CHARACTERIZATION OF POLYPHENOLOXIDASE FROM WILD PEAR (*PYRUS ELAEGRIFOLIA*)

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ABSTRACT

*Wild pear polyphenoloxidase (PePPO) was extracted and purified using a Sepharose 4B-L-tyrosine-*p-*amino benzoic acid affinity column. Optimum conditions for pH, temperature and heat inactivation were determined. At the optimum pH and temperature, K_M and V_{max} values for PePPO with catechol and pyrogallol were determined. The V_{max}/K_M showed that PePPO has the greatest activity toward catechol. Optimum pH for PePPO was pH 6.0 using catechol as substrate. Optimum temperatures of PePPO for pyragallol and catechol were 65 and 35C, respectively. Enzyme activity decreased because of heat denaturation with increasing temperature. Inhibition of PePPO was investigated using* p-*aminobenzoic acid, ethyleneglycol, L-cysteine, L-tyrosine, sodium azide,* p-*aminobenzenesulfonamide, β-mercaptoethanol and dithiothreitol and catechol as substrate. Competitive-type inhibition was obtained with ethyleneglycol, L-cysteine, L-tyrosine,* p-*aminobenzenesulfonamide and dithiothreitol. Uncompetitive inhibition was obtained with β-mercaptoethanol, sodium azide and* p-*aminobenzoic acid. These results show that the most effective inhibitor for PePPO was dithiothreitol and that the type of inhibition depended on the origin of PPO.*

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PRACTICAL APPLICATIONS

In this present work, the properties of polyphenoloxidase in *Pyrus elaegrifolia*, including optimum temperature, optimum pH, substrate specificity and response to inhibitors, were studied.

INTRODUCTION

Wild pear (*Pyrus elaegrifolia*) is a member of the Rosaceae family and is native to western Turkey. The fruits are hard when ripe and they become brown, soft, sweet and edible after harvesting (Rıvas and Whitaker 1973; Wissemann and Lee 1981). They are widely consumed as preserves and occasionally pickled and dried. The fruits are also used as folk medicines, primarily in the treatment of diarrhea and in poisonous snake bites for detoxification. It is deciduous, a part of the star chestnut family, growing up to 10 m. It is also one of the first flowering trees in spring. The flowers, white/pink clusters on the ends of branches, appear before the leaves. An infusion of the bark is used to treat intestinal ulcers, nausea and palpitations. A decoction is used for hemorrhoids, intestinal upsets and diarrhea, and to hasten the onset of labor while a colic remedy is made from the root. All of these properties make the wild pear very important in the food industry. Another important point is that this fruit contains the enzyme polyphenoloxidase (PPO).

PPO (EC 1.14.18.1) is a copper-containing enzyme, widely distributed in nature, responsible for melanization in animals and browning in plants (Gowda and Paul 2002; Shellby and Popham 2006). PPO also catalyzes the ortho-hydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones (Gowda and Paul 2002). *P. elaegrifolia* is used as a material for pickled fruit, and it is consumed all over the world. When it is stored in a refrigerator, the fruit develops unpleasant colors and flavors, and loses nutrients when it browns. Therefore, it is necessary to characterize the PPO to develop more effective methods for controlling browning in *P. elaegrifolia*. Enzymatic browning of fruits is related to oxidation of phenolic endogenous compounds into highly unstable quinones, which are later polymerized to brown, red and black pigments (Blumenthal *et al.* 2000). The degree of browning depends on the nature and amount of endogenous phenolic compounds, on the presence of oxygen, reducing substances, metallic ions, on pH and temperature and on the activity of PPO, the main enzyme involved in the reaction (Nunez-Delicado *et al.* 2005). Enzymatic browning is also an economic problem for processors and consumers (Marshall *et al.* 2000; Gowda and Paul 2002). 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 (Pizzocaro *et al.* 1993). Enzymatic browning has been studied in several plant tissues such as artichoke (Aydemir 2004), *Thymus longicaulis* var. Subisophyllus (Dogan *et al.* 2003), oregano (Dogan *et al.* 2005), apples (Murata *et al.* 1995), bananas (Galeazi *et al.* 1981; Kahn and Andrawis 1985), peaches (Flurkey and Jen 1980), grapes (Wissemann and Lee 1985; Lamikandra *et al.* 1992), plums (Siddig *et al.* 1992), herbs (Arslan *et al.* 1997), spinach (Golbeck and Cammarata 1981), broad beans (Huntcheson and Buchanan 1980; Flurkey 1989), field beans (Paull and Gowda 2000), wild potatoes (Kowalski *et al.* 1992), Jerusalem artichoke (Zawistowski *et al.* 1988a), cabbages (Fujita *et al.* 1995), tea leaves (Takeo and Baker 1972; Halder *et al.* 1998) and pears (Amiot *et al.* 1995; Siddig and Cash 2000; Nishimura *et al.* 2003; Kim *et al.* 2005).

Enzymatic browning can be controlled in different ways. In addition to heat treatment and acidification, a wide range of chemicals inhibit PPO activity. However, a limited number of them are considered to be acceptable when compared to consumer safety and/or cost, and could act as potential alternatives to sulfites, which are very effective in controlling browning but are subject to regulatory restrictions (Lattanzio *et al.* 1994). In this work, purification and characterization of PPO from wild pear (*P. elaegrifolia*) fruit were studied in terms of substrate specificity, optimum pH and temperature, heat inactivation and degrees of inhibition by general PPO inhibitors. This information may be useful in devising effective methods for inhibiting browning during storage.

MATERIALS AND METHODS

Materials

P. elaegrifolia fruits used in this study were harvested in November from a field near Balikesir in Turkey. All chemicals used in this study were the best grade available. Affinity gel used in this study was synthesized according to Arslan *et al.* (2004).

Extraction and Purification Procedure

The extraction procedure was adopted from Wesche-Ebeling and Montogomery (1990). Wild pear fruits were washed with distilled water three times. Crude extract was prepared from unpeeled sample tissue/10 g by cutting quickly into thin slices and homogenizing in a Waring blender (Torrington, CT) for 2 min using 100 mL 0.1 M phosphate buffer (pH 6.5) containing 5%

poly(ethylene glycol) and 10 mM ascorbic acid. The homogenate was purified with affinity chromotography. The affinity gel used was synthesized according to the method of Arslan *et al.* (2004). The enzyme solution was applied to the affinity column (1×10 cm), equilibrated with 5 mM phosphate buffer (pH 6.5). The affinity gel was washed with the same buffer. PPO was eluted with a solution of 5 mM phosphate buffer (pH 8.5) containing 1 M NaCl.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the method of Laemmli (1970). Samples were applied to 12% 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 (Sigma-Aldrich, Milan, Italy).

Spectrophotometric Assays

Kinetic assays were carried out by measuring the increase in absorbance at 420 nm for catechol and at 320 nm for pyrogallol, with a Carry |1E|g UV-visible spectrophotometer (Biotech Engineering, UK). The temperature was kept at 25C using a Tecne B12 water bath with Tempette Junior TE-8J 0-85C heater element, serial no. 76740-8 (Sygenta, UK). The reaction was carried out in a 1 cm light path quartz cuvette. The sample cuvette contained 2.8 mL of substrates at various concentrations prepared in the homogenization buffer (pH 6.5) and 0.2 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 substrate, with a final volume of 3 mL (Arslan *et al.* 1997).

Determination of Protein Content

The protein content was determined according to the Bradford method using bovine serum albumin as standard (Bradford 1976).

Enzyme Kinetics and Substrate Specificity

PPO activity was assayed using pyrogallol and catechol as substrate. The rate of reaction was measured as 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, Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) were determined according to the method of Lineweaver–Burk.

Effect of pH

PPO activity as a function of pH was determined using catechol as substrate (0.1 M stock concentration). The buffers used were 0.1 M acetate (pH 4.5–6.0) and 0.1 M phosphate (pH 6.0–9.5) adjusted with 0.1 M NaOH and HNO_3.

Effect of Temperature

The optimum temperature for PPO was measured at different temperatures in the range of 20–80C using pyrogallol and catechol as substrates. 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 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 (0.02 M final concentration), 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.

Heat Inactivation of PPO

Thermal inactivation of the partially purified enzyme was studied at 40, 50, 60, 70 and 80C. 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 25C (Chutintrasri and Noomhorm 2006).

Inhibition of *P. elaegrifolia* PPO (PePPO) Activity

IC_{50} and K_i values of different inhibitors (*p*-aminobenzoic acid, ethyleneglycol, L-cysteine, L-tyrosine, sodium azide, *p*-aminobenzenesulfonamide, β-mercaptoethanol, dithiothreitol) were determined on PePPO. In order to determine the IC_{50} values, 10 mM catechol was used as substrate. Activity was first measured without inhibitor and labeled control. Activities for the inhibitors were then compared to the control at different inhibitor concentrations. In order to determine the IC_{50}, graphs were drawn comparing percent activity versus inhibitor concentration. The IC_{50} values were determined from these graphs. This way was also followed to determine the K_i values. In the reaction mixture with or without inhibitor, the substrate concentrations were 0.02, 0.0266, 0.033 and 0.04 M. For this purpose, the substrate was used between

0.6 and 1.2 mL. Inhibitor solutions were added to the reaction medium as five different concentrations. The Lineweaver–Burk graphs were obtained, and K_i values were calculated.

RESULTS AND DISCUSSION

Extraction and Purification of PePPO

In this study, it is the first time PePPO was purified with affinity chromatography. The purification procedures are summarized in Table 1. As seen in Table 1, PePPO was purified up to 31.5-fold. Different purification protocols have been used for PPO enzyme from different sources (Weemaes *et al.* 1998; Jiang 1999). Some purification methods for PPO from different sources used methods such as Triton X-100, ammonium sulfate precipitation, dialysis, affinity chromatography, Sephadex G-200, Phenyl Sepharose hydrophobic chromatography (Weemaes *et al.* 1998; Jiang 1999; Arslan *et al.* 2004). However, PePPO was purified generally in two steps, while other purification methods usually required two or more steps such as Triton X-100, ammonium sulfate precipitation, dialysis and acetone precipitation (Weemaes *et al.* 1998; Siddig and Cash 2000; Carbanaro and Mattera 2001).

The molecular weight of PPO was estimated on SDS-PAGE as a single band of approximately 35 kDa (Fig. 1). The molecular mass of PPO from other species has been reported as follows: cabbages, 39 kDa (Fujita *et al.* 1995); sago palm, 53 kDa (Onsa *et al.* 2000); sunflower seeds, 42 kDa (Raymond *et al.* 1983); and field bean seeds, 120 kDa (Paull and Gowda 2000). These results indicate that the molecular mass of *P. elaegrifolia* was similar to cabbages, but different from those of sago palm, sunflower seeds and field bean seeds. In addition, it was reported that the molecular weight of pear PPO was found to be 750 kDa (Weemaes *et al.* 1998).

Substrate Specificity and Enzyme Kinetics

The PPO activity of partially purified enzyme was examined with regard to its diphenolase activity. The substrate specificity of the enzyme

TABLE 1.
PURIFICATION OF POLYPHENOLOXIDASE FROM *PYRUS ELAEGRIFOLIA*

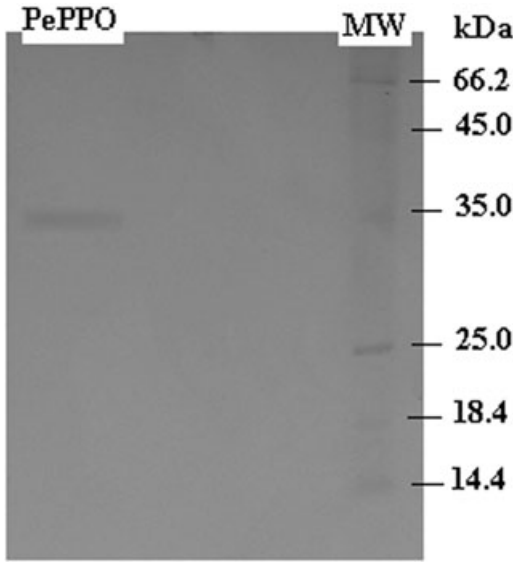| Purification step	Volume (mL)	Total activity	Activity (U/mL·min)	Total protein (mg)	Specific activity (U/mg protein)	Purification fold
Extract	5	20,950	4,190	1.44	14,558	–
Affinity chromatography	20	26,600	1,330	0.06	458,620	31.50

FIG. 1. SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS OF
PYRUS ELAEGRIFOLIA POLYPHENOLOXIDASE (PePPO) PURIFIED BY AFFINITY GEL
MW, molecular weight marker.

was investigated by using the two chemicals pyrogallol and catechol. Lineweaver–Burk plots for PePPO showed K_m values of 0.0011 and 0.0057 mM for pyrogallol and catechol, respectively. Previous studies found that the K_m values for mulberry PPO were 1.24 and 19.81 mM with pyrogallol and catechol as substrates, respectively (Arslan *et al.* 2004). In this study, the values of K_m for PPO from *P. elaegrifolia* for the substrates assayed were different from those reported in the literature: artichoke (10.2 mM) (Aydemir 2004), tea leaves (12.5 mM) (Halder *et al.* 1998), field bean seeds (10.5 mM) (Paull and Gowda 2000), Amasya apples (34 mM) (Oktay *et al.* 1995), thymus (18 mM) (Dogan and Dogan 2004), cabbages (682.5 mM) (Nagai and Suzuki 2001) and Stanley plums (20 mM) (Siddig *et al.* 1992) with catechol as a substrate. The V_{max}/K_m ratio referred to as "catalytic power" is a better parameter for evaluating the most effective substrate (Dogan *et al.* 2005). Considering the ratio V_{max}/K_m, it can be said that catechol is the most suitable substrate for PePPO activity. Similar results were found for *Ferula* sp. (Erat *et al.* 2006) and artichoke (Aydemir 2004). In addition, some pear cultivars (*Pyrus communis* L.) catalyzed different substrates than PePPO. It was found that 4-methylcatechol, followed by catechol and dopamine, was the most readily oxidized substrate of PPO from
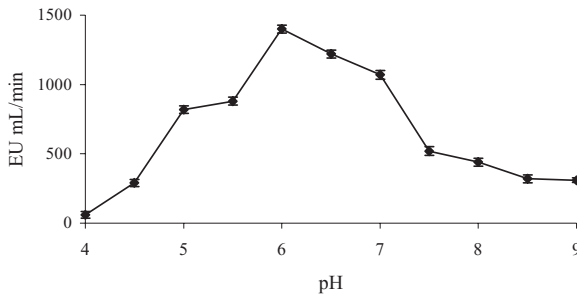
FIG. 2. THE EFFECT OF pH ON THE PURIFIED *PYRUS ELAEGRIFOLIA* POLYPHENOLOXIDASE ACTIVITY

pear cultivars (Siddig and Cash 2000). The large range in the apparent K_m values of PPO reported may be because of different reasons: different assay methods used, different varieties, different origins of the same variety and different extraction pH (Rocha *et al.* 1998).

Optimum pH

The enzyme activity exhibits a significant dependency on the pH value of the medium. With rising pH values, the activity increases to a maximum (pH optimum) and drops to zero in the alkaline region, which is expressed in a bell-shaped optimum curve. The optimum pH value for PePPO was determined in the pH range of 4.5–9.0. As seen in Fig. 2, it was found that the optimum pH value for PePPO was 6.0 for catechol as substrate. Different optimum pH values for PPO obtained from various sources are reported in the literature. For example, it was reported that the optimum pH values are 5.5 for strawberries (Wesche-Ebeling and Montogomery 1990); 6.0 for DeChaunac grapes (Lee *et al.* 1983); 7.0 for Amasya apples (Oktay *et al.* 1995), *Anethum graveolens* L. (Arslan and Tozlu 1997) and aubergines (Dogan *et al.* 2002); 7.5 for *Allium* sp. (Arslan *et al.* 1997); and 8.5 for dog rose (Sakiroglu *et al.* 1996) using catechol as a substrate, respectively. In addition, it was reported that the optimum pH values of pear cultivars for d'Anjou and Bartlett (*P. communis* L.) were found to be 4.7 and 5.5 (Siddig and Cash 2000). However, another study showed that the optimum pH value of pear PPO was found to be 7.0 (Weemaes *et al.* 1998).

Optimum Temperature

Figure 3A shows the effect of temperature on the activity of the enzyme. When catechol and pyragallol were used as the substrates, PPO showed
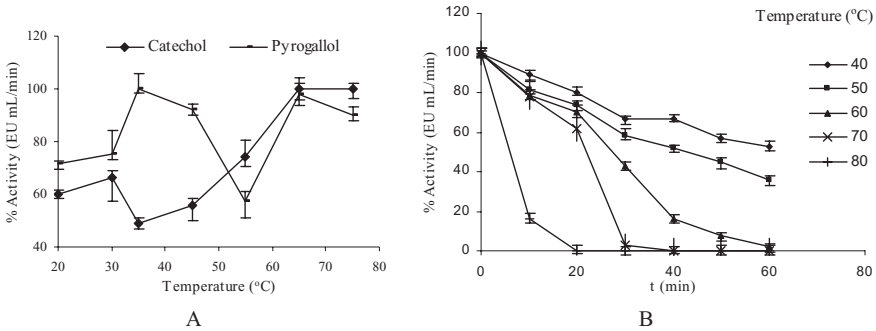
FIG. 3. THE EFFECT OF TEMPERATURE ON THE PURIFIED *PYRUS ELAEGRIFOLIA*
POLYPHENOLOXIDASE ACTIVITY
(A) Using different substrates. (B) Heating inactivation.

maximum activity at 35 and 65C, then decreased gradually with increasing temperatures. The optimum temperatures are substrate dependent. The optimum temperatures for dog rose PPO of 20C for 4-methylcatechol as substrate, and 15C for pyrogallol as substrate were found (Sakiroglu *et al.* 1996). In addition, it was reported that the optimum temperatures were 40C for Chinese cabbage (Nagai and Suzuki 2001), 12C for *Ferula* sp. (Erat *et al.* 2006) and 25C for artichoke (Aydemir 2004) using catechol as substrate.

Thermal Inactivation

The thermal stability profile for PePPO, presented as percent residual activity, is shown in Fig. 3B. The thermal inactivation for PePPO was determined using catechol as substrate, which has the best catalytic power for PePPO. The enzyme activity decreased because of heat denaturation of the enzyme with increasing temperature and incubation time. Figure 3B shows that temperatures above 40C resulted in loss of enzyme activity. In another study, pear PPO inactivation becomes progressive at about 60–65C (Weemaes *et al.* 1998). At high temperature, the enzyme activity was rapidly lost. For instance, when the temperature was increased from 40 to 60C, the activity of PePPO decreased from 75 to 15%. This indicated that the enzyme was rapidly inactivated at higher temperatures. The times required for 50% inactivation of activity at 70 and 80C were found to be 15 and 5 min, respectively. It has been reported that *Allium* sp. PPO is stable at 40C for 30 min (Arslan *et al.* 1997), Stanley plum (Siddig *et al.* 1992) and banana PPOs are stable at 70C for 30 min (Yang *et al.* 2000) and Jerusalem artichoke PPO is stable at 60C for 30 min (Zawistowski *et al.* 1988a,b).

TABLE 2.
EFFECT OF INHIBITORS ON THE ACTIVITY OF *PYRUS ELAEGRIFOLIA*
POLYPHENOLOXIDASE WITH CATECHOL AS SUBSTRATE

Inhibitor	IC_{50} (mM)	Type of inhibition	K_i (mM)
p-Aminobenzoic acid	0.847	Uncompetitive	$0.3 \pm 3 \times 10^{-3}$
Ethyleneglycol	2.01	Competitive	$7.6 \pm 5 \times 10^{-3}$
L-Cysteine	6.49	Competitive	$1.1 \pm 2 \times 10^{-4}$
L-Tyrosine	0.143	Competitive	$0.1 \pm 1 \times 10^{-4}$
Sodium azide	0.005	Uncompetitive	$0.01 \pm 6 \times 10^{-4}$
p-Aminobenzenesulfonamide	0.0017	Competitive	$2 \times 10^{-4} \pm 1 \times 10^{-4}$
B-Mercaptoethanol	0.002	Uncompetitive	$0.01 \pm 2 \times 10^{-3}$
Dithiothreitol	0.001	Competitive	$2 \times 10^{-5} \pm 1 \times 10^{-5}$

Inhibition of PPO

Inhibition of PePPO by *p*-aminobenzoic acid, ethyleneglycol, L-cysteine, L-tyrosine, sodium azide, dithiothreitol, β-mercaptoethanol and *p*-aminobenzenesulfonamide was investigated. It was found that the presence of all chemicals caused the inhibition of PePPO (Table 2). 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. A competitive-type inhibition was obtained with ethyleneglycol, L-cysteine, L-tyrosine, dithiothreitol and *p*-aminobenzenesulfonamide using catechol as substrate. Similar results were found for field bean seed PPO using L-cysteine and dithiothreitol as inhibitors and catechol as substrate (Paull and Gowda 2000). Figure 4A shows the effect of L-tyrosine and sodium azide inhibitors on PePPO using catechol as substrate (other figures are not shown).

The percent inhibition and K_i values for the inhibitors are given in Table 2 for catechol as substrate. 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. L-Cysteine can easily form complexes with quinones, and therefore, inhibit secondary oxidation and polymerization reactions (Davis and Pierpoint 1975). L-Cysteine, which can also act as a reducing agent (Wesche-Ebeling and Montogomery 1990), was a poor inhibitor for PePPO (IC_{50} 6.49 mM). However, it was reported that L-cysteine was a more effective inhibitor of some pear cultivars, namely d'Anjou and Bartlett (Siddig and Cash 2000). Sodium azide toxicity toward a metal enzyme, especially in the case of a copper enzyme, is mainly because of 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
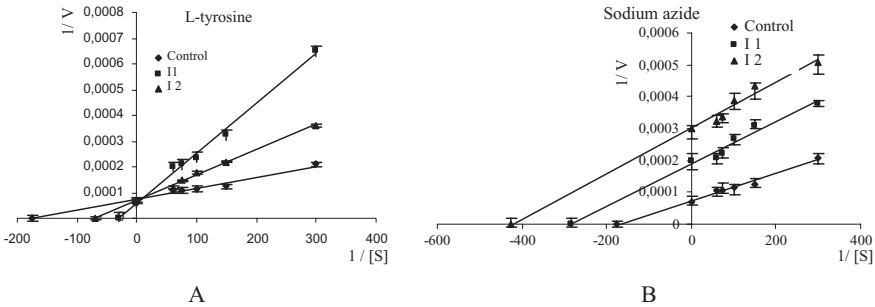
FIG. 4. INHIBITION OF *PYRUS ELAEGRIFOLIA* POLYPHENOLOXIDASE BY (A)
L-TYROSINE AND (B) SODIUM AZIDE WITH CATECHOL AS SUBSTRATE

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 (Schwartz *et al.* 2001). Paull and Gowda observed a competitive-type inhibition for field bean PPO with cysteine–HCl inhibitors and with catechol as substrate. From this, the type of inhibition does not depend on the origin of the PPO studied. Other studies investigating the inhibition on pear PPO include using sodium metabisulfite, ascorbic acid, thiourea, citric acid, potassium sorbate and heated onion (Siddig and Cash 2000; Kim *et al.* 2005).

Walker and Wilson suggested the existence of two distinct sites on the enzyme: one for binding of the substrate, and another adjacent site for binding of inhibitor. Even though some authors have found competitive inhibition of PPO using 4-methylcatechol as substrate (Walker and Wilson 1975; Gunata *et al.* 1987; Janovitz-Klapp *et al.* 1990), other differences in type and degree of inhibition for various PPOs were reported (Pifferi *et al.* 1974; Kermasha *et al.* 1993).

CONCLUSIONS

In this study, an uncompetitive-type inhibition was obtained with *p*-aminobenzoic acid, sodium azide and β-mercaptoethanol using catechol as substrate. Figure 4B shows the effect of sodium azide inhibitor on PePPO using catechol as substrate (other figures are not shown). The percent inhibition and K_i values for the uncompetitive inhibitors were determined and presented in Table 2 for catechol as substrate. When comparing K_i values from these tables, the most effective inhibitor for PePPO with catechol as substrate was dithiothreitol followed by *p*-aminobenzenesulfonamide, β-mercaptoethanol and sodium azide. Dithiothreitol in this study was the most effective inhibitor of PePPO because of its low K_i value.

REFERENCES

AMIOT, M.J., TACCHINI, M., AUBERT, S.Y. and OLESZEK, W. 1995. Influence of cultivar, maturity stage, and storage-conditions on phenolic composition and enzymatic browning of pear fruits. J. Agric. Food Chem. *43*(5), 1132–1137.

ARSLAN, O. and TOZLU, I. 1997. Substrate specificity, heat inactivation and inhibition of polyphenol oxidase from *Anethum graveolens* L. Ital. J. Food Sci. *9*(3), 249–253.

ARSLAN, O., TEMUR, A. and TOZLU, I. 1997. Polyphenol oxidase from *Allium* sp. J. Agric. Food Chem. *45*, 2861–2863.

ARSLAN, O., ERZENGIN, M., SINAN, S. and OZENSOY, O. 2004. Purification of mulberry (*Morus alba* L.) polyphenol oxidase by affinity chromatography and investigation of its kinetic and electrophoretic properties. Food Chem. *88*(3), 479–484.

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

BLUMENTHAL, M., GOLDBERG, A. and BRINCKMAN, J. 2000. *Herbal Medicine: Expanded Commission E Monographs*, American Botanical Council, Austin, TX.

BRADFORD, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. *72*, 248–254.

CARBANARO, M. and MATTERA, M. 2001. Polyphenoloxidase activity and polyphenol levels in organically and conventionally grown peach (*Prunus persica* L., cv. Regina bianca) and pear (*Pyrus communis* L., cv. Williams). Food Chem. *72*, 419–424.

CHUTINTRASRI, B. and NOOMHORM, A. 2006. Thermal inactivation of polyphenoloxidase in pineapple puree. LWT *39*, 492–495.

DAVIS, R. and PIERPOINT, W.S. 1975. 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. *3*, 671.

DOGAN, S. and DOGAN, M. 2004. Determination of kinetic properties of polyphenol oxidase from thymus (*Thymus longicaulis* subsp. Chaubardii var. Chaubardii). Food Chem. *88*, 69–77.

DOGAN, M., ARSLAN, O. and DOGAN, S. 2002. Substrate specificity, heat inactivation and inhibition of polyphenol oxidase from different aubergine cultivars. Int. J. Food Sci. Technol. *37*, 415–423.

DOGAN, S., DOGAN, M. and ARSLAN, O. 2003. Characterization of polyphenol oxidase from thymus (*Thymus longicaulis* var. Subisophyllus). Adv. Food Sci. *25*(2), 56–64.

DOGAN, S., ARSLAN, O. and OZEN, F. 2005. Polyphenol oxidase activity of oregano in different stages. Food Chem. *91*, 341–345.

ERAT, M., AKIROGLU, H. and KUFREVIOGLU, O.I. 2006. Purification and characterization of polyphenol oxidase from *Ferula* sp. Food Chem. *95*, 503–508.

FLURKEY, W.H. 1989. Polypeptide composition and aminoterminal sequence of broad bean polyphenoloxidase. Plant Physiol. *91*, 481–483.

FLURKEY, W.H. and JEN, J.J. 1980. Purification of peach polyphenoloxidase in the presence of added protease inhibitors. J. Food Biochem. *4*, 829.

FUJITA, S., SAARI, N.B., MAEGAWA, M., TETSUKA, T., HAYASHI, N. and TONO, T. 1995. Purification and properties of polyphenol oxidase from cabbage (*Brassica oleracea* L.). J. Agric. Food Chem. *43*, 1138–1142.

GALEAZI, M.A.M., SGARBIERI, V.C. and CONSTANTINIDES, S.M. 1981. Isolation, purification and physicochemical characterization of polyphenoloxidase (PPO) from a dwarf variety of banana (*Musa cavendishii* L.). J. Food Sci. *46*, 150–155.

GOLBECK, J.H. and CAMMARATA, K.V. 1981. Spinach thylakoid polyphenol oxidase. Isolation, activation and properties of the native chloroplast enzyme. Plant Physiol. *67*, 877–884.

GOWDA, L.R. and PAUL, B. 2002. Diphenol activation of the monophenolase and diphenolase activities of field bean (*Dolichos lablab*) polyphenol oxidase. J. Agric. Food Chem. *50*, 1608–1614.

GUNATA, Y.Z., SAPIS, J.C. and MOUTONET, M. 1987. Substrates and aromatic carboxylic and inhibitors of grape polyphenoloxidases. Phytochemistry *26*, 1573–1575.

HALDER, J., TAMULI, P. and BHADURI, A.N. 1998. Isolation and characterization of polyphenol oxidase from Indian tea leaf (*Camellia sinensis*). J. Nutr. Biochem. *9*, 75–80.

HUNTCHESON, S.W. and BUCHANAN, B.B. 1980. Polyphenol oxidation by *Vicia faba* chloroplast membranes. Plant Physiol. *66*, 1150–1154.

JANOVITZ-KLAPP, A.H., RICHARD, F.C., GOUPY, P.M. and NICOLAS, J.J. 1990. Kinetic studies on apple polyphenol oxidase. J. Agric. Food Chem. *38*, 1437–1441.

JIANG, Y.M. 1999. Purification and some properties of polyphenol oxidase of longan fruit. Food Chem. *66*, 75–79.

KAHN, V. and ANDRAWIS, A. 1985. Inhibition of mushroom tyrosinase by tropolone. Phytochemistry *24*, 905–908.

KERMASHA, S., GOETHEBEUR, M. and MONFETTE, A. 1993. Studies on inhibition of mushroom polyphenol oxidase using chlorogenic acid as substrate. J. Agric. Food Chem. *41*, 526–531.

KIM, M.J., KIM, C.Y. and PARK, I. 2005. Prevention of enzymatic browning of pear by onion extract. Food Chem. *89*(2), 181–184.

KOWALSKI, S.P., EANNETTA, N.T., HIRZEI, A.T. and STEENS, J.C. 1992. Purification and characterization of polyphenol oxidase from glandular trichomes of *Solanum berthaultii*. Plant Physiol. *100*, 677–684.

LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

LAMIKANDRA, O., SHARON, D.K. and MITWE, N.M. 1992. Muscadine grape polyphenol oxidase: Partial purification by high-pressure liqid chromatography and some properties. J. Food Sci. *57*, 686–689.

LATTANZIO, V., CARDINALL, A., DI VENERE, D., LINSALATA, V. and PALMIERI, S. 1994. Browning phenomena in stored artichoke (*Cynara scolymus* L.) heads: Enzymic or chemical reactions? Food Chem. *50*, 1–7.

LEE, C.Y., SMITH, N.L. and PENNESI, A.P. 1983. Polyphenol oxidase from DeChaunac grapes. J. Sci. Food Agric. *34*, 987–991.

MARSHALL, M.R., KIM, J. and WEI, C. 2000. Enzymatic browning in fruits, vegetables and seafoods. http://www.fao.org/ag/Ags/agsi/ENZYMEFINAL/Enzymatic%20Browning.html (accessed March 10, 2008).

MURATA, M., TSURUTANI, M., TOMITA, M., HOMMA, S. and KANEKO, K. 1995. Relationship between apple ripening and browning: Changes in polyphenol content and polyphenol oxidase. J. Agric. Food Chem. *43*, 1115.

NAGAI, T. and SUZUKI, N. 2001. Partial purification of polyphenol oxidase from Chinese cabbage (*Brassica rapa* L.). J. Agric. Food Chem. *49*, 3922–3926.

NISHIMURA, M., FUKUDA, C., MURATA, M. and HOMMA, S. 2003. Cloning and some properties of Japanese pear (*Pyrus pyrifolia*) polyphenol oxidase, and changes in browning potential during fruit maturation. J. Sci. Food Agric. *83*(11), 1156–1162.

NUNEZ-DELICADO, E., SANCHEZ-FERRER, A., GARCIA-CARMONA, F.F. and LOPEZ-NICOLAS, J.M. 2005. Effect of organic farming practices on the level of latent polyphenol oxidase in grapes. J. Food Sci. *70*(1), 74–85.

OKTAY, M., KUFREVIOGLU, I., KOCACALISKAN, I. and SAKIROGLU, H. 1995. Polyphenol oxidase from Amasya apple. J. Food Sci. *60*, 495–499.

ONSA, G.H., BIN SAARI, N., SELAMAT, J. and BAKAR, J. 2000. Latent polyphenol oxidases from sage log (*Metroxylon sagu*): Partial purification, activation, and some properties. J. Agric. Food Chem. *48*, 5041–5045.

PAULL, B. and GOWDA, L.R. 2000. Purification and characterization of a polyphenol oxidase from the seeds of field bean (*Dalichos lablab*). J. Agric. Food Chem. *48*, 3839–3846.

PIFFERI, P.G., BALDASSARI, L. and CULTERA, R. 1974. Inhibition by carboxylic acids of an *o*-diphenol oxidase from *Prunus avium* fruits. J. Sci. Food Agric. *25*, 263–270.

PIZZOCARO, F., TORREGGIANI, D. and GILARDI, G. 1993. Inhibition of apple polyphenol oxidase by ascorbic acid, citric acid and sodium chloride. J. Food Process. Preserv. *17*, 21–30.

RAYMOND, J., RAKARIYATHAN, N. and AZANZA, J.L. 1983. Purification and some properties of polyphenol oxidases from sunflower seeds. Photochemistry *34*, 927–932.

RIVAS, N.D.J. and WHITAKER, J.R. 1973. Purification and some properties of two polyphenol oxidases from Bartlett pear. Plant Physiol. *52*, 501–507.

ROCHA, A.M.C., PILAR CANO, M., GALEAZZI, M.A.M. and MORARIS, A.M.M.B. 1998. Characterization of "Starking" apple polyphenoloxidase. J. Sci. Food Agric. *77*, 527–534.

SAKIROGLU, H., KUFREVIOGLU, I.O., KOCACALISKAN, I., OKTAY, M. and ONGANER, Y. 1996. Purification and characterization of Dogrose (*Rosa dumalis* Rechst.) polyphenol oxidase. J. Agric. Food Chem. *44*, 2982–2986.

SCHWARTZ, B., OLGIN, A.K. and KLINMAN, J.P. 2001. The role of copper in topa quinone biogenesis and catalysis, as probed by azide inhibition of a copper amine oxidase from yeast. Biochemistry *40*, 2954–2963.

SHELLBY, K.S. and POPHAM, H.J.R. 2006. Plasma phenoloxidase of the larval tobacco budworm, *Heliothis virescens*, is virucidal. J. Insect Sci. *13*, 2442–2448.

SIDDIG, M. and CASH, J.N. 2000. Physico-chemical properties of polyphenol oxidase from d'Anjou and Bartlett pears (*Pyrus communis* L.). J. Food Process. Preserv. *24*(5), 353–364.

SIDDIG, M., SINHA, N.K. and CASH, J.N. 1992. Characterization of polyphenol oxidase from Stanley plums. J. Food Sci. *57*, 1177–1179.

TAKEO, T. and BAKER, J.E. 1972. Changes in multiple forms of polyphenol oxidase during maturation of tea leaves. Phytochemistry *12*, 21–24.

WALKER, J.R.L. and WILSON, E.L. 1975. Studies on the enzymatic browning of apples. Inhibition of apple *o*-diphenol oxidase by phenolic acids. J. Sci. Food Agric. *26*, 1825–1831.

WEEMAES, C.A., LUDIKHUYZE, L.R., BROECK, I.V., HENDRICKX, M.E. and TOBBACK, P.P. 1998. Activity, electrophoretic characteristics and heat inactivation of polyphenoloxidases from apples, avocados, grapes, pears and plums. Lebensm.-Wiss. Technol. *31*, 44–49.

WESCHE-EBELING, P. and MONTOGOMERY, M.W. 1990. Strawberry polyphenol oxidase: Extraction and partial characterization. J. Food Sci. *55*, 1320–1325.

WISSEMANN, K.W. and LEE, C.Y. 1981. Characterization of polyphenoloxidase from Ravat 51 and Niagara grapes. J. Food Biochem. *46*, 506.

WISSEMANN, K.W. and LEE, C.Y. 1985. Characterization of polyphenoloxidase from Ravat 51 and Niagara grapes. J. Food Sci. *46*, 506–508.

YANG, C.P., FUJITA, S., ASHRAFUZZAMAN, M.A., NAKAMURA, N. and HAYASHI, N. 2000. Purification and chracterization of polyphenol oxidase from banana (*Musa sapientum* L.) pulp. J. Agric. Food Chem. *48*, 2732–2735.

ZAWISTOWSKI, J., BILLIADERIS, C.G. and MURRAY, E.D. 1988a. Purification and characterization of Jerusalem artichoke (*Helianthus tuberosus* L.) polyphenol oxidase. J. Food Biochem. *12*, 1–22.

ZAWISTOWSKI, J., BILLIADERIS, C.G. and MURRAY, E.D. 1988b. Isolation and some properties of an acidic fraction of polyphenol oxidase from Jerusalem artichoke (*Helianthus tuberosus* L.). J. Food Biochem. *12*, 23–35.