL of 0.1 M phosphate buffer (pH 6.5) containing 10 mM ascorbic acid and 5% poly(ethylene glycol). The 0.1 M concentration was chosen to avoid the influence of enzymatic extract ionic strength on PPO activity, as described by Angleton and Flurkey (*29*). The homogenate was filtered, and the filtrate was centrifuged at 15000*g* for 30 min at 4 °C. The supernatant obtained was used as crude extract. The supernatant was brought to 80% $(NH_4)_2SO_4$ saturation with solid $(NH_4)_2SO_4$. The preciptated PPO was separated by centrifugation at 15000*g* for 60 min. The precipitate was dissolved in a small amount of homogenization buffer and dialyzed at 4 °C in the same buffer for 24 h with three changes of buffer during dialysis. The dialyzed sample was used as the PPO enzyme source in the following experiments (*19*). After dialysis, the active fraction was purified with affinity chromotography. The affinity gel used was synthesized according to the method of Arslan and Erzengin (*30*).

2.3. Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the method of Laemmli (*31*). Samples were applied to 10% polyacrylamide gels. The slab gels of 1.5 mm thickness were run at a constant current of 180 mV. Gels were stained for protein using a standard Coomassie Blue method.

2.4. Molecular Mass Determination. The molecular mass of the purified enzyme was determined by SDS-PAGE. Affinity chromatography was done according to the method of Arslan and Erzengin (*30*). SDS-PAGE was carried out using an SDS marker protein kit as standard.

2.5. Determination of Total Phenolic Compound Content. Total phenolics were determined using the Folin−Ciocalteu reagent (*32*). Samples (2 g) were homogenized in 80% aqueous ethanol at room temperature and centrifuged in cold at 10000*g* for 15 min, and the supernatant was saved. The residue was re-extracted twice with 80% ethanol, and the supernatants were pooled, put into evaporating dishes,

and evaporated to dryness at room temperature. The residue was dissolved in 5 mL of distilled water. One hundred microliters of this extract was diluted to 3 mL of the water, and 0.5 mL of Folin−Ciocalteu reagent was added. After 3 min, 2 mL of the 20% of sodium carbonate was added, and the contents were mixed thoroughly. The color was developed and the absorbance measured at 650 nm in a Carry |1E|g UV−visible spectrophotometer after 60 min using catechol as a standard. The result was expressed as milligrams of catechol per 100 g of fresh weight material.

2.6. Determination of Protein Content. The protein content was determined according to the Bradford method using bovine serum albumin as standard (*33*).

2.7. Spectrophotometric Assays. Kinetic assays were carried out by measuring the increase in absorbance at 420 nm for catechol and 4-methylcatechol, at 320 nm for pyrogallol, and at 460 nm for L-dopa with a Carry |1E|g UV−visible spectrophotometer (Varian). Temperature was kept at 25 °C using a Tempette Junior TE-85 circulating water bath with a heater/cooler. The reaction was carried out in a 1 cm light path quartz cuvette. The sample cuvette contained 2.9 mL of substrates in various concentrations prepared in the homogenization buffer and 0.1 mL of the enzyme. For each measurement, the volume of solution in the quartz cuvette was kept constant at 3 mL. The reference cuvette contained all of the components except the substrate, with a final volume of 3 mL (*14*, *34*).

2.8. Enzyme Kinetics and Substrate Specificity. PPO activity was assayed using 4-methylcatechol, pyrogallol, catechol, L-dopa, *p*-cresol, L-tyrosine, and resorsinol as substrates. The rate of the reaction was measured in terms of the increase in absorbance at the absorption maxima of the corresponding quinone products for each substrate. One unit of enzyme activity was defined as the amount of enzyme causing a change of 0.001 in absorbance per minute. For each substrate, the kinetic data were plotted as 1/activity versus 1/substrate concentration, according to the method of Lineweaver−Burk, and Michaelis−Menten constant (K_m) and maximum velocity (V_{max}) were determined with variable substrate concentrations in the standard reaction mixture. Substrate specificity (V_{max}/K_m) was calculated by using the data obtained on a Lineweaver−Burk plot (*20*).

2.9. Effect of pH. PPO activity as a function of pH was determined using 4-methylcatechol, pyrogallol, and catechol as substrates. The buffers used were 0.1 M acetate (pH 4.0−6.0) and 0.1 M phosphate (pH 6.0−9.0) adjusted with 0.1 M NaOH and HNO_3 (*34*).

2.10. Effect of Temperature. For determining the optimum temperature values of the enzyme, PPO activity was measured at different temperatures in the range of 10−60 °C using three different substrates as indicated above. The effect of temperature on the activity of PPO was tested by heating the standard reaction solutions (buffer and substrate) to the appropriate temperatures before introduction of the enzyme. The desired temperatures were provided by using a Tempette Junior TE-85 temperature controller attached to the cell-holder of the spectrophotometer. Once temperature equilibrium was reached, enzyme was added and the reaction was followed spectrophotometrically at constant temperature at given time intervals. The reaction mixture contained 0.6 mL of substrate, 2.3 mL of 0.1 M buffer solution, and 0.1 mL of enzyme solution. As mentioned, each assay mixture was repeated twice using the same stock of enzyme extract (*18*).

2.11. Heat Inactivation of PPO. The thermal denaturation of the partially purified enzyme was studied at 35, 55, and 75 °C. For the study, 1 mL of enzyme solution in a test tube was incubated at the required temperature for fixed time intervals. At the end of the required time interval, the test tube was cooled in an ice bath. The activity of the enzyme was then determined at 25 °C (*14*).

3. RESULTS AND DISCUSSION

3.1. Total Phenolics. It was found that the level of total phenolic compounds in the artichoke extracts was approximately 425 mg per 100 g of fresh weight. Similar results were found for vegetables such as mint (400 mg), black carrots (350 mg), aonla (349 mg), and beet root (323 mg) (*35*). However, the total phenolic contents of vegetables such as fresh turmeric (176 mg), broccoli (88 mg), tomato (68 mg), and yam (92 mg) are lower than those obtained in artichoke. As seen above, it can be said that artichoke has a rich phenolic compound content.

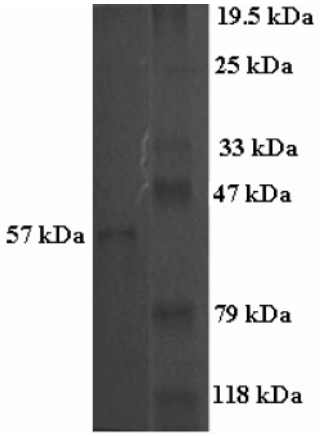

Figure 1. SDS-PAGE of artichoke PPO.

Table 1. Purification of Artichoke PPO

type of extract	vol of extract (mL)	protein concn (μg mL^{-1})	activity (EU mL^{-1})	specific activity (EU mg^{-1} of protein)	purifn -fold
crude extract	75	576	3215	5.6	
$(NH_4)_2SO_4$ preciptation	12	1171	6588	5.6	1.00
dialysis	12	926	8506	9.2	1.63
affinity	2	4.2	1659	394.4	42.95

3.2. Molecular Mass Determination. The purification procedures are summarized in **Table 1**. As seen in **Table 1**, finally, PPO was purified up to 43-fold. The molecular mass of PPO was estimated on SDS-PAGE with a single band of ~57 kDa (**Figure 1**). The molecular mass of PPO from other species has been reported as follows: *Vicia faba* L., 59 kDa (*36*); sago palm, 53 kDa (*37*); mushroom, 58 kDa (*38*); *Lactuca sativa*, 56 kDa (*39*); tea leaf, 72 kDa (*40*); sunflower seeds, 42 kDa (*41*); apple, 65 kDa (*42*, *43*); banana, 41 and 62 ± 2 kDa (*44*); cabbage, 39 kDa (*45*); field bean seed, 120 ± 3 kDa (*46*). Our results indicate that the molecular mass of *C. scolymus* L. was similar to those of *V. faba* L., sago palm, mushroom, and *L. sativa* but different from those of tea leaf, sunflower seeds, apple, banana, cabbage, and field bean seed.

3.3. Substrate Specificity and Enzyme Kinetics. PPO activity in partially purified extracts was examined with regard to its monophenolase, diphenol, and triphenol oxidase activities. The substrate specificity of the enzyme was investigated by using seven chemicals (4-methylcatechol, pyrogallol, catechol, L-dopa, *p*-cresol, resorsinol, and L-tyrosine) as substrates. Artichoke PPO showed no activity toward L-tyrosine (the monophenolase), resorsinol, and *p*-cresol, suggesting the absence of monophenolase (cresolase). Therefore, in this study, 4-methylcatechol, pyrogallol, catechol, and L-dopa were used as substrates. The enzyme is an *o*-diphenol oxidase as no cresolase activity was present. Some plant polyphenol oxidases, for example, mushroom, potato, and broadbean, catalyze both the hydroxylation of monophenols and the oxidation of *o*-diphenols. However, many polyphenol oxidases lack monophenol activity (*47*−*49*). Similar results were found for aubergine by Doğan et al. (*14*) and for Yali pear by Zhou and Feng (*50*). L-Dopa

Table 2. Substrate Specificity of Artichoke PPO

substrate	V_{max} (EU min^{-1})	K_m (mM)	V_{max}/K_m (EU min^{-1} mM^{-1})	optimum temp (°C)	optimum pH
catechol	7457	10.7	697	40	7
4-methylcatechol	16158	11.6	1393	<10	5
pyrogallol	6390	5.2	1220	20	8
L-dopa	4600	45	102		

was not used for the optimum pH and temperature, thermal denaturation, and inhibition studies. Michaelis constants (K_m) and maximum reaction velocities (V_{max}) were determined using these substrates at various concentrations. The Lineweaver−Burk plot analysis of this enzyme preparation showed K_m values of 11.6, 5.2, 10.7, and 45 mM for 4-methylcatechol, pyrogallol, catechol, and L-dopa, respectively (**Table 2**). We had previously found that the K_m value for *Thymus* PPO was 9.8 mM with 4-methylcatechol as substrate (*18*). In this study, the values of K_m for PPO obtained from *C. scolymus* L. for the substrates assayed were similar to those reported in the literature: Aydemir (*26*) reported the K_m values for artichoke variety as 12.4, 14.3, 10.2, and 37.7 mM for 4-methylcatechol, pyrogallol, catechol, and L-dopa, respectively. This value obtained with catechol was similar to that of aubergine (8.7−9.3 mM) (*14*), tea leaf (12.5 mM) (*40*), and field bean seed (10.5 mM) (*46*). The artichoke K_m value is lower than the 34 mM for Amasya apple (*51*), 18 mM for *Thymus* (*19*), 682.5 mM for cabbage (*52*), and 20 mM for Stanley plum (*53*) with catechol as a substrate. On the other hand, the K_m value for pyrogallol was different from that of spinach, 15.7 mM (*21*), cabbage, 15.4 mM (*52*) and tea leaf, 17.8 mM (*40*), but was similar to that of *Thymus*, 5.5 (*19*). The V_{max}/K_m ratio is called the "catalytic power", and it is a better parameter to find the most effective substrate. Considering the ratio V_{max}/K_m, it can be said that 4-methylcatechol is the most suitable substrate for *C. scolymus* L. PPO activity, followed by pyrogallol, catechol, and L-dopa (**Table 2**). Similar results were found for aubergine (*14*) and medlar fruits (*54*). The large ranges in the apparent K_m values of PPO reported in this study may be due to different reasons: different assay methods used, different varieties, different origins of the same variety, and different values of pH of extraction (*55*). On the other hand, it is generally assumed that the pH undoubtedly affects the apparent K_m values. Janovitz-Klapp et al. (*56*) showed that the apparent K_m values of Red Delicious apple for 4-methylcatechol, chlorogenic acid, and (+) catechin remained almost constant between pH 3.5 and 5.0, but increased above pH 5.0. In this state, it can be said that the enzyme is a diphenol oxidase as no cresolase activity was present.

3.4. Optimum pH. Enzyme activity exhibits a significant dependency on the pH value of the medium. With rising pH values, activity increases to a maximum (pH optimum) and drops to zero in the alkaline region, which is expressed in a bell-shaped optimum curve. Optimum pH values for artichoke PPO were determined in the pH range of 4−9. As seen in **Figure 2**, it was found that optimum pH values for artichoke PPO were 5, 8, and 7 for 4-methylcatechol, pyrogallol, and catechol as substrates, respectively. Different optimum pH values for PPO obtained from vairous sources are reported in the literature. For example, it is reported that optimum pH values are 4.5 for strawberry (*57*), 6.0 for aubergine (*14*), and 8.5 for Dog rose (*58*) using 4-methylcatechol as substrate; 7.0 for Dog rose (*58*) and 8.6 for Amasya apple (*51*) using pyrogallol as substrate; and 5.5 for strawberry (*57*), 6.0 for DeChaunac grape (*59*), 7.0 for Amasya apple (*51*), *Anethum graveolens* L. (*60*), and aubergine (*14*), 7.5 for *Allium* sp. (*34*), and 8.5 for Dog rose (*58*) using catechol as a substrate, respectively. Alyward and Haisman (*61*) reported that the optimum pH for maximum PPO activity in plants varies depending on the exraction method, the substrates used for assay, and the localization of the enzyme in the plant cell.

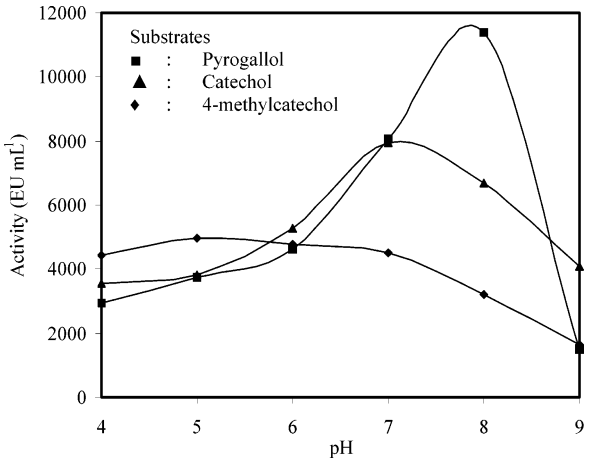

Figure 2. Effect of pH on PPO activity.

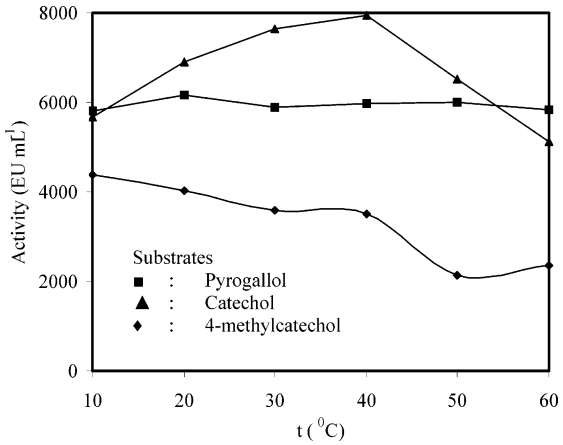

Figure 3. Effect of temperature on PPO activity.

3.5. Optimum Temperature. Figure 3 shows the effect of temperature on the activity and stability of the enzyme. When catechol was used as the substrate, PPO showed maximum activity at 40 °C and then decreased gradually with increasing temperatures. When pyrogallol was used as the substrate, however, PPO showed fluctuations in activity with increasing temperature even as high as 60 °C. The plot for pyrogallol demonstrated that the enzyme was very thermostable between 10 and 60 °C. Walker (*62*) also found a high inactivation temperature for PPO extracted from Sturmer Pippin apples. When 4-methylcatechol was used as the substrate, the optimum temperature was not observed in the studied temperature range, but it was found that enzyme was stable at low temperature. As seen above, optimum temperatures are substrate-dependent. It is reported that optimum temperature values are 40 °C for Chinese cabbage (*52*) using catechol as substrate; 20 °C for Dog rose (*58*) using 4-methylcatechol as substrate; and 15 °C for Dog rose (*58*) using pyrogallol as substrate.

3.6. Thermal Inactivation. The thermal stability profile for PPO, presented in the form of the residual percentage activity, is shown in **Figure 4**. The enzyme was incubated at different temperatures for 60 min at pH 6.5, and after cooling, the residual enzyme activity was measured using 4-methylcatechol, pyrogallol, and catechol as substrates. PPO showed similar behavior for 4-methylcatechol and pyrogallol substrates. The enzyme

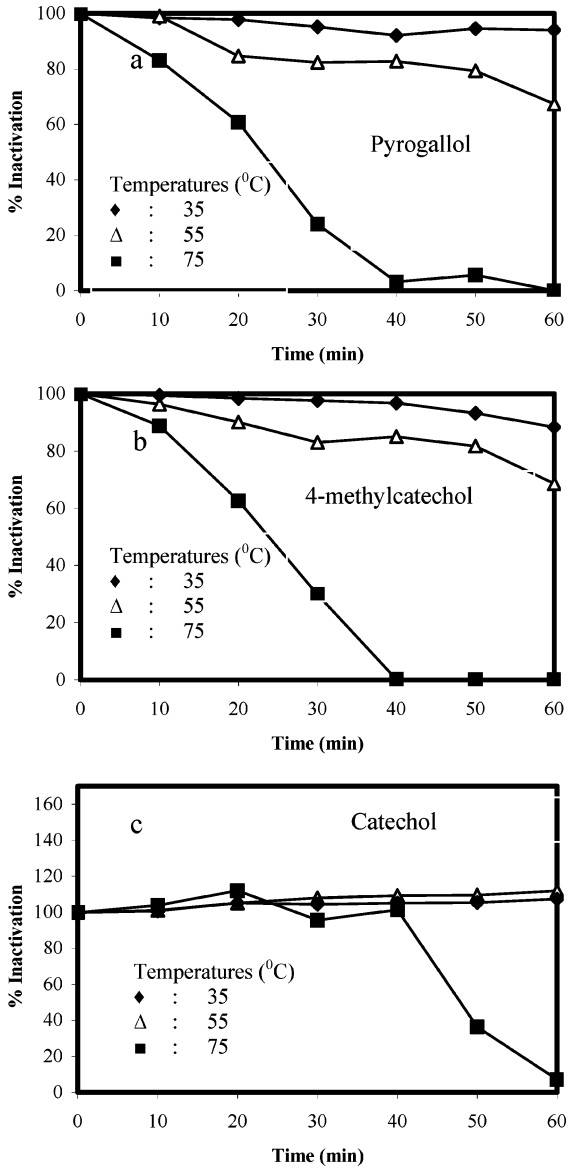

Figure 4. Change of PPO activity as a function of temperature and time.

activity decreased due to heat denaturation of the enzyme with increasing temperature and inactivation time for 4-methylcatechol and pyrogallol substrates. The drop in percentage residual activity at high temperatures can actually be due to the unfolding of the tertiary structure of the enzyme to form the secondary structure. For instance, when the temperature was increased from 55 to 75 °C, the activation of PPO decreased from 82 to 24% for 30 min and from 67 to 0% for 60 min with 4-methylcatechol as substrate; and the activation decreased from 83 to 30% for 30 min and from 69 to 0% for 60 min with pyrogallol as substrate. This indicated that the enzyme was rapidly denatured at higher temperatures.

Results of all inactivation experiments for catechol showed that the activity of artichoke PPO increased with mild heating, reached a maximum, and, then, decreased with time. As heating progressed, activity decreased, first gradually and then rapidly. An activation−inactivation curve was obtained by plotting the change in activity versus heating time at 35, 55, and 75 °C (**Figure 4c**). The first portion of the curves represents activation of PPO followed by both activation and inactivation. The final lines of curves indicate only an inactivation. The activation effect of heating was dependent not only on temperature but also on exposure time of the enzyme to various temperatures. The reduced effect of elevated temperatures on activation indicated that activation and inactivation occurred at the same time but that inactivation predominated at higher temperatures and with time. The observed increase in activity of artichoke PPO by heating could, in part, be due to a releasing of latent PPO. Kahn (*63*) demonstrated latent PPO in crude and partially purified preparations from avocado cultivars. Vamos-Vigyazo (*13*) reported the presence of latent PPO in apple peel extracts. Lee et al. (*64*) indicated that heating at 60 °C activated latent PPO in cocoa bean, but no activation was observed at higher temperatures. Mathew and Parpia (*65*) attributed the activation of PPO to protein association and dissociation. It has been noted that heat stability of the enzyme may be related to ripeness of the fruit and molecular forms of the enzyme, and in some cases it is also dependent on pH (*50*).

3.7. Inhibition of PPO. Many substances may alter the activity of an enzyme by combining with it in a way that influences the binding of substrate and/or its turnover number. Substances that reduce an enzyme's activity in this way are known as inhibitors. Many inhibitors are substances that structurally resemble their enzyme's substrate but either do not react or react very slowly compared to the substrate. Such inhibitors are commonly used to probe the chemical and conformational nature of a substrate-binding site as part of an effort to elucidate the enzyme's catalytic mechanism (*66*).

Enzymatic browning of vegetables may be delayed or eliminated by removing the reactants such as oxygen and phenolic compounds or by using PPO inhibitors. Complete elimination of oxygen from vegetables during processing is difficult because oxygen is ubiquitous (*67*). In this study, inhibition of artichoke PPO by L-cysteine, EDTA, ascorbic acid, gallic acid, D,L-dithiothreitol, tropolone, glutathione, sodium azide, benzoic acid, salicylic acid, and 4-aminobenzoic acid has been investigated. 4-Methylcatechol, pyrogallol, and catechol were used as substrates. It was found that the presence of EDTA, 4-aminobenzoic acid, salicylic acid, gallic acid, and benzoic acid from experimental results did not cause the inhibition of artichoke PPO (**Table 3**). The inhibiton of browning can be the result of (i) inactivation of PPO, (ii) elimination of one of the substrates (O_2, polyphenols) for the reaction, and (iii) the action of inhibitors on reaction products of enzyme action to inhibit the formation of colored products in secondary reactions (*68*). The prevention of enzymatic browning by a specific inhibitor may involve a single mechanism or may be the result of interplay of two or more mechanisms of inhibitor action. There are various mechanisms through which enzyme inhibitors can act.

Competitive Inhibition. The Lineweaver−Burk equation for competitive inhibition is

$$\frac{1}{v_0} = \left(\frac{\alpha K_m}{V_{max}}\right)\cdot\frac{1}{[S]} + \frac{1}{V_{max}} \quad (1)$$

where

$$\alpha = \left(1 + \frac{[I]}{K_i}\right) \quad (2)$$

A plot of this equation is linear and has a slope of $\alpha K_m/V_{max}$, a $1/[S]$ intercept of $-1/\alpha K_m$, and a $1/v_0$ intercept of $1/V_{max}$. The double-reciprocal plots for a competitive inhibitor at various concentrations of I intersect at $1/V_{max}$ on the $1/v_0$ axis; this is the diagnostic for competitive inhibition as compared with other types of inhibition (*66*).

Table 3. Change of PPO Activity with Various Inhibitor Concentrations

inhibitor	[I] (mol L^{-1})	substrate	activity (EU mL^{-1} min^{-1})	inhibitor	[I] (mol L^{-1})	substrate	activity (EU mL^{-1} min^{-1})
EDTA		4-methylcatechol	2960	salicylic acid		4-methylcatechol	2960
	6.67×10^{-4}		2930		3.33×10^{-5}		2961
	2.00×10^{-3}		2900		6.67×10^{-4}		2830
					2.00×10^{-3}		2910
		pyrogallol	4120				
	3.33×10^{-4}		3910			pyrogallol	4800
	1.00×10^{-3}		4160		1.00×10^{-3}		4730
	1.67×10^{-3}		4170		1.67×10^{-3}		4820
		catechol	7480			catechol	8420
	3.33×10^{-4}		7340		2.00×10^{-3}		8290
	2.00×10^{-3}		7460		2.67×10^{-3}		8100
benzoic acid		4-methylcatechol	3400	4-aminobenzoic acid		4-methylcatechol	3440
	3.33×10^{-4}		3400		3.33×10^{-4}		3310
	1.00×10^{-3}		3300		1.33×10^{-3}		3440
	2.00×10^{-3}		3310		2.00×10^{-3}		3400
		pyrogallol	5570			pyrogallol	2828
	3.33×10^{-4}		5570		3.33×10^{-5}		2780
	1.67×10^{-3}		5690		6.67×10^{-5}		2730
	2.67×10^{-3}		5680		1.00×10^{-4}		2850
		catechol	8620			catechol	8300
	2.00×10^{-3}		8400		3.33×10^{-4}		8240
	2.33×10^{-3}		8410		2.00×10^{-3}		8310
gallic acid		4-methylcatechol	3000				
	1.67×10^{-3}		3100				
	3.67×10^{-3}		2960				
		pyrogallol	5100				
	3.33×10^{-4}		5130				
	2.00×10^{-3}		4980				
		catechol	8150				
	1.67×10^{-3}		8100				
	3.67×10^{-3}		8175				

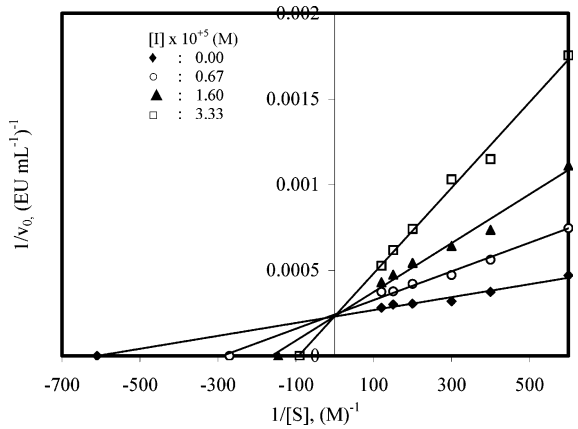

Figure 5. Effect of tropolone inhibitor on artichoke PPO using pyrogallol as a substrate.

A competitive-type inhibition was obtained with sodium azide, L-cysteine, and D,L-dithiothreitol inhibitors using 4-methylcatechol as substrate; with L-cysteine, tropolone, D,L-dithiothreitol, ascorbic acid, and sodium azide inhibitors using pyrogallol as substrate; and with L-cysteine, tropolone, D,L-dithiothreitol, and ascorbic acid inhibitors using catechol as substrate. Similar results was found for field bean seed PPO using L-cysteine, D,L-dithiothreitol, and ascorbic acid as inhibitors and catechol as substrate (*46*). **Figure 5** shows the effect of tropolone inhibitor on artichoke PPO using pyrogallol as substrate (other figures not shown). Percent inhibition and K_i values for the inhibitors we used have been given in **Table 4** for 4-methylcatechol, in **Table 5** for pyrogallol, and in **Table 6** for catechol as substrates. A substance that competes directly with a normal substrate for an enzymatic binding site is known as a competitive inhibitor. Such an inhibitor usually resembles the substrate to the extent that it specifically binds to the active site but differs from it so as to be unreactive. In inhibition of this type, the inhibitor does not affect the turnover number of the enzyme (*69*). The enzymatic browning by a specific inhibitor may involve a single mechanism or may be the result of interplay of two or more mechanisms of inhibitor action. Ascorbic acid acts by reducing the quinones formed by PPO action back to polyphenols while they are themselves oxidized. They therefore provide only temporary prevention of browning. They can also cause inactivation of the enzyme when used in high concentrations (*70*). L-Cysteine can easily form complexes with quinones and, therefore, inhibit secondary oxidation and polymerization reactions, thus consuming the substrate present (*71*). L-Cysteine can also act as a reducing agent (*57*). Inhibition by thiol compounds is attributed to either the stable colorless products formed by an addition reaction with *o*-quinones (*72*) or the binding to the active center of PPO, like metabisulfite (*73*). Sodium azide's toxicity toward a metal enzyme, especially in the case of a copper enzyme, is mainly due to its strong coordination ability with the metal within the active site, which provokes changes in the coordination number and conformation of the active site and depredates the active center metal. The reaction between the copper amine oxidase and azide probably hinders the bond of the precursor tyrosine to the copper. This prevents the formation of this key intermediate and inhibits the activity of the oxidase (*74*). Glutathione does not appear to affect the

Table 4. Inhibition Type, K_I, I_{50}, and Inhibition Percent Values of Artichoke PPO with 4-Methylcatechol as a Substrate

inhibitor	[I] (M)	K_I (M)	K_I' (M)	R^2	type of inhibition	inhibition%	$[I_{50}]$ (M)
glutathione	6.66×10^{-5}	4.8×10^{-4}		0.9994	mixed	12	1.74×10^{-4}
	1.33×10^{-4}	4.8×10^{-4}		0.9934		20	
	1.66×10^{-4}	4.1×10^{-4}		0.9967		24	
L-cysteine	1.00×10^{-4}	1.1×10^{-4}		0.9988	competitive	35	1.25×10^{-4}
	1.33×10^{-4}	8.3×10^{-5}		0.9931		54	
tropolone	3.33×10^{-6}	4.8×10^{-6}	4.9×10^{-6}	0.9991	noncompetitive	31	1.09×10^{-5}
	6.66×10^{-6}	5.4×10^{-6}	5.3×10^{-6}	0.9983		47	
	11.66×10^{-6}	5.3×10^{-7}	5.3×10^{-7}	0.9979		65	
D,L-dithiothreitol	1.00×10^{-4}	2.2×10^{-4}		0.9764	competitive	18	1.47×10^{-4}
	1.33×10^{-4}	1.8×10^{-4}		0.9968		32	
ascorbic acid	2.50×10^{-4}	8.6×10^{-4}	8.5×10^{-4}	0.9987	noncompetitive	23	3.57×10^{-4}
	3.33×10^{-4}	5.8×10^{-4}	5.8×10^{-4}	0.9980		39	
sodium azide	3.33×10^{-4}	6.3×10^{-4}		0.9992	competitive	29	1.31×10^{-3}
	1.00×10^{-3}	7.3×10^{-4}		0.9988		50	
	1.66×10^{-3}	5.8×10^{-4}		0.9883		71	

Table 5. Inhibition Type, K_I, I_{50}, and Inhibition Percent Values of Artichoke PPO with Pyrogallol as a Substrate

inhibitor	[I] (M)	K_I (M)	K_I' (M)	R^2	type of inhibition	unhibition%	$[I_{50}]$ (M)
glutathione	3.33×10^{-5}	1.4×10^{-4}	1.4×10^{-4}	0.9997	noncompetitive	18	3.35×10^{-4}
	1.33×10^{-4}	2.6×10^{-4}	2.6×10^{-4}	0.9999		34	
	3.33×10^{-4}	2.9×10^{-4}	2.9×10^{-4}	0.9994		52	
L-cysteine	1.00×10^{-4}	1.2×10^{-4}		0.9927	competitive	26	6.37×10^{-4}
	3.33×10^{-4}	1.6×10^{-4}		0.9986		45	
	1.00×10^{-3}	1.9×10^{-4}		0.9989		66	
tropolone	6.66×10^{-6}	5.5×10^{-6}		0.9932	competitive	24	5.39×10^{-5}
	1.66×10^{-5}	6.0×10^{-6}		0.9872		34	
	3.33×10^{-5}	5.9×10^{-6}		0.9946		46	
D,L-dithiothreitol	1.66×10^{-5}	2.4×10^{-5}		0.9877	competitive	25	3.29×10^{-5}
	2.33×10^{-5}	7.9×10^{-6}		0.9965		40	
ascorbic acid	3.33×10^{-5}	2.6×10^{-5}		0.9925	competitive	21	8.18×10^{-5}
	6.66×10^{-5}	1.5×10^{-5}		0.9976		49	
	8.33×10^{-5}	8.5×10^{-6}		0.9985		68	
sodium azide	3.33×10^{-4}	2.1×10^{-4}		0.9956	competitive	31	1.03×10^{-2}
	6.66×10^{-3}	2.3×10^{-3}		0.9994		44	
	1.00×10^{-2}	2.0×10^{-3}		0.9947		56	

enzyme directly, and oxygen uptake may be stimulated or inhibited depending upon the particular phenol being oxidized (*12*). Gunata et al. (*75*) observed a competitive-type inhibition for grape PPO with cinnamic and benzoic acid inhibitors with 4-methylcatechol as substrate; Doğan et al. (*15*) observed a competitive-type inhibition for *Thymus* PPO with glutathione inhibitor with 4-methylcatechol, pyrogallol, and catechol as substrates; Paul and Gowda (*46*) observed a competitive-type inhibition for field bean PPO with tropolone, ascorbic acid, and cysteine−HCl inhibitors with catechol as substrate; and Robert et al. (*76*) observed a competitive-type inhibition for palmito PPO with benzoic acid inhibitor with 4-methylcatechol as substrate. As seen above, the type of inhibition depends not only on the origin of the PPO studied but also on the substrate used. Walker and Wilson (*77*) suggested the existence of two distinct sites on the enzyme: one for the binding of the substrate and another, adjacent, site for binding of the inhibitor. Even though some authors have found a competitive inhibitory effect on PPO, using 4-methylcatechol as substrate (*75*, *77*, *78*), differences in type and degree of inhibition of various PPOs were reported (*79*, *80*).

Mixed Inhibition. In inhibition of this type, presumably a mixed inhibitor binds to enzyme sites that participate in both substrate binding and catalysis. The Lineweaver−Burk equation for mixed inhibition is

$$\frac{1}{v_0} = \left(\frac{\alpha K_m}{V_{max}}\right)\cdot\frac{1}{[S]} + \frac{\alpha'}{V_{max}} \quad (3)$$

where

$$\alpha' = 1 + \frac{[I]}{K_I'} \quad (4)$$

The plot of this equation consists of lines that have slope $\alpha K_m/V_{max}$ with $1/v_0$ intercept of α'/V_{max} and a 1/[S] intercept of $-\alpha'/\alpha K_m$). Algebraic manipulation of this equation for different values of [I] reveals that this equation describes a family of lines that intersects to the left of the $1/v_0$ axis (*66*).

In each case, the type of inhibition was deduced from Lineweaver−Burk double-reciprocal plots. As seen in **Table 4**, a mixed-type inhibition was obtained with glutathione inhibitor using 4-methylcatechol as substrate. A typical example of mixed-type inhibition is shown in **Figure 6** for glutathione inhibitor using 4-methylcatechol a substrate. The results lead to a series of lines, which intersect to the left of the vertical

Table 6. Inhibition Type, K_I, I_{50}, and Inhibition Percent Values of Artichoke PPO with Catechol as a Substrate

inhibitor	[I] (M)	K_I (M)	K_I' (M)	R^2	type of inhibition	inhibition%	$[I_{50}]$ (M)
glutathione	2.00×10^{-4}	5.6×10^{-4}	5.6×10^{-4}	0.9951	noncompetitive	25	3.23×10^{-4}
	3.00×10^{-4}	2.9×10^{-4}	3.0×10^{-4}	0.9999		48	
	3.33×10^{-4}	2.2×10^{-4}	2.3×10^{-4}	0.9856		56	
	4.00×10^{-4}	1.5×10^{-4}	1.5×10^{-4}	0.9928		69	
L-cysteine	6.66×10^{-5}	1.1×10^{-4}		0.9901	competitive	19	1.50×10^{-4}
	1.33×10^{-4}	6.2×10^{-5}		0.9931		40	
	2.00×10^{-4}	3.3×10^{-5}		0.9955		63	
tropolone	1.66×10^{-6}	4.75×10^{-6}		0.9954	competitive	39	2.97×10^{-5}
	3.66×10^{-6}	8.35×10^{-6}		0.9967		62	
D,L-dithiothreitol	6.66×10^{-5}	1.1×10^{-4}		0.9972	competitive	19	1.35×10^{-4}
	1.00×10^{-4}	7.3×10^{-5}		0.9935		31	
	1.33×10^{-4}	3.8×10^{-5}		0.9957		51	
ascorbic acid	1.66×10^{-4}	2.0×10^{-4}		0.9981	competitive	19	3.30×10^{-4}
	2.50×10^{-4}	1.2×10^{-4}		0.9969		35	
	3.33×10^{-4}	6.7×10^{-5}		0.9959		54	
sodium azide	6.66×10^{-4}	4.6×10^{-3}	4.7×10^{-3}	0.9975	noncompetitive	12	4.32×10^{-3}
	1.33×10^{-3}	3.6×10^{-3}	3.6×10^{-3}	0.9983		26	
	1.66×10^{-3}	2.8×10^{-3}	2.8×10^{-3}	0.9985		37	
	2.50×10^{-3}	2.7×10^{-3}	2.8×10^{-3}	0.9997		40	

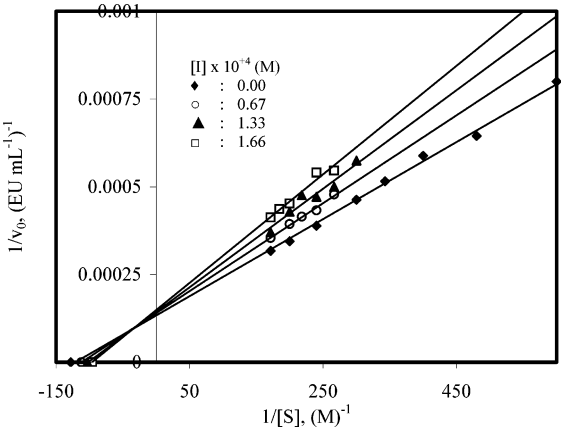

Figure 6. Effect of glutathione inhibitor on artichoke PPO using 4-methylcatechol as a substrate.

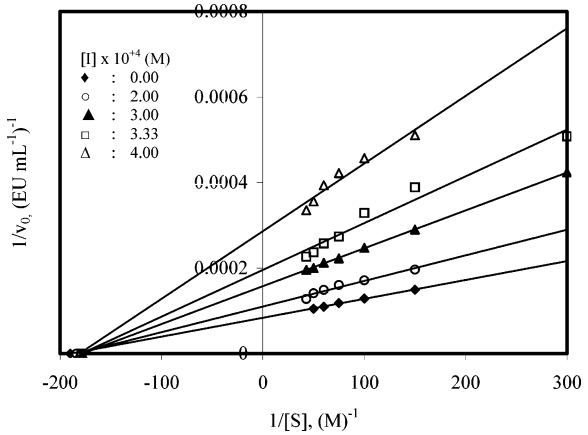

Figure 7. Effect of glutathione inhibitor on artichoke PPO using catechol as a substrate.

axis and above the horizontal axis, with a decrease in V_{max} and, conversely, an increase in K_m, indicating that all inhibitors tested were mixed-type inhibitors. The type of inhibition also depends on the origin of the PPO studied. With cinnamic acid, a mixed-type inhibition was observed for potato PPO (*81*). A mixed type of inhibition with tropolone was reported for soluble potato PPO (*82*) and mushroom tyrosinase (*83*).

For the particular case in which $K_I = K_I'$ ($\alpha = \alpha'$), the intersection is, in addition, on the 1/[S] axis, a situation which, in an ambiguity of nomenclature, is sometimes described as noncompetitive inhibition. **Figure 7** shows the effect of glutathione inhibitor on artichoke PPO using catechol as substrate. The two inhibition constants were obtained by fitting the experimental data to eq 3. When values of K_I and K_I' were of the same order of magnitude, the mixed-type system could be considered to be a noncompetitive inhibition. As seen from **Tables 4**−**6**, the fact that K_I and K_I' values obtained were of the same order of magnitude shows that inhibition type was noncompetitive inhibition for tropolone and ascorbic acid inhibitors using 4-methylcatechol as substrate, for glutathione inhibitor using pyrogallol as a substrate, and for glutathione and sodium azide inhibitors using catechol as a substrate.

A linear regression method was used to determine whether the experimental data fit with the inhibition equations. Linear regression coefficieints given in **Tables 4**−**6** were used to determine the inhibition types. As seen from these tables, the fact that regression coefficient values are in the range of 0.97−0.99 has shown that the experimental data fit better with related inhibition equations.

Tables 4−**6** show also I_{50} values for L-cysteine, ascorbic acid, D,L-dithiothreitol, tropolone, glutathione, and sodium azide using 4-methylcatechol, pyrogallol, and catechol as substrates. As seen from these tables, the sensitivity of PPO to inhibitors was different from one substrate to another. I_{50} values were also obtained for these inhibitors using 4-methylcatechol, pyrogallol, and catechol as substrates.

Again, as seen from K_I values in these tables, the most effective inhibitor for artichoke PPO with 4-methylcatechol as a substrate was tropolone, followed by L-cysteine, D,L-dithiothreitol, glutathione, sodium azide, and ascorbic acid; that with pyrogallol as substrate was tropolone, followed by D,L-dithiothreitol, ascorbic acid, L-cysteine, glutathione, and sodium azide; and that with catechol as substrate was tropolone, followed by L-cysteine, D,L-dithiothreitol, ascorbic acid, glutathione, and sodium azide, respectively. Tropolone in this study was the most effective inhibitor of artichoke PPO because of its low K_I value. Similar results were found for aubergine (*14*, *84*). Tropolone (2-hydroxy-2,4,6-cycloheptatrien-1-one), the progenitor of a

group of compounds called tropolones, is the most potent inhibitor, with apparent K_I values of $\sim 7.3 \times 10^{-6}$, 5.8×10^{-6}, and 6.5×10^{-6} M for artichoke PPO with 4-methylcatechol, pyrogallol, and catechol as substrate, respectively. It is both structurally analogous to the ortho-diphenolic substrates of PPO and an effective copper chelator (*85*), which explains the high inhibition potency.

LITERATURE CITED

(1) Adzet, T.; Puigmacia, M. High-performance liquid chromatography of caffeoylquinic acid derivatives of *Cynara scolymus* L. leaves. *J. Chromatogr.* **1985**, *348*, 447−452.

(2) Dranik, L. I.; Dolganenko, L. G.; Slapke, J.; Thoma, N. Chemical composition and medical usage of *Cynara scolymus* L. *Rastit Resur*. **1996**, *32*, 98−104.

(3) Nichiforescu, E. A. Composition of caffeoylquinic acid derivatives of artichoke (*Cynara scolymus* L.). *Plant Med. Phytother*. **1970**, *4*, 56−62.

(4) Wagenbreth, D. Evaluation of artichoke cultivars for growing and pharmaceutical use. *Beitr. Zuchtungsforsch.* **1996**, *2*, 400−403.

(5) Jimeanez-Escrig, A.; Dragsted, L. O.; Daneshvar, B.; Pulido, R.; Saura-Calixto, F. In vitro antioxidant activities of edible artichoke (*Cynara scolymus* L.) and effect on biomarkers of antioxidants in rats. *J. Agric. Food Chem*. **2003**, *51*, 5540−5545.

(6) Wang, M.; Simon, J. E.; Aviles, I. F.; He, K.; Zheng, Q. Y.; Tadmor, Y. Analysis of antioxidative phenolic compounds in artichoke (*Cynara scolymus* L.). *J. Agric. Food Chem***. 2003**, *51*, 601−608.

(7) Martino, V.; Caffini, N.; Phillipson, J. D.; Lappa, A.; Tchernitchin, A.; Ferraro, G.; Debenedelti, S.; Schilcher, H.; Acevedo, C. Identification and characterization of antimicrobial components in leaf extracts of globe artichoke (*Cynara scolymus* L.). *Acta Hortic.* **1999**, *501*, 111−114.

(8) Mcdougall, B.; King, P. J.; Wu, B. W.; Hostomsky, Z.; Manfred, G.; Robinsaon, W. E., Jr. Dicaffeoylquinic acid and dicaffeoyltartaric acid are selective inhibitors of human immunodeficiency virus type 1 integrase. *Antimicrob. Agents Chemother*. **1998**, *42*, 140−146.

(9) Kraft, K. Artichoke leaf extract. Recent findings reflecting effects on lipid metabolism, liver and gastrointestinal tracts. *Phytomedicine* **1997**, *4*, 369−378.

(10) Brown, J. E.; Rice-Evans, C. A. Luteolin-rich artichoke extract protects low-density lipoprotein from oxidation in vitro. *Free Radical Res*. **1998**, *29*, 247−255.

(11) Gowda, L. R.; Paul, B. Diphenol activation of the monophenolase and diphenolase activities of field bean (*Dolichos lablab*) polyphenol oxidase. *J. Agric. Food Chem.* **2002**, *50*, 1608−1614.

(12) Lee, C. Y.; Whitaker, J. R. *Enzymatic Browning and Its Prevention*; American Chemical Society: Washington, DC, 1995.

(13) Vamos-Vigyazo, L. Polyphenol oxidase and peroxidase in fruits and vegetables. *CRC Crit. Rev. Food Sci. Nutr*. **1981**, *15* (1), 49.

(14) Dogan, M.; Arslan, O.; Dogan, S. Substrate specificity, heat inactivation and inhibition of polyphenol oxidase from different aubergine cultivars. *Int. J. Food Sci. Technol.* **2002**, *37*, 415−423.

(15) Doğan, S.; Arslan, O.; Özen, F. Polyphenol oxidase activity of oregano in different stages. *Food Chem.* **2005**, *91*, 341−345.

(16) Arslan, O.; Temur, A.; Tozlu, İ. Polyphenol oxidase from Malatya apricot. *J. Agric. Food Chem*. **1998**, *46*, 1239−1241.

(17) Doğan, S.; Doğan, M.; Arslan, O. Characterization of polyphenol oxidase from *Thymus* (*Thymus longicaulis* var. *subisophyllus*). *Adv. Food Sci.* **2003**, *25* (2), 56−64.

(18) Doğan, S.; Doğan, M.; Arslan, O. Determination of some kinetic properties of polyphenol oxidase from *Thymus* (*Thymus zygioides* Griseb. var. *lycaonicus* (Čelak) Ronninger). *Adv. Food Sci.* **2003**, *25* (4), 130−136.

(19) Dogan, S.; Dogan, M. Determination of kinetic properties of polyphenol oxidase from *Thymus* (*Thymus longicaulis* subsp. *chaubardii* var. *chaubardii*). *Food Chem.* **2004**, *88*, 69−77.

(20) Gündoğmaz, G.; Doğan, S.; Arslan, O. Some kinetic properties of polyphenol oxidase obtained from various *Salvia* species (*Salvia viridis* L., *Salvia virgata* Jacq. and *Salvia tomentosa* Miller). *Food Sci. Technol. Int.* **2003**, *9* (4), 309−315.

(21) Golbeck, J. H.; Cammarata, K. V. Spinach thylakoid polyphenol oxidase. Isolation, activation and properties of the native chloroplast enzyme. *Plant Physiol.* **1981**, *67*, 877−884.

(22) Zawistowski, J.; Biliaderis, C.; Murray, D. Purification and charcterization of Jerusalem artichoke (*Helianthus tuberosus* L.) polyphenol oxidase. *J. Food Biochem.* **1988**, *12*, 1−22.

(23) Lopez-Molina, D.; Hiner, A. N. P.; Tudela, J.; Garcia-Canovas, F.; Rodriguez-Lopez, J. N. Enzymatic removal of phenols from aqueous solution by artichoke (*Cynara scolymus* L.) extracts. *Enzyme Microb. Technol.* **2003**, *33*, 738−742.

(24) Espin, J. C.; Tudela, J.; Garcia-Canovas, F. Monophenolase activity of polyphenol oxidase from artichoke heads (*Cynara scolymus* L.). *Lebensm.-Wiss. -Technol*. **1997**, *30*, 819−825.

(25) Ziyan, E.; Pekyardımcı, S. Characterization of polyphenol oxidase from Jerusalem artichoke (*Helianthus tuberosus*). *Turk. J. Chem.* **2003**, *27*, 217−225.

(26) Aydemir, T. Partial purification and characterization of polyphenol oxidase from artichoke (*Cynara scolymus* L.) heads. *Food Chem.* **2004**, *87* (1), 59−67.

(27) Lattanzio, V.; Cardinall, A.; Di Venere, D.; Linsalata, V.; Palmieri, S. Browning phenomena in stored artichoke (*Cynara scolymus* L.) heads: enzymic or chemical reactions? *Food Chem.* **1994**, *50*, 1−7.

(28) Leoni, O.; Palmieri, S.; Lattanzio, V.; Van Sumere, C. F. Polyphenol oxidase from artichoke (*Cynara scolymus* L.). *Food Chem.* **1990**, *38*, 27−39.

(29) Angleton, E. L.; Flurkey, W. H. Activation and alteration of plant and fungal polyphenol oxidase isoenzymes in sodium dodecyl sulfate electrophoresis. *Phytochemistry* **1984**, *23*, 2723−2725.

(30) Arslan, O.; Erzengin, M. Purification of polyphenol oxidase from different sources by affinity chromatography. Presented at Euroanalysis XII (Gesellschaft Deutscher Chemicer E. V.) Analytical Chemistry, 2002.

(31) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head bacteriophage. *Nature* **1970**, *227*, 680−685.

(32) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol.* **1965**, *16*, 144−158.

(33) Bradford, M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(34) Arslan, O.; Temur, A.; Tozlu, İ. Polyphenol oxidase from *Allium* sp. *J. Agric. Food Chem*. **1997**, *45*, 2861−2863.

(35) Kaur, C.; Kapoor, H. C. Anti-oxidant activity and total phenolic content of some Asian vegetables. *Int. J. Food Sci. Technol.* **2002**, *37* (2), 153−161.

(36) Lanker, T.; King, T. G.; Arnold, S. W.; Flurkey, W. H. *Physiol. Plant.* **1987**, *69*, 323.

(37) Onsa, G. H.; Bin Saari, N.; Selamat, J.; Bakar, J. Latent polyphenol oxidases from sage log (*Metroxylon sagu*): Partial purification, activation, and some properties. *J. Agric. Food Chem.* **2000**, *48*, 5041−5045.

(38) Espin, J. C.; van Leeuwen, J.; Wichers, H. J. Kinetic study of the activation process of a latent mushroom (*Agaricus bisporus*) tyrosinase by serine proteases. *J. Agric. Food Chem.* **1999**, *47*, 3509−3517.

(39) Fujita, S.; Tono, T. Purification and properties of polyphenol oxidase in bead lettuce (*Lactuce sativa*). *J. Sci. Food Agric.* **1991**, *55*, 115−123.

(40) Halder, J.; Tamuli, P.; Bhaduri, A. N. Isolation and characterization of polyphenol oxidase from Indian tea leaf (*Camellia sinensis*). *J. Nutr. Biochem.* **1998**, *9*, 75−80.

(41) Raymond, J.; Rakariyathan, N.; Azanza, J. L. Purification and some properties of polyphenol oxidases from sunflower seeds. *Photochemistry* **1993**, *34*, 927−932.
(42) Murata, M.; Kurokami, C.; Homma, S. Purification and some properties of chlorogenic acid oxidase from apple (*Malus pumila*). *Biosci., Biotechnol., Biochem.* **1992**, *56*, 1705−1710.
(43) Murata, M.; Kurokami, C.; Homma, S.; Matsuhashi, C. Immunochemical and immunohistochemical study of apple chlorogenic acid oxidase. *J. Agric. Food Chem.* **1993**, *41*, 1385−1390.
(44) Galeazzi, M. A. M.; Sgarbieri, V. C.; Constantinides, S. M. Isolation, purification and physicochemical characterization of polyphenoloxidase (PPO) from a dwarf variety of banana (*Musa cavendishii*, L). *J. Food Sci.* **1981**, *46*, 150−155.
(45) Fujita, S.; Saari, N. B.; Maegawa, M.; Tetsuka, T.; Hayashi, N.; Tono, T. Purification and properties of polyphenol oxidase from cabbage (*Brassica oleracea* L.). *J. Agric. Food Chem.* **1995**, *43*, 1138−1142.
(46) Paul, B.; Gowda, L. R. Purification and characterization of a polyphenol oxidase from the seeds of field bean (*Dalichos lablab*). *J. Agric. Food Chem.* **2000**, *48*, 3839−3846.
(47) Benjamin, N. D.; Montgomery, M. W. *J. Food Sci.* **1973**, *38*, 799.
(48) Anosike, E. O.; Ayacbene, A. O. *Phytochemistry* **1981**, *20*, 2625.
(49) Rivas, N. J.; Whitaker, J. R. *Plant Physiol.* **1973**, *52*, 501.
(50) Zhou, H.; Feng, X. Polyphenol oxidase from Yali pear (*Pyrus bretschneiderl*). *J. Sci. Food Agric.* **1991**, *57*, 307−313.
(51) Oktay, M.; Küfrevioglu, I.; Kocacaliskan, I.; Sakiroglu, H. Polyphenol oxidase from Amasya apple. *J. Food Sci.* **1995**, *60*, 495−499.
(52) Nagai, T.; Suzuki, N. Partial purification of polyphenol oxidase from Chinese cabbage (*Brassica rapa* L). *J. Agric. Food Chem.* **2001**, *49*, 3922−3926.
(53) Siddiq, M.; Sinha, N. K.; Cash, J. N. Characterization of polyphenol oxidase from Stanley plums. *J. Food Sci.* **1992**, *57*, 1l77−1179.
(54) Dincer, B.; Colak, A.; Aydin, N.; Kadıoğlu, A.; Güner, S. Chracterization of polyphenoloxidase from medlar fruits (*Mespilus germanica* L., Rosaceae). *Food Chem.* **2002**, *77*, 1−7.
(55) Rocha, A. M. C. N.; Pilar-Cano, M.; Galeazzi, M. A. M.; Morais, A. M. M. B. Characterization of ''Starking" apple polyphenoloxidase. *J. Sci. Food Agric.* **1998**, *77*, 527−534.
(56) Janovitz-Klapp, A. H.; Richard, F. C.; Nicolas, J. J. Polyphenol oxidase from apple, partial purification and some properties. *Phytochemistry* **1989**, *28*, 2903−2907.
(57) Wesche-Ebeling, P.; Montgomery, M. W. Strawberry polyphenol oxidase: extraction and partial characterization. *J. Food Sci.* **1990**, *55*, 1320−1325.
(58) Sakiroglu, H.; Küfrevioglu, I. Ö.; Kocacaliskan, I.; Oktay, M.; Onganer, Y. Purification and characterization of Dog-rose (*Rosa dumalis* Rechst.) polyphenol oxidase. *J. Agric. Food Chem.* **1996**, *44*, 2982−2986.
(59) Lee, C. Y.; Smith, N. L.; Pennesi, A. P. Polyphenol oxidase from DeChaunac grapes. *J. Sci. Food Agric.* **1983**, *34*, 987−991.
(60) Arslan, O.; Tozlu, I. Substrate specificity, heat inactivation and inhibition of polyphenol oxidase from *Anethum graveolens* L. *Ital. J. Food Sci.* **1997**, *9* (3), 249−253.
(61) Alyward, F.; Haisman, D. R. Oxidation system in fruits and vegetables-their relation to the quality of pressured products. *Adv. Food Res*. **1969**, *17*, 1−76.
(62) Walker, J. R. Studies on the enzymic browning of apples. III. Purification of apple phenolase. *Phytochemistry* **1966**, *5*, 259−262.
(63) Kahn, V. Effects of proteins, proteinhydrolyzates and amino acids on *o*-dihydroxyphenolase activity of polyphenol oxidase of mushroom, avocado and banana. *J. Food Sci.* **1977**, *50*, 111−119.
(64) Lee, P. M.; Lee, K. H.; Karim, M. I. A. Biochemical studies of cocoa bean polyphenol oxidase. *J. Sci. Food Agric*. **1991**, *55*, 251−260.
(65) Mathew, A. G.; Parpia, H. A. B. Food browning as a polyphenol reaction. *Adv. Food Res*. **1971**, *19*, 75−145.
(66) Voet, D.; Voet, J. G. *Biochemistry*; Wiley: New York, 2003.
(67) Roudsari, M. H.; Signoset, A.; Crovzet, J. Eggplant polyphenol oxidase: purification, characterization and properties. *Food Chem.* **1981**, *7*, 227−235.
(68) Augustin, M. A.; Ghazali, H. M.; Hashim, H. Polyphenol oxidase from guava (*Psidium guajava* L.). *J. Agric. Food Chem.* **1985**, *36*, 1259−1265.
(69) Bisswanger, H. *Enzyme Kinetics*; Wiley-VCH Verlag: Weinheim, Germany, 2002.
(70) Golan, A.; Goldhirsh, A.; Whitaker, J. R. Effect of ascorbic acid, sodium bisulfite and thiol compounds on mushroom polyphenol oxidase. *J. Agric. Food Chem.* **1984**, *32*, 1003−1009.
(71) Davis, R.; Pierpoint, W. S. Problem of the reactive species from enzymic and chemical oxidation of *o*-diphenols: anomalies in the trapping of *o*-quinonoids with benzenesulfinic acid. *Biochem. Soc. Trans.* **1975**, *3*, 671.
(72) Ikediobi, C. O.; Obasuyi, H. N. Purification and some properties o-diphenolase from white yam tubers. *Phytochemistry* **1982**, *21*, 2815−2820.
(73) Valero, E.; Garcia-Carlmona, F. Hysteresis and cooperative behaviors of a latent plant polyphenol oxidase. *Plant Physiol*. **1992**, *98*, 774−776.
(74) Schwartz, B.; Olgin, A. K.; Klinman, J. P. The role of cooper in topa quinone biogenesis and catalysis, as probed by azide inhibiton of a copper amine oxidase from yeast. *Biochemistry* **2001**, *40*, 2954−2963.
(75) Gunata, Y. Z.; Sapis, J. C.; Moutonet, M. Substrates and aromatic carboxylic and inhibitors of grape polyphenoloxidases. *Phytochemistry* **1987**, *26*, 1573−1575.
(76) Robert, C.; Rouch, C.; Cadet, F. Inhibition of palmito (*Acanthophoenix rubra*) polyphenol oxidase by carboxylic acids. *Food Chem.* **1997**, *59* (3), 355−360.
(77) Walker, J. R. L.; Wilson, E. L. Studies on the enzymatic browning of apples. Inhibition of apple *o*-diphenol oxidase by phenolics acids. *J. Sci. Food Agric*. **1975**, *26*, 1825−1831.
(78) Janovitz-Klapp, A. H.; Richard, F. C.; Goupy, P. M.; Nicolas, J. J. Kinetic studies on apple polyphenol oxidase. *J. Agric. Food Chem*. **1990**, *38*, 1437−1441.
(79) Pifferi, P. G.; Baldassari, L.; Cultrera, R. Inhibition by carboxylic acids of an *o*-diphenol oxidase from *Prunus avium* fruits. *J. Sci. Food Agric*. **1974**, *25*, 263−270.
(80) Kermasha, S.; Goetghebeur, M.; Monfette, A. Studies on inhibition of mushroom polyphenol oxidase using chlorogenic acid as substrate. *J. Agric. Food Chem*. **1993**, *41*, 526−531.
(81) Macrae, A. R.; Duggleby, R. G. Substrates and inhibitors of potato tuber phenolase. *Phytochemistry* **1968**, *7*, 855−861.
(82) Sanchez-Ferrer, A.; Laveda, F.; Garcia-Carmona, F. Cresolase activity of potato tuber partially purified in a two-phase partition system. *J. Agric. Food Chem.* **1993**, *41*, 1225−1228.
(83) Kahn, V.; Andrawis, A. Inhibition of mushroom tyrosinase by tropolone. *Phytochemistry* **1985**, *24*, 905−908.
(84) Perez-Gilabert, M.; Garcia-Carmona, F. Characterization of catecholase and cresolase activities of eggplant polyphenol oxidase. *J. Agric. Food Chem* **2000**, *48*, 695−700.
(85) Bryant, B. E.; Fernelius, W. C.; Douglas, E. Formation constants of metal complexes of tropolone and its derivatives 1. Tropolone. *J. Am. Chem. Soc*. **1953**, *75*, 3784−3790.

Received for review June 11, 2004. Revised manuscript received October 24, 2004. Accepted November 24, 2004.

JF049053G