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Characterization of polyphenoloxidase (PPO) and total phenolic contents in medlar (*Mespilus germanica* L.) fruit during ripening and over ripening

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Abstract

Characterization of polyphenoloxidase (PPO) enzyme and determination of total phenolic concentrations during fruit ripening and over ripening in medlar (*Mespilus germanica* L.) were determined. During ripening, PPO substrate specificity, optimum pH and temperature, optimum enzyme and substrate concentrations were determined. Among the five mono- and di-phenolic substrates examined ((*p*-hydroxyphenyl) propionic acid, L-3,4-dihydroxyphenylalanine, catechol, 4-methylcatechol and tyrosine), 4-methylcatechol was selected as the best substrate for all ripening stages. A range of pH 3.0–9.0 was also tested and the highest enzyme activity was at pH 7.0 throughout ripening. The optimum temperature for each ripening stage was determined by measuring the enzyme activity at various temperatures over the range of 10–70 °C with 10 °C increments. The optimum temperatures were found to be 30, 20 and 30 °C, respectively, for each ripening stage. Optimum enzyme and substrate concentrations were found to be 0.1 mg/ml and 40 mM, respectively. The V_{max} and K_m value of the reaction were determined during ripening and found to be 476 U/mg protein and 26 mM at 193 DAFB (days after full bloom) – stage 1, 256 U/mg protein and 12 mM at 207 DAFB – stage 2, 222 U/mg protein and 8 mM at 214 DAFB – stage 3. For all ripening stages sodium metabisulfite markedly inhibited PPO activity. For stage 1 of ripening, Cu^{2+}, Hg^{2+} and Al^{3+}, for stage 2, Cu^{2+} and Hg^{2+}, and for stage 3, Cu^{2+}, Hg^{2+}, Al^{3+} and Ca^{2+} strongly inhibited diphenolase activity. Accordingly, it can be concluded that as medlar fruit ripen there is no significant changes in the optimum values of polyphenoloxidases, although their kinetic parametres change. As the fruit ripening progressed through ripe to over-ripe, in contrary to polyphenoloxidase activity, there was an apparent gradual decrease in total fruit phenolic concentrations, as determined by using the aqueous solvents and water extractions.

Keywords: Polyphenoloxidase; *Mespilus germanica*; Phenolics; Medlar; Enzyme; Ripening

1. Introduction

Polyphenoloxidases (PPO, EC 1.14.18.1) are a group of copper-containing enzymes (Robb, 1984) catalyzing oxidation of polyphenolic compounds in the presence of molecular oxygen which are responsible for enzymatic browning reactions occuring during harvesting, handling, processing and storage of many plant materials. They possess three different activities connected with each other as (i) catechol oxidase or *o*-diphenol:oxygen oxidoreductase (EC 1.10.3.1); (ii) laccase or *p*-diphenol:oxygen oxidoreductase (EC 1.10.3.2) and (iii) cresolase or monophenol monooxygenase (EC 1.18.14.1) (Sheptovitsky & Brudwig, 1996).

PPO and phenolics are directly responsible for some of the enzymatic browning in fruits and vegetables

Abbreviations: PPO, polyphenoloxidase; PHPPA, (*p*-hydroxyphenyl) propionic acid; 4-MC, 4-methylcatechol; DAFB, day after full bloom; PMSF, phenylmethylsulfonyl fluoride; L-DOPA, L-3,4-dihydroxyphenylalanine; MBTH, 3-methyl-2-benzothiazolinone hydrazone; DMF, dimethylformamide; PVPP, polyvinylpolypyrolidone; DIW, deionized water; TPC, total phenolic content.

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(Mayer & Harel, 1979). It has been reported that levels of PPO and phenolics may change during fruit development and ripening which may influence the potential damage in loquat fruit (Sánchez-Ferrer, Bru, & Garcia-Carmona, 1989; Vamos-Vigyazo & Nadudvari-Markus, 1982).

The medlar (*Mespilus germanica* L., Fam: Rosaceae) fruit is brown, sometimes reddish tinged, pear and apple-shaped fruits ranging from 1.5 to 3 cm in diameter and weighing from very small (about 10 g) to large (more than 80 g) (Bignami, 2000; Browicz, 1972).

The medlar is a typical climacteric fruit which has gained a value in human consumption and commercial importance in recent years, attracting researches to study its chemical or nutrient compositions. Contents of fructose and glucose (Glew et al., 2003a, 2003b; Romero-Rodriguez, Simal-Lozano, Vazguez-Oderiz, Lopez-Hernandez, & Gonzalez-Castro, 2000), linoleic acid and palmitic acid (Ayaz et al., 2002), malic acid and citric acid (Glew et al., 2003a, 2003b), aspartate and glutamate (Glew et al., 2003b) and potassium (Glew et al., 2003c) were determined in high levels in the mature medlar fruits.

Medlar fruit is widely consumed in Turkey and especially in northeast Anatolia (Turkey) where it is one of the unique places where the people grow the wild and alternative cultivars to consume their fruits in different ways. A long list of recipes utilizing medlar fruits such as in jams and jellies are well known (Baytop, 1999; Bignami, 2000). The astringency of the fruit is well known and it has been reported that bletted pulp (bletting or blet is a process certain fleshy fruits undergo when, beyond ripening, they have started to decay and ferment) or syrup of the fruit was a popular remedy against enteritis and has many human healing properties (Baytop, 1999; Bignami, 2000). Medlar fruits are harvested through October and November, however, the fruits are not appropriate for market sale and home uses.

PPO characterization and total phenolics content of medlar fruit used in the present study were conducted at three ripening stages. These times were selected to coincide with the season to consume the fruit by the local people and which are available in the local markets or for export.

Aydin and Kadioglu (2001) reported PPO activity, total soluble sugar and ascorbic acid contents, and Dincer, Colak, Aydın, Kadioglu, and Güner (2002) have also performed PPO characterization in medlar fruits prior to ripening and over-ripening. However, no information regarding the changes of PPO characterization have been reported during ripening and over ripening parallel with total phenolics content in medlar. The aims of this study were to (1) characterize PPO activity during ripening of medlar fruit and (2) find a relationship in PPO activity and total phenolic concentrations during the rapid ripening leading to over-ripening parallel with enzymatic browning.

2. Materials and methods

2.1. Plant material

Medlar (*Mespilus germanica* L., wild type) fruit was randomly harvested from fourteen 20-year-old trees in the early morning from various single genotypes of bulk populations in the native habitat of the species located in the grounds of hillsides of several towns in Trabzon (Turkey) about 500–600 m above sea level, northeast Anatolia (Turkey). A half kg fruit (40 ± 1) was collected from each single genotype in triplicate, and the harvests were brought together and harmonized. Triplicate samples were then separately prepared for analysis. Full bloom of the medlar source orchard occurred on 10 May 2003 and the three ripening stages were sampled at 193, 207 and 214 DAFBs (days after full bloom). Within the DAFBs, the fruits were divided into three distinct specific maturities, mature (193 DAFB, in the middle of November), ripe (207 DAFB, at the beginning of December) and over ripe (214 DAFB, in the middle of December), respectively. Samples were made at 2-week and 1-week intervals through the middle of November and December (Table 1). One kg of medlar fruit was gathered in triplicate at each sampling time. The harvested fruit was stored at −80 °C and freeze-dried. After lyophilization, the hard, dried fruits were crushed with a steel hammer and then ground to a fine powder using a stainless steel mill.

2.2. Reagents and chemicals

All chemicals and reagents used were analytical grade. Sodium acetate, sodium and potassium phosphate, polyvinylpolypyrolidone (PVPP), 3-methyl-2-benzothiazolinone hydrazone (MBTH), Tris–HCl, EDTA, dimethylformamide (DMF) and $MgCl_2$ were purchased from Merck A.G. (Darmstadt, Germany). (*p*-hydroxyphenyl) Propionic acid (PHPPA), L-3,4-dihydroxyphenylalanine (L-DOPA), Triton X-114, tyrosine, 4-methylcatechol (4-MC), catechol and phenylmethylsulfonyl fluoride (PMSF) were purchased

Table 1
Variation in the color of the skin and pulp of medlar (*M. germanica* L.) fruit during ripening and over ripening

Harvest date	Days after full bloom (DAFB)	State of ripeness, fruit skin and pulp color
18 November 2003	193	Ripe, skin brownish, pulp white, fruit hard
2 December 2003	207	Ripe, skin completely brown, pulp white (60%) and partly brownish (40%) around core, fruit half soft
9 December 2003	214	Over ripe, skin completely dark brown, pulp fully dark brown and fully soft

from Sigma Chem. Co. (St. Louis, USA). Deionized water (DIW) was used during preparation of solutions and buffers as well reaction mixture during spectrophotometric measurements.

2.3. Crude enzyme preparation

Crude enzyme fractions were prepared as reported previously (Colak, Özen, Dincer, Güner, & Ayaz, 2005; Dincer et al., 2002). Medlar fruits (10 g), in triplicate, were placed in a dewar flask under liquid nitrogen for 10 min in order to disrupt the cell membranes. The cold medlar fruits were homogenized by using blender in cold 10 ml of 50 mM acetate buffer (pH 4.0 and pH 5.0), phosphate buffer (pH 6.0 and pH 7.0), Tris–HCl buffer (pH 8.0 and pH 9.0) separately, containing 2 mM EDTA, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% (w/v) PVPP and 6% (w/v) Triton X-114, for 2 min at 4 °C. The homogenate was filtered using three layers of cheese cloth before centrifugation in a Sigma 2–16 K centrifuge at 15,000 rpm for 20 min at 4 °C. The supernatant was used as the crude enzyme extract and stored at −20 °C.

2.4. Protein determination

Soluble protein content in crude enzyme of triplicate extractions was determined by Lowry, Rosebrough, Farr, and Randall (1951), with bovine serum albumin as standard. The absorbances obtained were evaluated by graphic interpolation on a calibration curve at 650 nm.

2.5. Enzyme assay

PPO activity was assayed by spectrophotometric procedure (Colak et al., 2005; Dincer et al., 2002). The activity was determined by using different substrates from the triplicate extracts, measuring the increase in absorbance at 494 nm for 4-methylcatechol (4-MC) and 500 nm for all other subsrates (Espin, Trujano, Tudela, & Garcia-Canovas, 1997) at pH 7.0 and room temperature. Tyrosine and (*p*-hydroxyphenyl) propionic acid (PHPPA) were assayed as monophenolic substrates and 4-methylcatecol, catechol and L-3,4-dihydroxyphenylalanine (L-DOPA) as diphenolic substrates with 3-methyl-2-benzothiazolinone hydrazone (MBTH) in 50 mM potassium phosphate buffer at pH 7.0. Enzyme activity was assayed using a 1 ml sample cuvette containing 100 μl substrates (stock 100 mM), an equal volume of MBTH (stock 10 mM), and 20 μl of dimethylformamide (DMF). The solution was diluted to 950 μl with buffer and 50 μl enzyme extract were added to start the reaction. One unit of PPO activity was defined as 1 μM of product formed per minute in 1 ml of reaction mixture. Specific activity was defined as units of enzyme activity per mg of protein (Kolcuoglu, Colak, Sesli, Yildirim, & Saglam, 2007).

2.6. Evaluation of enzyme properties

2.6.1. Substrate specificity

Catechol, 4-MC, L-DOPA, PHPPA and tyrosine, at the stock concentration of 100 mM, were used to monitor PPO activity. The rate of the reaction was measured in terms of the increase in absorbance at the absorption maxima of the corresponding quinone products for each substrate (Espin et al., 1997).

2.6.2. Effect of pH on PPO activity

PPO activity, as a function of pH, was determined using 100 mM of 4-MC as the substrate. The buffers were used to obtain the required pH ranges were: acetate (pH 4.0 and 5.0), phosphate (pH 6.0 and 7.0) and Tris–HCl (pH 8.0 and 9.0). For each ripening stage, all assays were performed in triplicate.

2.6.3. Effect of temperature on PPO activity

The optimum temperature for PPO activity was determined by measuring the enzyme activity at various temperatures over range 10–70 °C with 10 °C increments, using a circulation water bath. The substrate and buffer were incubated for 5 min at various temperatures as indicated above, prior to the addition of the enzyme solution. For each stage, all assays were performed in triplicate using the separate three extractions.

2.6.4. Effect of substrate concentration on PPO activity and enzyme kinetics

PPO activity was evaluated at 494 nm by mixing the total enzymatic crude extracts with 4-MC at different final concentrations, 0.5, 1, 5, 10, 20, 30, 40 and 50 mM, respectively. Determinations were carried out using the triplicate extractions for each ripening stages.

The kinetic data were plotted as 1/specific activity ($1/V$) versus 1/substrate concentration (1/[S]). The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) parameters were obtained 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 (Lineweaver & Burk, 1934).

2.6.5. Effect of inhibitors on PPO activity

Inhibition of PPO by sodium azide (1–60 mM), sodium metabisulfite (0.01–5 mM), ascorbic acid (0.01–5 mM) and thiourea (0.01–5 mM) were determined at 494 nm for 4-MC. Percent activity graphs were drawn from these triplicate results to find IC_{50} values at constant inhibitor concentrations, which shows 50% inhibition of PPO.

2.6.6. Effect of metal ions on PPO activity

K^+, Ni^{2+}, Zn^{2+}, Hg^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+},Ca^{2+}, Al^{3+} and Fe^{3+} were used as metal ions to measure PPO activity. Final concentration of each metal ion in the PPO assay was 1 mM. The percentage remaining activities were expressed

by comparison with standard assay mixture with no metal ion added.

2.6.7. Electrophoresis

Prior to electrophoresis, an aliquot of crude enzyme from the triplicate extracts was lyophilized. The lyophilizate was dissolved in sample buffer for native-PAGE which was carried out as described by Laemmli (1970). Polyacrylamide gel electrophoresis was run on 8% gels. After electrophoresis, the gel was stained in 24 mM L-DOPA for PPO activity.

2.6.8. Extraction of total phenolics (TPC)

A 10 g fresh mesocarp sample was collected from 10 separate fruits with six replications for each ripening stages. The fresh samples were crushed in small pieces and refluxed in *n*-hexane (3 × 30 ml) for 30 min. The phenolic compounds from the de-fatted fruit sample were extracted by homogenizing the sample in 20 ml (3 × 20 ml) acetone (80%), methanol (80%), ethanol (80%) and water, respectively, using a pestle and mortar. Fruit samples were further homogenized using the solvents with an Ultra-Turrax T25 homogenizer (Janke & Kunkel, IKA-Labortechnik, Germany) for 3 min at 10,000 rpm and subsequently centrifuged (5000*g*) and supernatants collected. The supernatants were combined and concentrated to 10 ml using rotary evaporator at 40 °C.

2.6.9. Determination of total phenolic compounds (TPC)

TPCs were determined according to the method of Moyer, Hummer, Finn, Frei, and Wrolstad (2002) with slight modifications. One hundred times diluted fruit extracts were mixed with the reaction mixture, incubated at 40 °C, and the absorbance of the mixture at 755 nm was measured on a UV–Vis spectrophotometer (Techcomp 8500 II, South Korea). TPCs was expressed as mg of catechin equivalents per 100 g of fresh weight (mg of CE/100 g fw).

3. Results and discussion

During medlar fruit ripening, rapid enzymatic browning has been observed as the fruit soften. Throughout the ripening and including over-ripening, considerable changes were observed in the color of the mesocarp, state of maturity and the skin color, throughout 193, 207 and 214 DAFBs (Table 1). Crude enzyme preparations extracted from medlar fruits were used to characterize the properties of the PPO activity originating from a combination of isoenzymes. PPO isoforms were located by the native polyacrylamide gel electrophoresis (8%), 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. In general, variations in PPO isoenzymes ranging from two to four bands have been detected in different fruits such as papaya (Cano, Lobo, De Ancos, & Galeazzi, 1996), medlar (Dincer et al., 2002), cherry laurel (Colak et al., 2005). In the present study, using L-DOPA as a substrate, two PPO isoforms in the first (193 DAFB) and third (214 DAFB) stages, and five PPO isoforms in the second stage were determined (Fig. 1). In some cases, cultivar variations affecting the distribution of PPO isoforms has been reported. For example in four raspberry cultivars, an isoenzyme band was

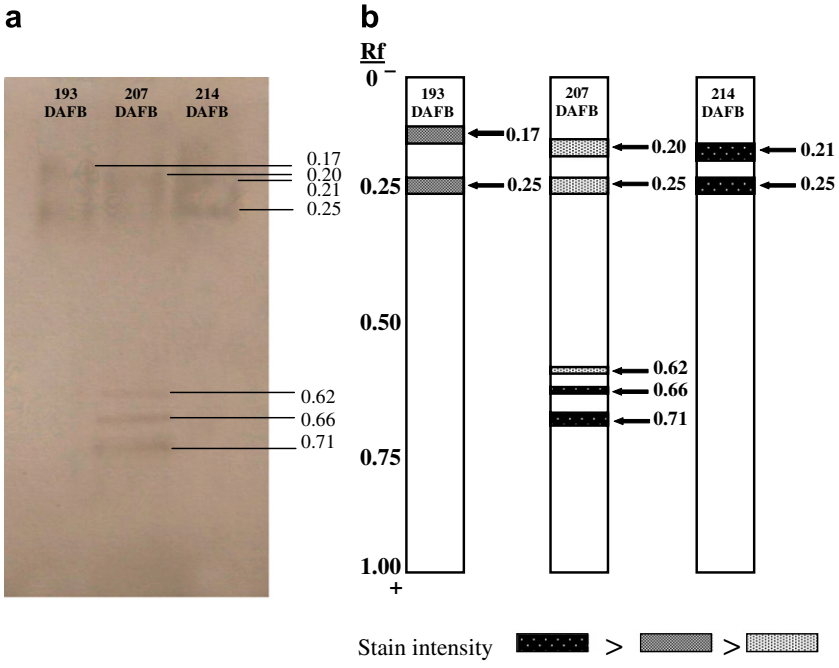

Fig. 1. L-DOPA staining for PPO from medlar (*M. germanica* L.) fruit at three stages of ripening. (a) Native polyacrylamide gel electrophoretic pattern of soluble PPO activity, (b) Diagramatic representation of PPO isoenzymes.

Table 2
Substrate specificity of crude PPO from medlar (*M. germanica* L.) fruit during ripening and over ripening[a]

Substrates (100 mM)	Wavelength (nm)	Relative activity (%)		
		Days after full bloom (DAFB)		
		193	207	214
Monophenols				
L-Tyrosine	500	10 ± 0.1	4 ± 0.3	10 ± 0.1
PHPPA	500	51 ± 0.2	13 ± 0.1	6 ± 0.1
Diphenols				
4-Methylcatechol	494	100 ± 0.0	100 ± 0.0	69 ± 0.2
Catechol	500	52 ± 0.1	72 ± 0.1	100 ± 0.0
L-DOPA	500	12 ± 0.1	6 ± 0.1	3 ± 0.1

[a] Values are means ± standard deviation of triplicate extractions and determinations ($n = 3$).

detected with a mobility $R_f = 0.25$ (Gonzalez, De Ancos, & Cano, 1999). Previously, Aydin and Kadioglu (2001) had reported that PPO activity, L-DOPA used as substrate, in medlar gradually decreased during fruit development beginning from 15 July to 15 October, and then a slight increase in the middle of October and an abrupt increase in the middle of November were recorded. Later Dincer et al. (2002) detected four PPO isoforms in medlar fruits which were harvested earlier (in the first week of November) than in this present study. However, both studies did not follow the PPO activity or characterization after this period which leads to the development of the over-ripening stage, where the main enzymatic browning and fruit texture changes occur.

The absence or presence of different medlar PPO isoforms may be explained by the period of fruit maturities during ripening which were held in different harvest periods. There was 18 days difference in harvesting between medlar fruits in this study and that of Dincer et al. (2002). They harvested the fruit in the first week of November in which the medlars, are not generally appropriate for market sale and home use. The three harvest stages 193, 207 and 214 DAFBs (18 November, 2 and 9 December) used in the present study are considered by the local people as favorable times or seasons to consume the fruit and to sell by the local markets or for export. Differentiation in genotype of medlar fruits as well as other fruits can also affect distribution of PPO isoforms. The genotype of medlar fruits previously used in PPO characterization (Dincer et al., 2002) and in the present study is not from the same bulk populations. It can be conluded that harvest time or season or even state of maturity in ripening may have a considerable effect in the distribution of PPO isoforms in medlar during the fruit's rapid ripening and enzymatic browning stages, depending on different genotypes.

3.1. Substrate specificity

Diphenolic substrates 4-MC, catechol and L-DOPA and monophenolic substrates tyrosine and PHPPA were used to test for substrate specificity. In this study, 4-MC was used as the substrate because of its greatest activity at all stages (Table 2). There was very little PPO activity with monophenolic compounds and indicates that the PPO enzyme from medlar fruits contain a diphenolase which is responsible for the oxidation. This result is consistent with previous reports from other fruit such as loquat (Ding, Chachin, Ueda, & Imahori, 1998), eggplant (Perez-Gilabert & Garcia-Carmona, 2000), medlar (Dincer et al., 2002), 'Jonagored' apple (Rocha & Morais, 2001), cherry laurel (Colak et al., 2005).

3.2. Kinetic parameters

For medlar fruit PPO activity, the maximum reaction velocity (V_{max}), Michaelis–Menten constant (K_m) and V_{max}/K_m values were calculated using Lineweaver and Burk (1934) graph (Table 3). Using 4-MC as a diphenolic substrate V_{max} and K_m were determined for each stages and shows that the K_m values were 26 mM for stage 1, 12 mM for stage 2 and 8 mM for stage 3. In addition, V_{max} values were 476, 256 and 222 μM/dak mg protein, respectively (Table 3). It can be extracted from the substrate saturation curves for each maturity stages, the optimum activity values were obtained at 40 mM 4-MC concentrations for each stages. All characterization studies were carried out using these substrate concentrations.

3.3. Effect of pH

The activity of medlar PPO was measured at different pH, ranging from 3.0 to 9.0, using 4-MC as a substrate

Table 3
Biochemical characteristics of PPO from medlar (*M. germanica* L.) fruit during ripening and over ripening

	DAFB (days after full bloom)		
	193	207	214
V_{max} (U/mg)	476	256	222
K_m (mM)	26	12	8
V_{max}/K_m	0.018	0.021	0.028
Optimum pH	7.0	4.0	5.0
Optimum temperature (°C)	30	20	30

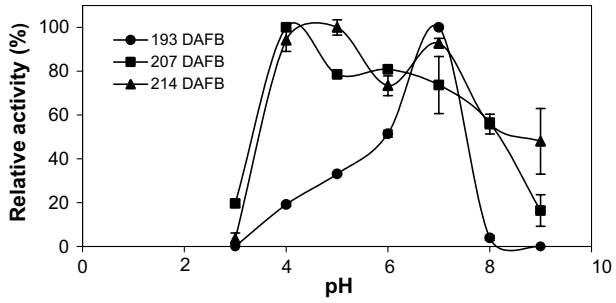

Fig. 2. pH activity profiles for medlar (*M. germanica* L.) fruits at three stages of ripening maturities. The buffers were used acetate (pH 4.0 and 5.0), phosphate (pH 6.0 and 7.0) and Tris-HCl (pH 8.0 and 9.0). The reaction was carried out at room temperature. Reaction mixture contained 4-MC (stock 100 mM), an equal volume of MBTH (stock 10 mM), 20 DMF, and the solution was diluted to 950 μl with buffer and 50 μl enzyme extract was added. Data are means of three replicates ± standard deviation.

(Fig. 2). The results show that for stage 1, there was one peak at pH 7.0, for stage 2, there were two peaks at pH 5.0 and 7.0 and for stage 3, and there were two peaks at pH 4.0 and 7.0 (Fig. 2). These results show that the PPO from medlar fruits is almost inactivating below pH 4.0 and above 8.0 in all stages. From these results, pH 7.0 was chosen to continue other works to analyze alteration of PPO enzyme activity for each stage of fruit. The optimum pH for PPO activity in fruits is highly dependent on the enzyme source and the nature of substrate used (Duangmal & Owusu Apenten, 1999). Generally the maximum PPO activity in most plants is at or near neutral pH values (Siddiq, Sinha, & Cash, 1992; Wong, Luh, & Whitaker, 1971). It has been shown that different fruits types exhibited different optimum pH values. This pH optimum was 7.0 for Amasya apple (Oktay, Küfrevioglu, & Sakiroglu, 1995) and cherry laurel (Colak et al., 2005), and 6.0 for DeChaunac grape (Lee, Smith, & Pennesi, 1983).

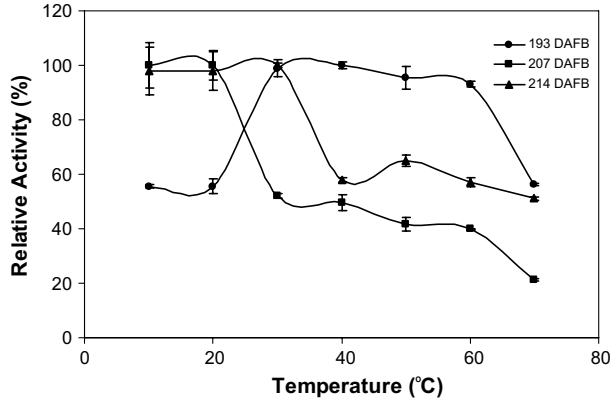

Fig. 3. Temperature-activity profiles for medlar (*M. germanica* L.) at three stages of ripening maturities. The reaction mixture all the reagents except crude enzyme was incubated for 5 min at indicated temperatures. After the enzyme was added, the activity was measured spectrophotometrically at 494 nm as rapidly as possible. Data are means of three replicates ± standard deviation.

3.4. Effect of temperature

The effects of assay temperature between 10 and 70 °C were measured using 4-MC as a substrate (Fig. 3). It is clear that the optimum temperature is 30 °C for stages 1 and 3, and 20 °C for stage 2 (Fig. 3). These values were considered as 100% of the relative activity. Enzymatic activity began to decrease more strongly below 60, 20 and 30 °C for each stages, respectively. Variations in optimal temperature for fruit PPO activity ranging between 18 and 37 °C have also reported by some other authors (Ding et al., 1998; Duangmal & Owusu Apenten, 1999; Oktay et al., 1995; Sakiroglu, Küfrevioglu, Kocacaliskan, Oktay, & Onganer, 1996; Siddiq et al., 1992).

3.5. Effect of various inhibitors

Enzymatic browning of plants may be reduced by using appropriate inhibitors. In this study, four inhibitors (sodium metabisulfite, sodium azide, ascorbic acid and thiourea) were used to monitor prevention of enzymatic browning. Their potential for the inhibition of 4-MC oxidation by the medlar PPO are presented as IC_{50} values (Table 4). Sodium metabisulfite was the most effective inhibitor at all stages and followed by ascorbic acid and sodium metabisulfite exhibited nearly complete inhibition on the medlar PPO. As seen in Table 3, sodium metabisulfite has very low IC_{50} value for each stage. These results were consistent with the earlier reports such as De Chaunac grapes (Lee et al., 1983), dog rose (Sakiroglu et al., 1996), loquat (Ding et al., 1998), cherry laurel (Colak et al., 2005).

In general the mechanism of inhibition differs depending on the compound use. Inhibition assays indicate that thiol compounds such as sodium metabisulfite are one of the potent inhibitors of PPO enzyme (Ding et al., 1998; Duangmal & Owusu Apenten, 1999).

3.6. Effect of various metal ions

The effect of metal ions on medlar PPO is shown in Table 5. For ripening stage 1, Cu^{2+}, Hg^{2+} and Al^{3+}, for stage 2, Cu^{2+} and Hg^{2+}, and for stage 3, Cu^{2+}, Hg^{2+}, Al^{3+} and Ca^{2+} inhibited diphenolase activity very strongly. Although, stimulation of the diphenolase activity was measured using Mn^{2+} and Zn^{2+} for stage 1, and Al^{3+}, Ni^{2+} and

Table 4
Inhibition of medlar (*M. germanica* L.) fruits' diphenolases by some general PPO inhibitors during ripening and over ripening

Substrate	IC_{50} (mM)		
	193 DAFB	207 DAFB	214 DAFB
Sodium azide	23.8	41	9
Sodium metabisulfite	0.06	0.06	0.05
Ascorbic acid	0.09	0.1	0.08
Thiourea	1.5	1.73	1.49

Table 5
Effects of various metal ions on medlar (*M. germanica* L.) fruit's PPOs during ripening and over ripening[a]

Metal ions	Relative activity (%)		
	193 DAFB	207 DAFB	214 DAFB
None	100.0 ± 1.0	100.0 ± 1.0	100.0 ± 1.0
K^{+}	77.7 ± 1.0	88.6 ± 1.0	85.8 ± 1.0
Cd^{2+}	73.2 ± 2.1	91.3 ± 2.3	87.9 ± 1.5
Ca^{2+}	94.8 ± 1.3	88.3 ± 2.1	59.0 ± 1.3
Ni^{2+}	67.1 ± 1.6	122.2 ± 2.6	63.6 ± 2.0
Zn^{2+}	113.7 ± 4.0	92.4 ± 1.9	98.9 ± 1.6
Hg^{2+}	38.7 ± 1.1	3.5 ± 1.1	3.4 ± 1.0
Mn^{2+}	108.4 ± 3.2	91.7 ± 1.0	70.5 ± 2.4
Cu^{2+}	12.9 ± 1.1	6.7 ± 1.1	0.6 ± 0.5
Al^{3+}	13.4 ± 1.1	103.7 ± 1.4	56.4 ± 1.1
Fe^{3+}	74.5 ± 1.7	117.6 ± 2.9	4.2 ± 1.1

[a] Values are means ± standard deviation of triplicate extractions and determinations ($n = 3$).

Fe^{3+} for stage 2, there was no activation in stage 3. These differences among inhibitions are presented in Table 5.

3.7. Effect of fruit ripening on phenolic contents

The variation of total phenolics during ripening and over ripening extracted using acetone, methanol, ethanol (80%) and water are illustrated in Table 6. The results show that as the fruit ripens from ripe to over-ripe maturities (193–214 DAFB), there was an apparent gradual decrease in phenolics, as determined by using the aqueous solvents and water alone. The effectivenes of different solvents for the extraction of total phenolics from medlar fruit was determined in high levels by using acetone (80%), comprising 564.7, 436.5 and 251 mg CE/100 g fw through ripening. The aqueous actone served as an efficient system for recovery of maximum amount of total phenolics from the mesocarp of medlar fruit. The least levels of total phenolics was yielded using water alone (Table 6).

A correlation between phenolics and PPO activity in fruits during pre- and post-harvest handling, processing and among species/cultivar variations have been reported. For instance, a dramatic decrease in phenolics and PPO activity in a loquat cultivar (cv. Mogi) during fruit development ripening has been reported (Ding et al., 1998). An increase in PPO activity after fruit set, which reaches very high levels, and a steeply accumulation in phenolics have been observed with the development and ripening in Algerie loquat variety (Vela, Marchart, Lucas, & Martinez, 2002). These authors also found the highest levels of PPO and phenolics occurred together at harvest. This coincidence of high PPO and phenolics enabled the fruits to become more susceptible to enzymatic browning. In 15 peach cultivars, the concentration of individual phenolic compounds decreased steadily during maturation and remained low until harvest time (Lee, Kagan, Jaworski, & Brown, 1990). In the same fruits, PPO activity followed a pattern similar to that of the phenolics during ripening. Both phenolic contents and PPO activity in the cultivars, were closely found correlated with the degree of browning (Lee et al., 1990). The data obtained from medlar fruit in this study coincided with these observations. In the present study, an adverse relation in results between total phenolics content and PPO activity in medlar fruit was found. As the ripening progressed, total content of phenolics decreased, while PPO activity increased in the fruit. This reverse change between PPO activity and total phenolics in medlar fruit during ripening and over ripening can be attributed to an increase of the catalytic efficency of enzyme during ripening (Table 3).

Table 6
Total phenolic concentrations (mg catechin equivalent/100 g fresh weight) in medlar (*Mespilus germanica* L.) fruit during ripening and over ripening

Extraction solvents	Days after full bloom (DAFB)		
	193	207	214
Acetone (80%)	564.7 ± 3.1[a]	436.5 ± 14.6	351.7 ± 5.5
Methanol (80%)	239.6 ± 4.7	197.3 ± 1.7	48.7 ± 1.1
Ethanol (80%)	156.4 ± 2.7	126.7 ± 8.7	38.4 ± 4.1
Water	45.1 ± 5	32.4 ± 1.2	16.2 ± 2.7

[a] Values are means ± standard deviation of six separate extractions and determinations ($n = 6$).

Wide variations in seasonal and genotypic variations in phenolic concentrations across all fruits have been reported (Burda, Oleszek, & Lee, 1990; Lima et al., 2005; Macheix, Fleuriet, & Billot, 1990). In acerola fruit at three maturity stages, harvested in either dry or rainy seasons, showed a genotype selectivity among 12 cultivar genotypes (Lima et al., 2005). Four of these 12 genotypes, total phenolic contents decreased during progressing maturation (Lima et al., 2005). They concluded that genotype is a significant factor in determining PPO. Macheix et al. (1990) suggested there are generally fairly good correlations between the phenolic levels during ripening and the activity of the enzymes responsible for biosynthesis (phenylalanine ammonialyase and CoA ligase) and degradation of these constituents.

Fleshy fruits are frequently harvested prior to physiological ripening and following harvest can have a relatively short shelf life during which they undergo profound changes in texture, color and flavour (Seymour, Manning, Eriksson, Popovich, & King, 2002). In this study, medlar fruit exhibited, there was profound changes in the texture, color and flavour during its short life of ripening, leading to over-ripening (Table 1). There were also phenomenal variations in PPO activity and characterization which have also been found among cultivars and wild types in medlar fruit (Aydin & Kadioglu, 2001; Dincer et al., 2002). It can be concluded that harvest time, season, fruit maturity and genotype difreneces during ripening, result in considerable effects in the distribution of PPO isoforms, activity, characterization and phenolics in medlar fruit. This study quantifies medlar fruit PPO activity, characterization and total phenolic contents during ripening and over-ripening period.

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