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Characterization of polyphenoloxidase from medlar fruits (*Mespilus germanica* L., Rosaceae)

Barbaros Dincer[a], Ahmet Colak[a], Nese Aydin[b], Asim Kadioglu[b], Saadettin Güner[a,*]

[a]*Department of Chemistry, Karadeniz Technical University, 61080 Trabzon, Turkey*
[b]*Department of Biology, Karadeniz Technical University, 61080 Trabzon, Turkey*

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Abstract

Crude extracts prepared from medlar fruits (*Mespilus germanica* L., Rosaceae) possess a diphenolase activity toward catechol, 4-methyl catechol, *L*-3,4-dihydroxyphenylalanine, epicatechin and 3-(3,4-dihydroxyphenyl)propionic acid. Among the substrates employed, the greatest substrate specificity was observed with 4-methylcatechol. The pH-activity optimum for the enzyme, in the presence of this substrate, was 6.5 and the pH-stability profile for the enzyme showed that 80% of the PPO activity was retained at physiological pH values. The temperature-activity optimum, for the enzyme in the presence 4-methyl catechol, was 35 °C. The enzyme was stable for 30 min at its optimum temperature and moderately stable at 60 °C. At higher temperatures, heat denaturation of the enzyme occurred after 10 min of incubation. Thermal inactivation parameters indicate that the medlar enzyme is very heat-labile. Moreover, the medlar PPO activity was very sensitive to some common PPO inhibitors, especially to cysteine and metabisulfite. All data indicate that medlar fruits have highly active PPO enzymes which possess similar biochemical and kinetic characteristics to other plant PPO enzymes.

Keywords: Polyphenol oxidase; 4-methylcatechol; Medlar; *Mespilus germanica*; Rosaceae

1. Introduction

Plant polyphenol oxidases (PPO) are responsible for the enzymatic browning reaction occurring during the handling, storage and processing of fruits and vegetables. PPOs catalyze the hydroxylation of monophenols to *o*-diphenols which are oxidized to the corresponding *o*-quinones and subsequently polymerized to brown, red or black pigments, depending on natural components present in a given plant material (Whitaker, 1972; Zawistowski, Biliaderis, & Eskin, 1991). In plant tissues, the browning pigments lead to organoleptic and nutritional modifications, thus depreciating the food product (Friedman, 1996; Matheis & Whitaker, 1984; Sanchez-Ferrer, Rodriguez-Lopez, Garcia-Carnovas, & Garcia-Carmona, 1995). Occasionally this colour change is desirable.

Medlar fruit (*Mespilus germanica* L.) is a member of the Rosaceae family and is native to southeastern Europe, Anatolia, Crimea, Caucasia and the northern parts of Iraq and Iran (Browicz, 1972). The fruits are hard when ripe, and they become brown, soft, sweet and edible after harvesting (Dirr, 1990). They are widely consumed as preserves and occasionally as pickle or liqueur. The fruits are also used as folk medicines, primarily in the treatment of constipation, in the removal of kidney and bladder stones, or as a diuretic (Baytop, 1999).

In this work, characterization of PPO from medlar fruit was studied in terms of substrate specificities, thermal activation and stability, pH optimum and stability, and degrees of inhibition by general PPO inhibitors, in order to help to predict the behaviour of the medlar enzyme.

2. Materials and methods

2.1. Plant materials and chemicals

Medlar fruits were harvested fresh from a local garden in the first week of November. The fruits were carried

* Corresponding author. Tel.: +90-462-377-2598; fax: +90-462-325-3195.
E-mail address: sguner@ktu.edu.tr (S. Güner).

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into the laboratory in liquid nitrogen, with a nitrogen dewar flask, and stored deep-frozen at −20 °C for 1–3 months until used in the experiments.

Substrates and 3-methyl-2-benzothiazolinone hydrazone (MBTH) were purchased from Sigma Chemical Co. (St. Louis, MO) and the other reagents were of analytical grade and used as obtained.

2.2. Crude polyphenoloxidase preparation

Medlar fruits (50 g) were placed in a dewar flask under liquid nitrogen for 10 min in order to decompose cell membranes. The cold fruits were homogenized by using the blender in 100 ml of 50 mM cold acetate buffer (pH 5.5), containing 2 mM EDTA, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonylfluoride (PMSF) and 6% (w/v) Triton X-114, for 2 min at 4 °C. The homogenate was filtered and kept at 4 °C for 60 min before being centrifuged at 20,000×***g*** for 30 min at 4 °C. The supernatant was used as crude enzyme extract which retained PPO activity for 1 month at 4 °C.

2.3. Protein determination

Protein quantity in extracts was assayed by the Lowry method with bovine serum albumin as the standard (Lowry, Rosebrough, Farr, & Randall, 1951). The values were obtained by graphic interpolation on a calibration curve at 650 nm.

2.4. Assay of polyphenol oxidase activity

PPO activity was determined by the method reported previously (Espin, Morales, Varon, Tudela, & Garcia-Canovas, 1995) using an ATI Unicam UV2-100 double beam UV-Vis spectrophotometer equipped with a quartz cell of 10 mm path length. The activity was determined using different substrates by measuring the increase in absorbance at 494 nm for 4-methylcatechol and 500 nm for all other substrates (Espin et al., 1995). For enzyme activity, 1-ml sample cuvettes contained various concentrations of substrates (stock 100 mM), an equal volume of 3-methyl-2-benzothiazolinone hydrazone (MBTH, stock 10 mM), and 20 μl dimethylformamide (DMF), and the solution was diluted to 950 μl with buffer and 50 μl enzyme extract was added. The blank contained the same concentration of the solution except 50 μl enzyme extract in 1 ml (Espin et al., 1995). One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001/min (Galeazzi & Sgarbieri, 1981). All the determinations were performed in triplicate and corresponding means were plotted.

Table 1
Optimization of medlar PPO-catalyzed oxidation reactions of various phenolic substrates

Phenolic substrate	λ_{max} (nm)[a]	Optimum pH	Optimum temperature (°C)	Relative activity (%)
4-methylcatechol	494	6.5	35	100.0
Catechol	500	8.5	55	67.9
DOPA	500	6.5	55	58.8
Epicatechin	500	5.5	25	46.8
DHPPA	500	5.5	35	5.5

[a] The values were previously reported by Espin, Trujano, Tudela, and Garcia-Canovas (1997).

2.5. Properties of medlar PPO

2.5.1. pH optimum and stability

Relative PPO activity was determined in a pH range of 4.5–5.5 in 50 mM acetate buffer, 6.5 in 50 mM phosphate buffer and 7.5–9.5 in 50 mM Tris–HCl buffer. PPO activity was assayed using catechol, 4-methylcatechol, *L*-3,4-dihydroxyphenylalanine (*L*-DOPA), 3-(3,4-dihydroxyphenyl)propionic acid (DHPPA) or epicatechin as substrates, with MBTH and DMF prepared in a buffer solution at various pH values.

In order to determine pH stability of the medlar PPO, 0.05 ml of crude enzyme solution was incubated in 0.73 ml buffer solution, ranging from pH 4.5 to 9.5 for 24 h at 4 °C. pH was varied over a pH range of 4.5–5.5 in 50 mM acetate buffer, 6.5 in 50 mM phosphate buffer and 7.5–9.5 in 50 mM Tris–HCl buffer. Residual PPO activity was determined in the form of percent residual PPO activity at the optimum pH by mixing 0.1 ml of 100 mM 4-methylcatechol as a substrate, 0.1 ml of 10 mM MBTH and 0.02 ml DMF with the incubated enzyme solution.

2.5.2. Thermal activity and stability

The PPO activity was determined at various temperatures controlled by a circulation water bath. The mixtures of buffer and each substrate solution were incubated for 5 min at various temperatures over the range of 5–85 °C at the optimum pH values of the substrates, prior to the addition of the enzyme solution. The relative activity of PPO at a specific temperature was determined spectrophotometrically by addition of enzyme extract to the mixture as rapidly as possible.

In order to determine the thermal stability of the PPO, the enzyme solution in 50 mM phosphate buffer, pH 6.5, within Eppendorf tubes, was incubated in a water bath at temperatures of 35, 40, 45, 60 and 80 °C for 30 min. After the mixture was cooled in an ice bath and brought to room temperature, 0.05 ml heated enzyme extract was mixed with 0.1 ml of 100 mM 4-methylcatechol, 0.1 ml of 10 mM MBTH, and 0.02 ml DMF, and residual PPO activity was determined spectrophotometrically. The percentage residual PPO activity was calculated by comparison with unheated enzyme.

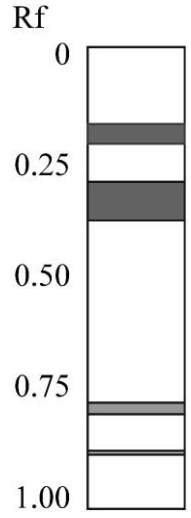

Fig. 1. Activity staining for PPO from medlar fruit with *L*-DOPA.

The data obtained from the thermal stability profile have been used to analyze some thermodynamic parameters related to medlar PPO activity in the crude extracts. The rate constant for the thermal inactivation reaction (and its temperature-dependence) was calculated by comparing the activity changes upon heat treatment with the unheated enzyme extract as reported (Amiza & Apenten, 1994; Duangmal & Owusu Apenten, 1999).

2.5.3. Enzyme kinetics and substrate specificity

PPO activity was assayed using catechol, 4-methylcatechol, *L*-DOPA, epicatechin and DHPPA with MBTH (Espin et al., 1995) in buffers at optimum pH values for each substrate. The rate of the reaction was measured in terms of the increase in absorbance at the absorption maxima of the corresponding chromophoric MBTH-quinone adducts for each substrate (Table 1). One unit of enzyme activity was defined as the amount of the 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 (Lineweaver & Burk, 1934), and the 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.

2.5.4. Effect of inhibitors

L-cysteine (20–30 μM), sodium azide (10–50 mM), benzoic acid (3–5 mM) and sodium metabisulfite (0.01–0.1 mM) were evaluated for their effectiveness as inhibitors of medlar PPO activity using 4-methylcatechol as the substrate. 1 ml reaction mixture contained 0.1 ml of 4-methylcatechol at various concentrations in 50 mM phosphate buffer (pH 6.5), 0.05 ml enzyme solution and 0.1 ml of inhibitor solutions at fixed concentrations. I_{50} values were calculated from the plots of inhibitor concentration versus percentage inhibition of 4-methylcatechol oxidation, and inhibition constants (K_i) were deduced from the Dixon plots for each inhibitor.

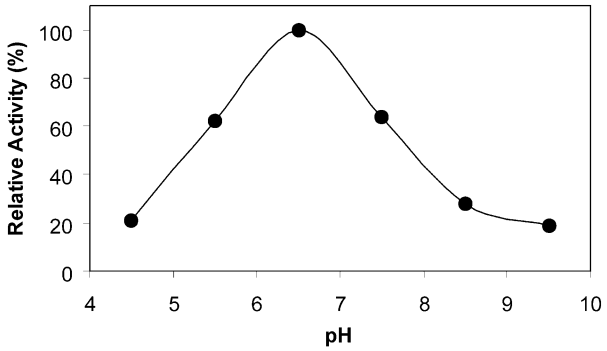

Fig. 2. pH-activity profile for PPO in in 50 mM acetate buffer (pH 4.5–5.5), in 50 mM phosphate buffer (pH 6.5) and in 50 mM Tris–HCl buffer (pH 7.5–9.5).

2.5.5. Native polyacrylamide gel electrophoresis (PAGE)

Electrophoresis was performed on an SE 600 Series Electrophoresis dual slab cell unit (Hoeffer). Polyacrylamide gels (8%) were prepared according to Laemmli (Laemmli, 1970) under native conditions (i.e. without sodium dodecyl sulfate). After running, gels were incubated in 15 mM *L*-DOPA in 0.1 M phosphate buffer (pH 7.0) at 37 °C for 1 h and then in 1 mM ascorbic acid solution until appearance of isoenzyme bands.

3. Results and discussion

Crude enzyme preparations extracted from medlar fruits have been used to characterize the properties of the polyphenol oxidase activity originating from a combination of isoenzymes. PPO isoforms were located by native electrophoresis, using *L*-DOPA as substrate (Fig. 1). Visualization of at least four bands on the activity-stained gels indicated the presence of different isoforms of PPO in the medlar fruits. Generally, two or three PPO isoenzymes have been detected in banana (Palmer, 1963), apple (Constantinides & Bedford, 1967), pear (Rivas & Whitaker, 1973), cherry (Pifferi & Cultrera, 1974), papaya (Cano, Lobo, de Ancos, & Galeazzi, 1996) and dog-rose fruit (Sakiroglu, Küfrevioglu, Kocacaliskan, Oktay, & Onganer, 1996).

3.1. pH optimum and stability

The pH activity profile for crude enzyme extract is shown in Fig. 2. The profile had a typical bell-shaped

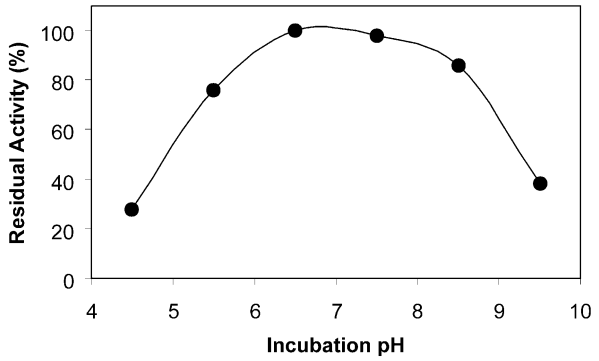

Fig. 3. pH stability of PPO from medlar fruit.

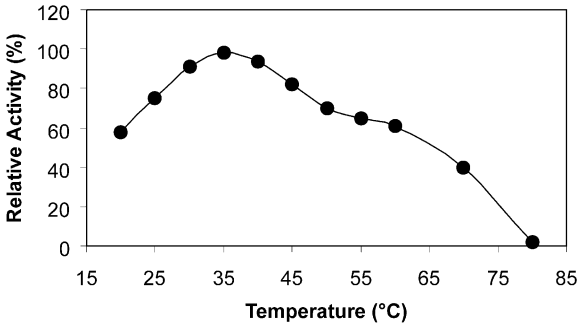

Fig. 4. Optimum temperature of PPO from medlar fruit in 50 mM phosphate buffer (pH 6.5).

Table 2
Thermodynamic parameters for thermal inactivation of the medlar PPO[a]

Temperature (°C)	$k \times 10^5$ (s^{-1})	ΔG (J mol^{-1})	ΔH (J mol^{-1})	ΔS (J $mol^{-1}K^{-1}$)
35	6.8	320,538	63,327	−257,212
40	25.1	329,186	63,286	−265,900
45	35.0	335,362	63,244	−272,118
50	45.7	341,395	63,202	−278,193
60	79.7	353,586	63,119	−290,467
80	277	378,648	62,953	−315,695

[a] E_a was calculated to be 65,890 J mol^{-1} from the plot of 1/T vs ln k and used for the calculation of ΔH.

curve with optimum activity at pH 6.5 for 4-methyl catechol. Medlar PPO was almost inactive below pH 4.5 and above 8.5 in the presence of this substrate. The optimum pH, for PPO activity in fruits, differs among plant sources. Differences in pH optima have been reported for partially purified PPOs from tea-leaf (Gregory & Bendall, 1966), strawberry (Wesche-Ebeling & Montgomery, 1990) and field bean (Paul & Gowda, 2000). It is around pH 7–8.5 for Malatya apricot (Arslan, Temur, & Tozlu, 1998) and dog-rose fruit PPO (Sakiroglu et al., 1996), pH 6–7 for PPO from kiwifruit (Park & Luh, 1985), apple (Murata, Kurokami, & Homma, 1992), pineapple (Das, Santhoor, & Gowda, 1997), and longan fruit (Jiang, 1999) and pH 4–5 for eggplant (Fujita & Tono, 1988) and cherry (Fraignier, Marques, Fleuriert, & Macheix, 1995).

The residual percentage activity of the enzyme from medlar fruits, for various pH values between 4.5 and 9.5, is shown in Fig. 3. The pH-stability profile for the enzyme shows that 80% of the PPO activity was retained at physiological pH values. The enzyme is not stable at pH below 4 and above 9.5.

3.2. Thermal activity and stability

Thermal activity of crude PPO from medlar fruit is presented in Fig. 4. The optimum temperature for activity of medlar PPO was at 35 °C. It has been reported that the optimum temperatures for PPO of peach (Jen & Kahler, 1974), grape (Cash, Sistrunk, & Stutte, 1976) and plum (Siddiq, Sinha, & Cash, 1992) were 20, 25 and 37 °C, respectively. At 65 °C, approximately 60% of PPO activity was lost. It appears that crude PPO is sensitive to the increase in assay temperature. Above 50 °C, the medlar PPO activity declined as the temperature increased and the enzyme was completely inactivated at 80 °C.

The thermal stability profile for crude PPO, presented in the form of the residual percentage activity, is shown in Fig. 5. The enzyme was stable for 30 min at its optimum temperature and moderately stable for 30 min at 60 °C. At higher temperatures, heat-denaturation of the enzyme occurred after 10 min of incubation. The drop in percentage residual activity at high temperatures is actually due to the unfolding of the tertiary structure of the enzyme to form the secondary structure. Although heating at 60 °C for 20 min resulted in partial (50–60%) inactivation, heating at 80 °C for the same period completely inactivated the enzyme (Duangmal & Owusu Apenten, 1999; Vamos-Vigyazo, 1981). Thermodynamic parameters (Table 2) related to medlar PPO have also shown that the enzyme behaves very similarly upon heat-inactivation, as observed for PPOs from other plants (Buchelli & Robinson, 1994; Mazzafera & Robinson, 2000; Murata et al., 1992; Robinson, Loveys, & Chacko, 1993), but PPO from medlar has greater heat-sensitivity, with a larger free energy change (ΔG) than those from potato or taro (Duangmal & Owusu Apenten, 1999).

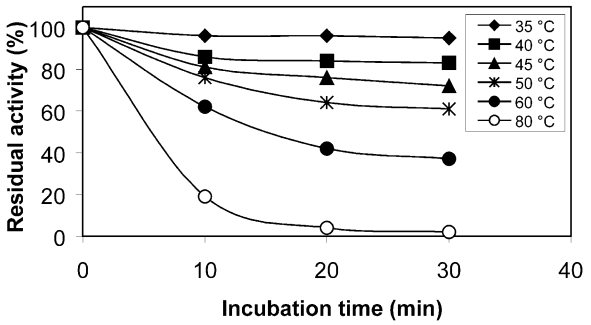

Fig. 5. Thermal stability of PPO from medlar fruit.

Table 3
Kinetic parameters for the oxidation of diphenolic substrates by the medlar PPO

Substrate	V_{max} (μM/min)	K_m (mM)	V_{max}/K_m (min^{-1})
Catechol	88.0	5.7	0.0154
4-methylcatechol	130	7.5	0.0173
DHPPA	7.2	1.9	0.0038
L-DOPA	76.2	4.7	0.0162
Epicatechin	60.6	4.0	0.0152

3.3. Substrate specificity

The relative activity of PPO, based on absorption at the wavelength maximum of the product, was compared with the activity in the presence of 4-methylcatechol as 100% (Table 1). All the substrates, i.e. catechol, 4-methylcatechol, *L*-DOPA and epicatechin, were oxidized significantly by the medlar enzyme, displaying simple Michaelis-Menten kinetics. Linear regression analysis of v versus [S] determined V_{max} and K_m values for each substrate (Table 3). Catalytic efficiency was the lowest but substrate binding was the highest with DHPPA. On the other hand, 4-methylcatechol is oxidized by medlar PPO at a much higher rate with the highest K_m value. In order to evaluate the substrate specificity, the V_{max}/K_m ratio was taken as the criterion (Palmer, 1995). Although the medlar enzyme seemed to have the highest affinity for DHPPA, 4-methylcatechol was found to be the most efficient phenolic substrate for medlar fruits when considering the ratio V_{max}/K_m. This result is consistent with a previous report indicating that 4-methylcatechol is usually the best substrate for plant PPOs (Walker, 1995). It appears that the medlar PPO has a substrate-binding site with a high affinity for small *o*-diphenols such as catechol, 4-methylcatechol or *L*-DOPA and less affinity for bulky *o*-diphenols such as epicatechin or DHPPA. Monohydroxyphenols are not oxidized by the medlar extracts, indicating that the enzyme utilizes only dihydroxyphenols as reported for PPOs from other plant sources (Cash et al., 1976; Ding, Chachin, Ueda, & Imahori, 1998; Perez-Gilabert & Carmona, 2000; Siddiq et al., 1992).

Table 4
Sensitivity of medlar PPO-catalyzed oxidation of 4-methylcatechol to some common PPO inhibitors

Inhibitor	I_{50} (mM)	K_i (mM)
Sodium azide	2.8	2.3
Benzoic acid	1.9	1.7
Cysteine	0.0125	0.0036
Sodium metabisulfite	0.095	0.085

3.4. Effect of inhibitors

The effects of four inhibitors, namely *L*-cysteine (20–30 μM), sodium azide (10–50 mM), benzoic acid (3–5 mM) and sodium metabisulfite (0.01–0.1 mM), were examined, to determine their potential for inhibition of 4-methylcatechol oxidation by the medlar PPO (Table 4). The percentage inhibition was compared with that of the control (100% activity). Among these inhibitors, cysteine and metabisulfite exhibited nearly complete inhibition of medlar PPO at a range of 0.1–1.0 mM. Cysteine was the most effective inhibitor, followed by metabisulfite.

All of the inhibitors used in this study exhibited complete inhibition on the medlar PPO. The inhibition constants for the reducing agents, cysteine and metabisulfite, are several-fold lower than those for benzoic acid and azide. Inhibition assays indicate that thiol compounds, such as cysteine and metabisulfite with very low K_i values, are potent inhibitors of the medlar enzyme, consistent with earlier reports (Ding et al., 1998; Duangmal & Owusu Apenten, 1999; Friedman & Bautista, 1995; Richard, Goupy, Nicolas, Lacombe, & Pavia, 1991; Yang, Fujita, Ashrafuzzaman, Nakamura, & Hayashi, 2000). The mechanism of inhibition differs, depending on the compound used. The general mechanisms of PPO inhibition have been reviewed previously (Duangmal & Owusu Apenten, 1999; Iyengar & McEvily, 1992; Martinez & Whitaker, 1995; Sapers, 1993; Valero, Varon, & Garcia-Carmona, 1991).

It can be concluded that crude extracts prepared from medlar fruits have a PPO activity very similar to that of other plants. The enzyme is a catecholase, active toward diphenols, and has the greatest substrate specificity towards 4-methyl catechol among the substrates tested. Consistent with previous results, obtained from other plant PPOs, pH and temperature optima for the enzyme, in the presence of this substrate, were 6.5 and 35 °C, respectively. Moreover, the medlar PPO activity was very sensitive to some of the general PPO inhibitors, especially to cysteine and metabisulfite.

Acknowledgements

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