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Affinity Purification and Characterization of Polyphenol Oxidase from *Helianthus tuberosus* L.

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Abstract

Polyphenol oxidase (PPO) of Jerusalem artichoke (*Helianthus Tuberosus* L.) tubers was purified using a Sepharose-4B-L-tyrosine-*p*-amino benzoic acid affinity gel. Both native- and SDS-gel electrophoresis analyses of the purified PPO gave a single band (ca. 65 kDa based on SDS-PAGE), indicating that it is a monomer. The purified PPO showed activity towards diphenolic and triphenolic substrates but not with the monophenolic substrates, suggesting that it lacks monophenolase activity. The optimum temperature and pH values vary between 20-35°C and 5.0-8.0, respectively, depending on the substrate used; for catechol, the optimum temperature and pH values were found to be 20°C and 7.0, respectively. The purified enzyme was relatively stable at 40°C but unstable at higher temperatures. Furthermore, IC_{50} values for various inhibitors and inhibition modes were also determined using catechol as a substrate; β-mercaptoethanol showed the strongest inhibition, followed by 2-mercapto benzothiazol, glutathione, L-cysteine and dithioerythritol, respectively.

INTRODUCTION

Browning reactions occuring in food systems may be broadly classified into non-enzymatic and enzymatic reactions. Non-enzymatic browning results from oxidation, caramelization, or the Maillard reaction. Enzymatic browning is due to oxidation of phenolic compounds by the action of oxidoreductase enzymes. Enzymatic browning in fruits and vegetables can cause undesirable quality changes during handling, processing and storage. Moreover, this reaction significantly diminish consumer acceptance, storage life and value of the plant products. Therefore, browning is important when food is processed and preserved. The main enzyme responsible for the brownig reaction is polyphenol oxidase (PPO). Polyphenol oxidase is a bifunctional copper-containing oxidoreductase enzyme that uses molecular oxygen to catalyse one or both of the following reactions: the hdyroxylation of monophenols to *o*-diphenols (E.C.1.14.18.1, monophenolase or cresolase activity), and the oxidation of *o*-diphenols to *o*-quinones (E.C.1.10.3.2, diphenolase or catecholase activity). In some cases, the absence of monophenolase activity can be due to latency [1]. Latent PPO can be activated by a variety of agents and treatments, including alcohols, urea, anionic detergents such as SDS, proteases (trypsin or proteinase K), sonication, heat, treatment with low and high pH

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levels, and exposure to fatty acids in incubation mixtures. The *o*-quinones which are unstable and electrophilic highly reactive molecules may condense to form darkened pigments and/or react non-enzymatically with other phenolic compounds, proteins, amino acids, peptides and other cellular constituents to form colored polymers. Reduction in the nutritive value of food proteins is due to the interactions of quinones with side chains of essential amino acids in plant proteins [2]. The –SH and $–NH_2$ groups of amino acids are mostly susceptible to binding or alkylation by quinones [2]. Moreover, quionones formed during PPO oxidation reactions may undergo redox recycling, which generate free radicals, and can damage DNA, proteins, amino acids or lipids. Phenolic compounds may function as inhibitor for bacterial growth or serve as precursors in the formation of physical polyphenolic barriers, limiting pathogen translocation. Polymeric polyphenols seem to be more toxic to potential phytopathogenes than are the phenolic monomers [3]. Monophenolase activity of PPO has been used in the removal of phenols from waste water. Moreover, monophenolase activity of PPO attracts scientific interest for use in the synthesis of L-Dopa, used for treatment of Parkinson's diseases [4]. PPO which is responsible for melanization in animals and browning in plants is widely distributed in higher plants, animals, and microorganisms, and many researchers are interested in the PPO isolated from various source such as apple [5], broccoli florets [6], banana [7], grape [8], quince [9], plums [10], herbs [4,11,12], seeds of field bean [13], mulberry [14], artichoke [3], Jerusalem artichoke [15,16], cabbage [17,18], and apricot [19]. Jerusalem artichoke (*Helianthus tuberosus* L.) is cultivated widely across the temperate world for its tuber, which is used in industry to make alcohol and as a root vegetable. This plant is a rich source of inulin which is a plant-derived carbohydrate with the benefits of soluble dietary fiber. It is not digested or absorbed in the small intestine, but is fermented in the colon by beneficial bacteria. The inulin can be converted into fructose, a sweet substance that is safe for diabetics to use. Complete hydrolysis of inulin yields about 95% fructose and 5% glucose. For this reason, Jerusalem artichoke tubers are one of the most important candidates for use as a raw material for the industrial production of biological fructose and inulin. Jerusalem artichoke is also a folk remedy for diabetes and rheumatism. Reported to be aperient, aphrodisiac, cholagogue, diuretic, spermatogenetic, stomachic, and tonic. Jerusalem artichokes are sold in the produce departments of many supermarkets and consumed as a fresh vegetable in Turkey and in other countries. Jerusalem artichoke PPO has been studied by some researchers [15,16] who purified PPO partially; but firstly, it was purified with affinity chromatography by us. The objective of this study was to achive affinity purification and a better understanding of the properties of the Jerusalem artichoke PPO that catalyzes the browning reaction during fruit storage and transportation. Therefore, the characterization of the enzyme could help develop more effective methods for controlling browning of Jerusalem artichoke and products.

MATERIALS AND METHODS

Materials

Jerusalem artichoke (*Helianthus tuberosus* L.) fruits used in this study were harvested at commercial maturity in winter from a field near Balikesir in Turkey and stored at 4°C until used in the study. Fruits were selected for uniformity of shape, colour and size. Any blemished or diseased fruits were discarded. Biochemicals were purchased from Sigma Chem. Co. and used without further purification. All other chemicals were of analytical grade. Enzyme assays were carried out with a UV-visible spectrophotometer (Varian).

Extraction and purification of PPO by affinity chromotography

All steps were carried out at 4°C. Jerusalem artichoke tubers were washed with distilled water and cut quickly into thin slices. To prepare the crude extract, 50 g of material was homogenized with 100 ml of 0.5 M phosphate buffer (pH 7.3) containing 5% polyethylene glycol and 10 mM ascorbic acid in a waring blender for 2 min. The homogenate was filtered through four layers of cheese cloth and filtrate was centrifuged at 20000 g for 60 min. The supernatanat was brought 0-80% saturation with $(NH_4)_2SO_4$ under continuous stirring. The precipitated PPO was separated by centrifugation at 20000 g for 60 min. The precipitate was dissolved in a small amount of 5 mM phosphate buffer (pH 6.3) and dialyzed at 4°C against the same buffer for 24 h (MW cut-off > 12000) with four changes of the buffer during dialysis. The dialyzed solution was loaded onto the affinity column (1x10 cm), previously equilibrated with 0.05 M phosphate buffer (pH 5.0 or 6.0, as indicated in the text). The affinity gel was washed with the equilibration buffer to remove proteins unbound to the column. Washing was contuinued until the effluent was free of proteins. The bound PPO was eluted with the solution of 0.05 M phosphate buffer (pH 8.0) containing 1 M NaCl. The flow rate was 1 $ml.min^{-1}$, and 3.0 ml fractions were collected. The fractions containing PPO were pooled and stored at 4°C, which was used as the enzyme source in the following experiments.

Assay of PPO activity

PPO activity was assayed by measuring the rate of increase in absorbance at a given wavelenght using a double beam model a UV-vis spectrophotometer. The activity was determined using different substrates by measuring the increase in absorbance at 420 nm for catechol, 4-methyl catechol, *p*-cresol and L-tyrosine substrates and 320 nm for pyrogallol substrate. The reaction mixture is consisted of 2.5 ml of 0.2 M sodium phosphate buffer (pH 7.0), 0.3 ml substrate at indicated concentrations and 0.2 ml enzyme solution in a final volume of 3.0 ml. The substrate solutions were prepared in 0.2 M sodium phosphate buffer (pH 7.0). The blank sample contained only 0.3 ml of substrate solution and 2.7 ml of 0.2 M buffer solution. The reaction was carried out in a 10 mm light path quartz cuvette. The temperature was kept constant to desired degree using a Beckmann Peltier temperature controller attached to the cell-holder of the spectrophotometer. The linear portion of the activity curve was used to express enzyme activity. One unit of PPO activity (EU) was defined as the amount of enzyme that causes an increase in absorbance of 0.001 ml^{-1} min^{-1} [14]. PPO activity was assayed in triplicate measurements.

Determination of protein

Protein content was determined according to the dye-binding method of Bradford [20], measuring optical density (OD) at 595 nm, using bovine serum albumin (BSA) as a standard. The values were obtained by graphic interpolation on a calibration curve. In chromatography studies, protein was expressed with absorbance at 280 nm. To monitor proteins in column eluates the absorbance at 280 nm was measured.

Electrophoresis

The enzyme purified by affinity chromatography was analyzed electrophoretically under denaturating conditions. Discontinuous polyacrylamide slab gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [21]. For SDS-PAGE, 3% stacking and 10% separating gels were used. The electrode buffer is consisted of Tris-HCl, glycine, SDS and water. Affinity purified enzyme sample was mixed with 10% SDS, glycerol, β-mercaptoethanol and bromophenol, and heated in boiling water for 3 minutes before being applied to the stacking gel. Electrophoresis was carried out at

4°C. Initially, an electric voltage (80 V) was applied until bromphenol dye reached the seperating gel. Then, it was increased to 150 V for 2 h. After completion of the run, the gel was stained with Coomassie brillant blue R-250 for 2 h to detect proteins. Later, the gel was destained by shaking for 1 h in acetic acid/methanol solution and then its photograph was taken. Molecular weight of PPO was determined by comparision with known standards. Molecular markers used were lysozyme (14.3 kDa), β-lactoglubulin (18.4 kDa), trypsinogen (24.0 kDa), pepsin (34.7 kDa), egg albumin (45.0 kDa), and bovine serum albumin (66.0 kDa). Native PAGE was performed by the same way but 2-mercaptoethanol was excluded from the sample buffer and heating wasn't done.

Effect of pH on enzyme activity

PPO activity as a function of pH was determined in the pH range of 4.5-9.0 by using 0.1 M citrate/0.2 M phosphate (pH 4.0-4.5), 0.2 M phosphate (pH 5.0-7.0) and 0.2 M Tris-HCl (pH 7.5-9.0) buffers; pHs were adjusted with 0.1 M NaOH or 0.1 M HCl at different temperatures in the range of 20-75°C. The optimum pH value for PPO activity was obtained using three different substrates (catechol, 4-methyl catechol and pyrogallol). In each measurement, the final volume of solution in quartz cuvette was 3.0 ml. The reaction mixture is consisted of 2.5 ml of 0.2 M buffer solution, 0.3 ml of 0.1 M substrate solution and 0.2 ml of enzyme solution. PPO activity was assayed in triplicate measurements [4]. The optimum pH value obtained for this enzyme was used in all other studies.

Effect of temperature on enzyme activity

To determine the optimum temperature of PPO, the enzyme activity was measured at different temperatures ranging from 20°C to 75°C by using three different substrates (catechol, 4-methyl catechol and pyrogallol) at different pH values, in the range of 4.5-9.0. The effect of temperature on the activity of PPO was tested by heating the standard reaction solution (buffer and substrate) to appropriate temperatures before introduction of the enzyme. The temperature was kept constant to desired degree using a Beckmann Peltier 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. In each measurement, the final volume of solution in quartz cuvette was 3.0 ml. The reaction mixture consisted of 2.5 ml of 0.2 M buffer solution, 0.3 ml of 0.1 M substrate solution and 0.2 ml of enzyme solution. PPO activity was assayed in triplicate measurements. The determined optimum temperature value was used in all other experiments.

Substrate specificity and enzyme kinetics

The substrate specificity of Jerusalem artichoke PPO was investigated for five different commercial grade substrates (p-cresol, L-tyrosine, catechol, 4-methylcatechol and pyrogallol) The kinetic parameters, Michaelis-Menten constants (K_M) and maximum velocities (V_{max}) were determined using three substrates (catechol, 4-methylcatechol and pyrogallol) in five different concentrations (3.33, 6.67, 10.00, 13.33 and 16.67 mM) at optimum pH and temperature values. The absorbance of the oxidation products were measured as described previously. K_M and V_{max} values of PPO, for each substrate, were calculated from a plot of 1/V vs. 1/[S] by the method of Lineweaver and Burk [12].

Heat dependent denaturation and renaturation

The effects of temperature and incubation time on PPO activity were also determined. Thermal denaturation property of the enzyme was determined by measuring PPO activity at different temperatures in the range of 40-80°C for 1 h duration at certain time intervals using catechol as a substrate. For this, enzyme extracts (5.0 ml) were

subjected to 40-80°C heating using a water bath. After the desired temperature was reached, the enzyme solution was immediately transferred into buffer solution containing catechol (10 mM) that were prewarmed to the corresponding temperatures. The reaction rates were measured for 1 h duration at certain time intervals, as previously described in 1 cm cuvettes around which water circulated at the respective reaction temperatures. Thermal renaturation property of the enzyme was investigated by measuring the enzyme activity by catechol within certain time intervals at room temperature. First of all, enzyme activity was measured using 10 mM catechol substrate at optimum conditions and this value was taken as 100% activity. Then, enzyme extracts (5.0 ml) were subjected to 40-80°C heating using a water bath for 1 h. After one hour incubation, the temperature of the enzyme solution was immediately lowered to ambient temperature and enzyme activity was measured within certain time intervals in 1h.

Activation Energy

Activation energy studies of the PPO enzyme action were run at 25, 35, 45, 55, 65 and 75°C for catechol, 4-methyl catechol and pyrogallol, at different pH values, in the range of 4.5-9.0. The activation energies were calculated from experimental results for enzyme rections by using the Arrhenius equation,

$$\ln A = \ln Z = \frac{E_a}{RT}$$

where, A is enzyme activity value, Z is frequency factor, E_a is activation energy and T is temperature. The lnA values were plotted versus the reciprocal of absolute temperature. The graph of lnA vs. 1/T was a straight line. The parameter Z is obtained from the intercept point at 1/T = 0 and the activation energies of reactions were calculated from the slope of lines [4].

Effect of various inhibitors on enzyme activity

To determine the effect of inhibitor, PPO activity was measured in the standard reaction medium in the presence or absence of the stated concentration of inhibitor [3]. The effect of inhibitor on PPO activity was measured by using β-mercaptoethanol, 2-mercapto benzothiazol, glutathione, L-cysteine, dithioerythritol, 2,4-dichloro-5-sulfamoil benzoic acid, p-aminobenzoic acid, benzoic acid, 4-carboxy benzene sulfanamid, sulfosalicilic acid, sodium azide and Na-EDTA. In order to determine the inhibitor concentration that reduced the enzyme activity by 50% (IC_{50}), enzyme activity was measured spectrophotometrically at 420 nm using catechol (10 mM) as a substrate at optimum temperature and pH. At first, the activity of enzyme was assayed without introducing any inhibitor to reaction medium. This measurement was accepted as 100% activity, and then enzyme activity was assayed with five different inhibitor concentrations. The IC_{50} values were determined from the plot of % activity *vs.* inhibitior concentration. At optimum temperature and pH, inhibition constants (K_i) and type of inhibitions were determined. Enzyme activity was measured spectrofotometrically at 420 nm by using catechol as a substrate. Using five different concentrations of the substrate, PPO activities were measured at three constant inhibitor concentrations. Three-milliliter reaction mixture contained substrate at various concentrations (1.67, 3.33, 5.00, 8.33, and 13.33 mM) in 0.2 M phosphate buffer pH 7.0, 0.2 ml of enzyme solution, required volume (ml) of 0.2 M phosphate buffer pH 7.0 and inhibitor solutions at three constant concentrations. The control study was performed without addition of any inhibitor. 1/V and 1/[S] values were employed to draw Lineweaver-Burk graphs. Finally, inhibition constants (K_i) and the type of inhibitions were deduced from Lineweaver-Burk graph for each inhibitor [14].

RESULTS AND DISCUSSION

Extraction and purification of PPO by affinity chromotography

Enzyme purification in plant extracts is hampered by the presence of a large variety and quantity of secondary products that can bind tightly to the enzymes and change their native characteristics. Crude extract of PPO from Jerusalem artichoke tubers was prepared in 0.5 M phosphate buffer (pH 7.3) containing 5% polyethylene glycol (PEG) and 10 mM ascorbic acid. PEG was used during extraction to bind the endogenous polyphenols which could inactivate the PPO. Oxidation of phenolic compounds by PPO produces quinones which would inhibit PPO, hence, ascorbic acid was also used to reduce quinones to their corresponding phenolic substrates during extraction [12]. Affinity chromotography has proven to be a powerful technique in the isolation and purification of biological molecules. In this study, PPO was purified from Jerusalem artichoke (*Helianthus Tuberosus* L.*)* tubers using the Sepharose 4B-L-tyrosine-*p*-aminobenzoic acid affinity gel [14]. Figure 1 shows the typical elution pattern of the enzyme activity on affinity column which is previously equilibrated with 0.05 M phosphate buffer at two different pH values (5.0 or 6.0).

In both cases, the enzyme activity showed a single peak and the peak fractions were pooled as purified PPO. The elution patterns for Jerusalem artichoke tuber PPO were reproducible. Purification scheme

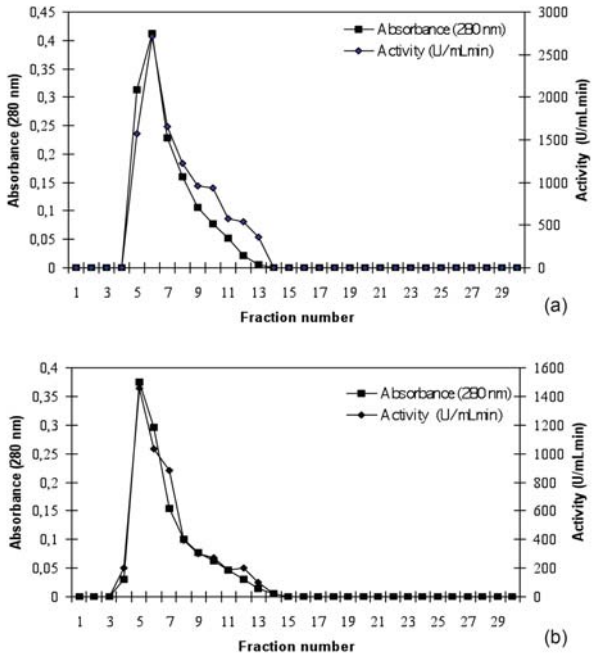

Figure 1. Sepharose 4B-L-tyrosine-*p*-aminobenzoic acid affinity chromatographic profile of PPO. a) Affinity gel equilibrated with 0.05 M phosphate buffer pH 5.0. b) Afinity gel equilibrated with 0.05 M phosphate buffer pH 6.0. The bound PPO was eluted with the solution of 0.05 M phosphate buffer at pH 8.0 containing 1 M NaCl. Proteins were monitored by UV absorbance at 280 nm and PPO activity was assayed at 420 nm in standard conditions using 10 mM catechol as a substrate.

of PPO from Jerusalem artichoke is given in Table 1. At the end of the chromatography on affinity column, Jerusalem artichoke PPO was purified 100-fold at pH 5.0 and 60-fold at pH 6.0, with a total yield of 81% and 36% respectively.

These purification values are higher than 44.8-, 43.33-, 37.0-, 18.0-, 10.8- and 4.9-fold obtained for iceberg lettuce [22], ferula sp.leaf [12], henryi chestnuts [23], Jerusalem artichoke [16], yali pear

Table 1. Purification scheme of PPO from Jerusalem artichoke.

Purification step	Total volume (mL)	Activity (U/mLmin)	Total activity	Protein (mg/mL)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Extract	5	8014	40070	0.95	4.75	8436	-	-
Affinity chromatography (pH 5.0)	12	2710	32520	0.0032	0.038	846875	81.16	100.39
Affinity chromatography (pH 6.0)	10	1460	14600	0.0029	0.029	503448	36.44	59.68

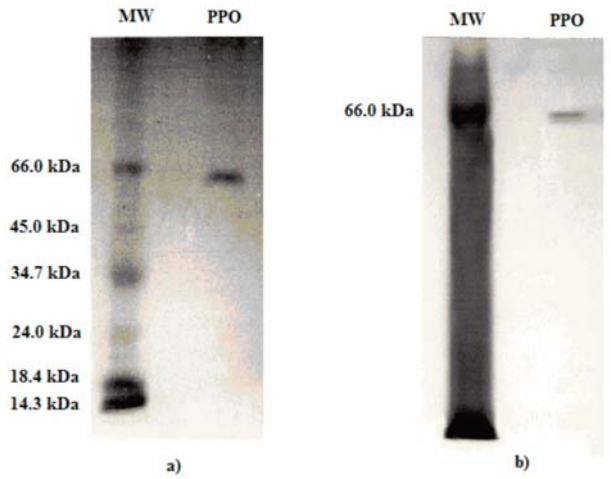

Figure 2. a) SDS-PAGE of the purified PPO from Jerusalem artichoke b) Native-PAGE of the purified PPO from Jerusalem artichoke. The line marked MW contained the molecular weight markers; lysozyme (14.3 kDa), β-lactoglobulin (18.4 kDa), trypsinogen (24 kDa), pepsin (34.7 kDa), egg albumin (45.0 kDa), and bovine serum albumin (66.0 kDa).

[24] and yam tubers [25], on the other hand, lower than the victoria grape PPO of 135-fold [8] and Monastrell grape PPO of 126-fold purification [26].

Electrophoresis

The purified PPO migrated as a single band during on native and SDS–polyacrylamide gel electrophoresis (Figure 2).

These results showed that the purified enzyme is a monomer. The molecular weight of PPO was estimated to be about 65.0 kDa. The molecular weight of PPO from other species has been reported as follows: broccoli florets, 51.3 kDa [6]; mulberry, 65 kDa [14]; chinese cabbage, 65 kDa [18]; cabbage, 39 kDa [17] and seeds of field bean, 120 kDa [13]. These results indicate that the molecular weights of PPO were similar to those of mulberry, chinese cabbage and banana but was different from those of field bean seed and cabbage.

Effect of pH on enzyme activity

The effect of pH on the activity of Jerusalem artichoke PPO was studied with catechol, 4-methyl catechol and pyrogallol substrates at different temperatures in the range of 20-75°C. As the temperature increases the optimum pHs gradually decreases for all substrates, this case is more clearly seen when catechol was used as a substrate. Optimum pH values of the enzyme were found to be 7.0 for catechol, 5.0 for 4-methyl catechol and 8.0 for pyrogallol substrate. The optimum pH of PPO enzyme varies widely with plant source but is generally in the range of 4.0-8.0. Optimum pH values of Jerusalem artichoke PPO considerably lies in this range. Different optimum pHs for PPO obtained from various sources are reported in literature. For example, it is reported that optimum pH values are 5.72 for broccoli florets [6], 7.0 for Amasya apple [5], ferula sp.leaf and stem [12], yali pear [24], mulberry [14], 6.0 for artichoke heads [3], 8.5 for Dog rose [27], Malatya apricot [19], 5.0 for Victoria grape [8], 6.5 for *thymus* [4] and 7.5 for *Allium* sp. [11] using catechol as a substrate; 6.0 for ferula sp.leaf and stem [12], artichoke heads [3], 5.0 for mulberry [14], 8.5 for Dog rose [27], 9.0 for Amasya apple [5], 5.5 for *thymus* [4] and 5.72 for broccoli florets [6] using 4-methyl catechol as a substrate; 7.5 for mulberry [14], 8.6 for Amasya apple [5], 7.0 for Dog rose [27], 6.5 for artichoke heads [3] and 6.5 for *thymus* [4] using pyrogallol as a substrate. The optimum pH is influenced by a number of experimental factors such as extraction methods, temperature, nature of the phenolic substrate, and buffer system used during activity determination [28]. The substrate dependent pH optimum exists in PPO from several sources [3,5,27]. This variation could be accounted for the differences in the binding ability of the substrates to the active site under acidic and alkaline conditions [1,28]. The rapid deactivation of the enzyme at pH>8.5 is presumably attributed to one or a combination of the following possibilities; conformational change of the enzyme under alkaline condition and/or the enzyme may react more rapidly with *o*-quinone through Maillard reaction and/or Strecker degradation and/or *o*-quinone itself

undergoes rapid secondary reactions which are known to be catalyzed by base [29]. The low activity observed at more acidic pH values may be due to enzyme instability at these pH values.

Effect of temperature on enzyme activity

The effect of temperature between 20 and 75°C on PPO activity at different pHs was assayed for each substrate. The optimum temperature for Jerusalem artichoke PPO activity has been found to vary with substrate of the enzyme. Optimum temperature values of the PPO enzyme for catechol, 4-methyl catechol and pyrogallol substrates were determined as 20°C, 20°C and 35°C, respectively. Optimum temperature of the activity is affected by the substrate used in the assay. It has been reported that optimum temperature values are 45°C for mulberry [14], 15°C for Amasya apple [5], 25°C for *thymus* [4], Dog rose [27], Victoria grape [8], artichoke heads [3], 40°C for Chinese cabbage [18] and 12°C for ferula sp. leaf and stem [12] using catechol as a substrate; 20°C for Dog rose [27], mulberry [14], 56°C for Amasya apple [5] and 25°C for *thymus* [4], ferula sp.leaf and stem [12] using 4-methyl catechol as a substrate; 70°C for Amasya apple [5], 20°C for Dog rose [27], mulberry [14], and 35°C for *thymus* [7] using pyrogallol as a substrate. Temperature is an important factor that significantly influences the catalytic activity of PPO. It is well known that a decrease in the kinetic energy of the reactant molecules at low temperatures corresponds to a slower reaction. In addition, integrity of the delicate three-dimensional structure of the enzyme molecule is subjected to disruption and denaturation at high temperatures [28]. Variations in temperature may also alter the solubility of oxygen, one of the substrates required for PPO to perform its catalytic activity [1,28].

Substrate specificity and enzyme kinetics

PPO extracts from different sources has been shown to have varying substrate specificity [3]. In this study, various monophenols, diphenols and triphenols were used to test substrate specificity. Jerusalem artichoke PPO showed activity with diphenolic and triphenolic substrates (catechol, 4-methyl catechol, pyrogallol) but not with the monophenolic substrates (p-cresol, L-tyrosine), suggesting the absence of monophenolase (cresolase) activity. Therefore, mulberry PPO could be a diphenol oxidase. This result is similar to those reported by Paul and Gowda [13], Gawlik-Dziki *et al*. [6], Rapeanu *et al*. [8], Arslan *et al*. [19], Erat *et al*. [12], Aydemir [3], Zhou and Feng [24] and Arslan *et al*. [14] for the seeds of field bean, broccoli florets, Victoria grape, Malatya apricot, ferula sp.leaf and stem, artichoke heads, yali pear and mulberry PPO, respectively. The number of hydroxyl groups and their positions in the benzene ring of the substrate affected oxidase activity [8]. PPO preparations from a number of plants have both types of activities while some plants lack the hydroxylation properties and act only on *o*-diphenols. Diphenolase activity is generally the most prevalent form of PPO in higher plants. When both monophenolase and diphenolase activities are present, the ratio of monophenolase to diphenolase activity varies from 1:10 to 1:40 depending on plant sources [30]. It has been reported that lack of monophenolase activity in some banana isoforms is due to inactivation during the extraction and purification processes [1]. In some cases, the absence of monophenolase activity can be due to latency. Latent monophenolase activity of some PPO preparations could be detected after activation by urea, anionic detergents such as SDS, proteases (trypsin or proteinase K), sonication, heat, treatment with low and high pH levels, and exposure to fatty acids in incubation mixtures. Michaelis-Menten constants (K_M) and maximum velocities (V_{max}) were determined using three substrates (catechol, 4-methyl catechol and pyrogallol) in five different concentrations (3.33, 6.67, 10.00, 13.33 and 16.67 mM) at optimum conditions of pH and temperature. For each

substrate Lineweaver-Burk graphs were drawn and K_M, V_{max} and V_{max}/K_M values of PPO, for each substrate, were calculated from the plots of 1/V vs 1/[S]. The K_M and V_{max} values were 6.12 mM and 4564.75 U/mL.min for catechol and 5.18 mM and 2177.37 U/mL.min for 4-methyl catechol and 4.79 mM and 1963.21 U/mLmin for pyrogallol. These results correspond well to those reported in the literature. V_{max}/K_M ratio is called the "catalytic power" and is a good parameter for finding the most effective substrate [4]. V_{max}/K_M values are $745.9x10^3$, $420.3x10^3$ and $409.9x10^3$ for catechol, 4-methyl catechol and pyrogallol, respectively. According to these values; the best substrate of PPO were found to be catechol followed by 4-methyl catechol and pyrogallol. K_M value of PPO depends on the type of the substrate, used buffer and the source of the plant. It has been reported that K_M values for catechol in literature are 4.54 mM for quince [9], 10.5 mM for the seeds of field bean [13], 18 mM for *thymus* [4], 2.64 mM for ferula sp. stem [12], 6.6 mM for Malatya apricot [19], 10.2 mM for artichoke heads [3], 12.34 mM for broccoli florets [6], 52.6 mM for Victoria grape [8], 19.81 mM for mulberry [14] and 34.0 mM for Amasya apple [5]. K_M values for 4-methyl catechol in literature are 4.0 mM for the seeds of field bean [13], for *thymus* [4], 6.78 mM for ferula sp. stem [12], 12.4 mM for artichoke heads [3], 21 mM for broccoli florets [6], 7.51 mM for Victoria grape [8], 9.18 mM for mulberry [14] and 3.1 mM for Amasya apple [5]. K_M values obtained for pyrogallol in literature are 7.35 mM for quince [9], 12.5 mM for the seeds of field bean [13], 5.5 mM for *thymus* [4], 14.3 mM for artichoke heads [3], 1.24 mM for mulberry [14] and 27 mM for Amasya apple [5].

Activation Energy

Activation energies were determined at 25°C, 35°C, 45°C, 55°C, 65°C and 75°C for catechol, 4-methyl catechol and pyrogallol, at different pH values, in the range of 4.5-9.0. Activation energy values were calculated from the plots of lnA vs 1/T. Activation energy values have changed in the range of -8.48 and 1.42 kcal/mol for catechol, -6.14 and 6.15 kcal/mol for 4-methyl catechol and -4.06 and 6.73 kcal/mol for pyrogallol substrates. Some of the activation energies are negative, this may be explained by inactivation of the PPO at high temperatures [4,5]. Activation energy values are, generally, not published, therefore comparisions are not possible. There are only a few data relate to activation energy. The calculated E_a values fit well with values obtained from *thymus* [4].

Heat dependent denaturation and renaturation

Thermal denaturation property of the Jerusalem artichoke PPO was determined by measuring PPO activity at different temperatures in the range of 40-80°C for 1 h duration at certain time intervals using catechol as a substrate (Figure 3(a)).

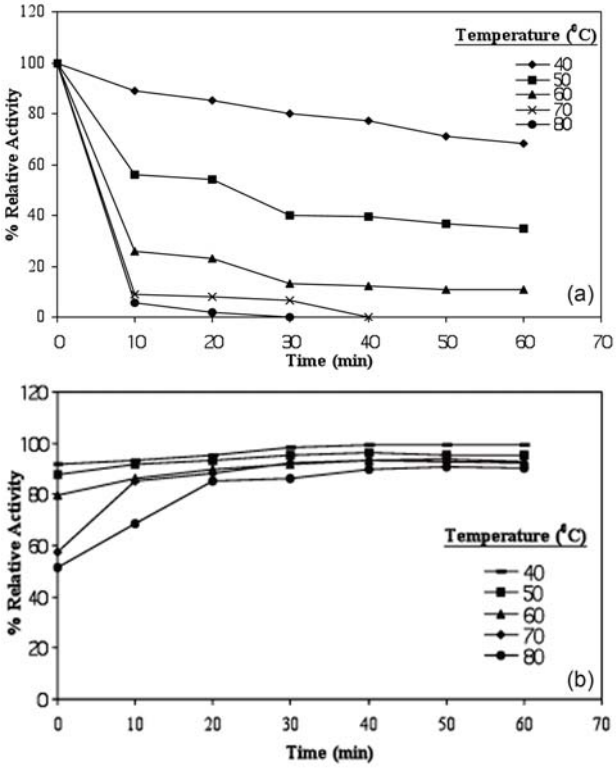

Figure 3. a) Denaturation property of Jerusalem artichoke PPO. Residual enzyme activity was measured in 0.2 M sodium phosphate buffer at pH 7.0 with 10 mM catechol as a substrate. b) Renaturation property of Jerusalem artichoke PPO. Residual enzyme activity was measured in 0.2 M sodium phosphate buffer at pH 7.0 with 10 mM catechol as a substrate.

The enzyme was stable at 40°C but unstable at higher temperatures. At the higher temperatures, the enzyme lost activity more rapidly. It is probable that during the heating process, at high temperatures, conformational changes of the enzyme taking place which lead to the observed deactivation of PPO. It is worth to note that, PPO activity is dependent not only on temperature but also on the length of exposure of the enzyme to various temperatures [29]. On the other hand, heat stability of the enzyme may be related to ripeness of the plant and in some cases it is also dependent on pH. In addition, different moleculer forms from the same source may have different thermostability [24]. Aydemir [3] reported a loss of about 85% of PPO activity for artichoke heads at 60°C for 60 min, while Rapeanu *et al*. [8] found a 50% reduction of PPO activity after 10 min at 60°C for Victoria grape. Thermal renaturation property of the Jerusalem artichoke PPO was also investigated by measuring the activity within certain time intervals by lowering the temperatures to ambient. First of all enzyme activity was measured with 10 mM catechol substrate at optimum conditions and this value was taken as 100% activity. Then, the enzyme solution was incubated at 40°C, 50°C, 60°C, 70°C and 80°C for 1 h. After one hour incubation, temperature of the enzyme solution was immediately lowered to ambient temperature and enzyme activity was measured within certain time intervals in 1 h. Examination of Figure 3 (b) shows that partially or completely denaturated PPO almost recover its original activity at the end of 1 h duration. Hence, we do not suggest the thermal inactivation method for preventing enzymatic browning of Jerusalem artichoke.

Effect of various inhibitors on enzyme activity

Control of enzymatic browning of vegetables and fruits has received a great deal of attention by researchers because of its importance to the food processing industry. In theory, PPO-catalysed browning of vegetables and fruits can be prevented by heat inactivation of the enzyme, exclusion or removal of one or both substrates (O_2 and phenols), lowering the pH to 2 or more units below the optimum, or adding compounds that inhibit PPO or prevent melanin formation [28]. There are a number of inhibitors used by researchers to prevent enzymatic browning [3-6,8,9,12-14,19]. Lineweaver-Burk plots of 1/V and 1/[S] at three inhibitor concentrations were plotted to determine the type of inhibition. K_i values and inhibition modes for different inhibitors are given in Table 2. From the K_i constants, it was concluded that the inhibition mode of dithioerythritol is uncompetitive, and the other inhibitors are competitive, as illustrated in the Figure 4 (a)-(d).

Table 2. K_i values and inhibition modes of four inhibitors for PPO.

Inhibitors	I [mM]	K_i [mM]	Average values of K_i [mM]	Type of inhibition
dithioerythritol	0.079	7.07×10^{-6}	8.82×10^{-6}	uncompetitive
	0.012	9.07×10^{-6}		
	0.016	10.35×10^{-6}		
L-cysteine	2.50	2.12	2.19	competitive
	3.33	2.47		
	5.00	1.98		
	10.00	15.78		
sodium azide	15.00	6.95	9.58	competitive
	20.00	6.02		
sulfosalicylic acid	6.67	16.33	10.40	competitive
	10.00	10.19		
	11.67	4.67		

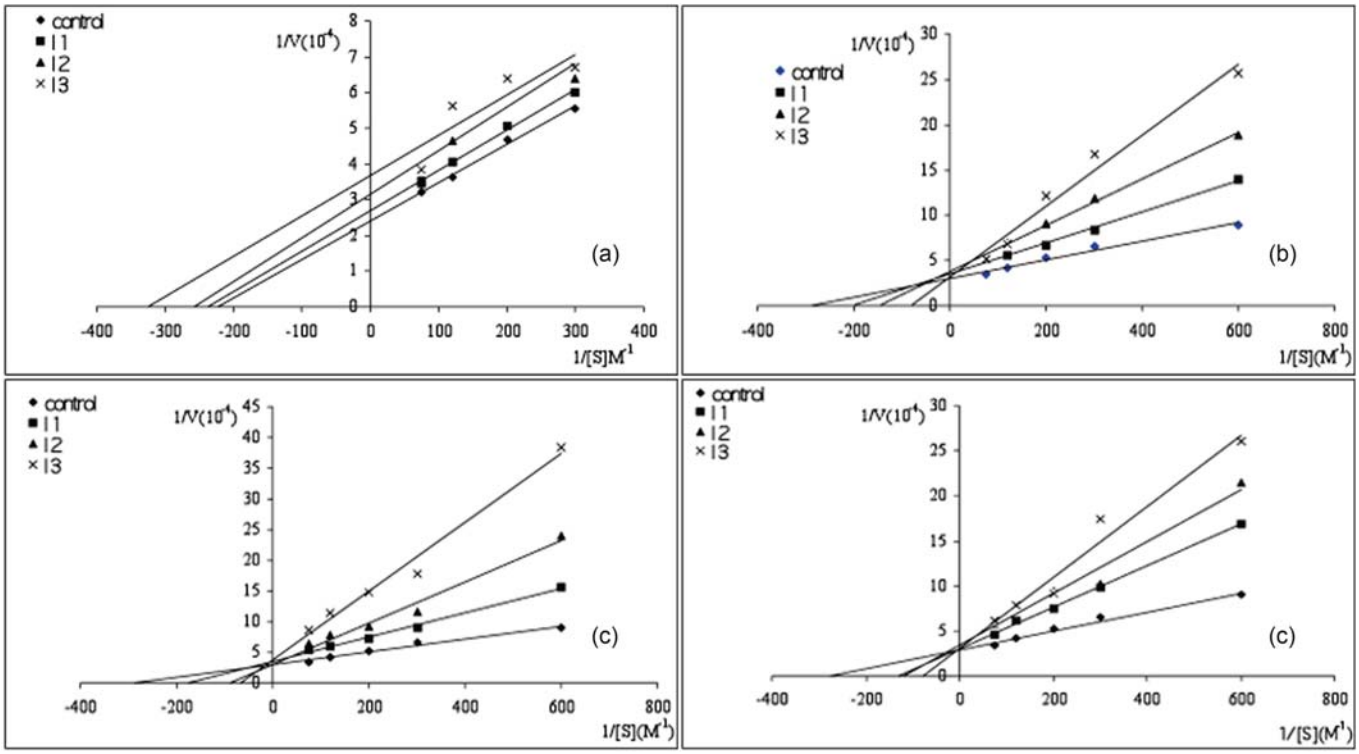

Figure 4. Linewear-Burk plots showing inhibition of Jerusalem artichoke PPO by a) dithioerythritol; $[I_1]$= 7.94x10^{-5}, $[I_2]$= 11.91x10^{-5}, $[I_3]$= 15.87x10^{-5} b) sulfosalicylic acid; $[I_1]$= 6.67x10^{-3}, $[I_2]$= 10.00x10^{-3}, $[I_3]$= 11.67x10^{-3} c) sodium azide; $[I_1]$= 1.0x10^{-2}, $[I_2]$= 1.5x10^{-2}, $[I_3]$= 2.0x10^{-2} d) L-cysteine; $[I_1]$= 2.5x10^{-3}, $[I_2]$= 3.33x10^{-3}, $[I_3]$= 5.0x10^{-3}.

It is known that inhibition type changes depending on the substrate used. IC_{50} values were obtained with various compounds as inhibitors using catechol as a substrate. The values were 27x10^{-3} mM, 86 x10^{-3} mM, 19 x10^{-2} mM, 24 x10^{-2} mM, 28 x10^{-2} mM, 3.07 mM, 4.10 mM, 8.18 mM, 10.26 mM, 10.48 mM, 16.26 mM and 169.04 mM for β-mercaptoethanol, 2-mercaptobenzothiazol, glutathione, L-cysteine, dithioerythritol, 2,4-dichloro-5-sulfamoil benzoic acid, p-aminobenzoic acid, benzoic acid, 4-carboxy benzene sulfanamide, sulfosalicylic acid, sodium azide and Na-EDTA, respectively. The strongest inhibitor was found to be β-mercaptoethanol followed by 2-mercaptobenzothiazol, glutathione, L-cysteine and dithioerythritol, respectively. On the other hand, inhibitory effects of 2,4-dichloro-5-sulfamoil benzoic acid, p-aminobenzoic acid, benzoic acid, 4-carboxy benzene sulfanamide, sulfosalicylic acid, sodium azide and Na-EDTA were found to be weaker. Therefore, these compounds can be used to prevent enzymatic browning in Jerusalem artichoke products. Rapeanu *et al*. [8] reported that for Victoria grape L-cysteine, ascorbic acid and sodium metabisulfite are stronger inhibitors for Victoria grape. On the other hand, dithioerythritol, sodium metabisulfite and ascorbic acid are found to be stronger inhibitors for artichoke heads [3]. The prevention of enzymatic browning by a specific inhibitor may involve a single mechanism or be the result of an interplay of two or more mechanisms of inhibitor action. There are various mechanisms through which enzyme inhibitors can act. For example, the action of cysteine is complex, it forms addition compounds with phenolic substrates. Cysteine also forms adducts with quinones. Inhibition by thiol compounds is attributed to either the stable colorless products formed trough an additional reaction with *o*-quinones or binding to the active center of PPO, like metabisulfite. 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 [3].

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