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Diphenolases from two cultivars of cherry laurel (*Laurocerasus officinalis* Roem.) fruits at an early stage of maturation

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Abstract

Diphenolases from two cherry laurel cultivars (*Laurocerasus officinalis* Roem. 'Globigemmis' and 'Oxygemmis') were highly active against 3-(3,4-dihydroxyphenyl)propionic acid (DHPPA) at acidic pH values with temperature optima of 50 and 40 °C, respectively. Although the pH-stability profiles showed that both enzymes were fully stable at pH 7.0, their stabilities decreased significantly at alkaline pH values. Thermal-stabilities of the cherry laurel diphenolases indicated that enzymes from the two cultivars share similar thermodynamic properties and heat-sensitivities as a result of heat-inactivation. In addition, ascorbate and metabisulfite, at 1 mM final concentrations, almost completely inhibited the oxidation of DHPPA by the enzymes, indicating the sensitivities of the cherry laurel diphenolases from the two cultivars towards general Polyphenol oxidases inhibitors. It can be concluded that the crude enzymes prepared from the cherry laurel fruits of the two cultivars, at an early stage of development, possess diphenolase activities sharing similar behaviours.

Keywords: *Laurocerasus officinalis*; Diphenolase; Polyphenoloxidase; Cherry laurel; Maturation; Rosaceae

1. Introduction

Polyphenol oxidases (PPOs) are a group of copper-proteins distributed widely through all the phylogenetic scale, from bacteria to mammals (Robb, 1984) and, in plants, these enzymes are responsible for the enzymatic browning reaction occurring during handling, storage and processing of fruits and vegetables. PPOs, utilizing molecular oxygen, catalyze two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase, EC 1.14.18.1) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase, EC 1.10.3.1). In plant tissues, the quinones are then polymerized to brown, red or black pigments (Zawistowski, Biliaderis, & Eskin, 1991) which lead to organoleptic and nutritional modifications that depreciate the food product (Friedman, 1996).

Cherry laurel fruit (*Laurocerasus officinalis* Roem.) is a member of Rosaceae family, native to the west of Asia and cultivated throughout northern Turkey for its edible fruits (Browicz, 1972). The fruits of cultivated plants are very poisonous at early stages. However, various alcoholic drinks, jam and marmalade can be prepared when ripe. The fruits are also consumed in fresh and dried forms (Ayaz, Kadioğlu, Reunanen, & Var, 1997b; Flint, 1983; Milan, 1984). But, the fruits of cultivated plants are not sufficiently known as a potential food source in Turkey. In addition, the nutritional value of cherry laurel fruit (*L. officinalis* Roem.) arises from its phenolic acid, fatty acid and sugar contents. The very ripe fruit is

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characterized by high levels of fructose and glucose as sugars, especially vanillic acid, as a phenolic acid, and linoleic acid, as an unsaturated fatty acid (Ayaz, Kadioğlu, Reunanen, & Var, 1997a; Ayaz et al., 1997b). The reason that cherry laurel can be astringent is because of soluble tannins contained in the fruits. The fruit and seeds of cherry laurel and its cultivated forms are used in the treatment of stomach ulcer, digestive system illness, bronchitis (seeds), eczemas and haemorrhoids and as a diuretic (fruits) in folk medicine in Turkey (Baytop, 1999). Moreover, it is also used externally for its analgesic and antipuriginous effects (Alpinar & Yazicioglu, 1991; Cubukcu, 1989).

In this work, characterization of diphenolases from two cultivars of cherry laurel fruits (*L. officinalis* 'Globigemmis and Oxygemmis') at an early stage of maturation 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 enzymes.

2. Materials and methods

2.1. Plant materials and chemicals

Cherry laurel fruits from both cultivars (*L. officinalis* Roem. 'Globigemmis' and 'Oxygemmis') were harvested directly from local gardens during early morning at the same altitude (150 m above sea level) in Trabzon (Turkey) on June 21st, 2003 (182th day of the year). The fruits were carried into the laboratory in liquid nitrogen, with a nitrogen Dewar flask, and stored deep-frozen at −20 °C for 1–2 months until used; About 0.5 kg of unripe and fully green cherry laurel fruit (ca. 250 fruits) was randomly collected in triplicate from each cultivar.

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

2.2. Crude polyphenoloxidase preparation

Crude enzyme fractions were prepared as reported previously (Dincer, Colak, Aydin, Kadioglu, & Güner, 2002; Özen, Colak, Dincer, & Güner, 2004). Cherry laurel fruits (ca. 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 a blender in 50 ml of 50 mM cold sodium phosphate buffer (pH 7.5), containing 2 mM EDTA, 1mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (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 17,000 rpm for 30 min at 4 °C. The supernatant was used as crude enzyme which retained PPO activity for at least 15 days at 4 °C.

2.3. Protein determination

Protein content in the enzyme extracts was determined according to the Lowry method with bovine serum albumin as a standard (Lowry, Rosebrough, Farr, & Randall, 1951). The values were obtained by graphic interpolation on a calibration curve at 650 nm and it was found that the crude extracts from *L. officinalis* 'Globigemmis' and *L. officinalis* 'Oxygemmis' had an average of 4.9 ± 1.1 and 4.2 ± 1.0 mg/ml of protein, respectively.

2.4. Assay of polyphenol oxidase activity

PPO activity was assayed by measuring the rate of increase in absorbance at a given wavelength, using a double beam model ATI Unicam UV2-100 spectrophotometer, as described previously (Dincer et al., 2002). 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, Morales, Varon, Tudela, & Garcia-Canovas, 1995). 4-Methylcatechol and 3-(3,4-dihydroxyphenyl) propionic acid (DHPPA) were assayed as diphenolic substrates and *L*-tyrosine, and (*p*-hydroxyphenyl)propionic acid (PHPPA) as monophenolic substrates with MBTH in 50 mM potassium phosphate buffer at pH 6.5.

The reaction mixture contained substrates (stock 100 mM), an equal volume of MBTH (stock 10 mM), and 20 μl dimethylformamide (DMF), and the solution was diluted to 950 μl with buffer and 50 μl enzyme extract were added. One unit of PPO activity was defined as the amount of enzyme causing 0.001 increase of absorbance per minute in 1 ml reaction mixture (Galeazzi & Sgarbieri, 1981).

2.5. Properties of cherry laurel PPO

2.5.1. pH optimum and stability

PPO activity, as a function of pH, was determined in a pH range of 4.0–5.0 in 50 mM acetate buffer, 6.0–7.0 in 50 mM phosphate buffer and 8.0–9.0 in 50 mM Tris–HCl buffer. The pH stability was determined by incubating 0.05 ml of crude enzyme solution in 0.73 ml buffer solution, ranging from pH 4.0 to 5.0 in 50 mM acetate buffer, 6.0–7.0 in 50 mM phosphate buffer, and 8.0–9.0 in 50 mM Tris–HCl buffer for 24 h at 4 °C. 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 DHPPA as a substrate, and 0.1 ml of 10 mM MBTH and 0.02 ml DMF, with the incubated enzyme solution (Dincer et al., 2002).

2.5.2. Thermal activity and stability

Temperature optimum for the cherry laurel PPO was determined by measuring the enzyme activity at various temperatures over the range 10–80 °C, using a circulation water bath. The mixtures of buffer and DHPPA solution were incubated for 5 min at the different temperatures indicated above at the optimum pH value of the enzyme from each cultivar, using DHPPA as a substrate. The enzyme extract was added to the mixture and the relative activity of PPO was determined spectrophotometrically at 500 nm as rapidly as possible.

Thermal stability of the PPO was determined by measuring enzyme activity in 50 mM acetate buffer at pH 5 for crude enzymes from each cultivar. The mixtures were kept within Eppendorf tubes which were incubated in a water bath at various temperatures of 20–80 °C with 10 °C increments 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 DHPPA, 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. The data obtained from the thermal stability profile were used to analyze some thermodynamic parameters related to cherry laurel PPO activity in the crude extracts from each cultivar (Amiza & Apenten, 1994; Dincer et al., 2002; Duangmal & Owusu Apenten, 1999).

2.5.3. Enzyme kinetics

Enzyme kinetics for the cherry laurel PPO from each cultivar were studied by using DHPPA as a substrate and the rate of the PPO reaction was measured at various DHPPA concentrations in the standard reaction mixture in terms of the increase in absorbance at the wavelength of maximum absorption for the corresponding chromophore (Espin, Trujano, Tudela, & Garcia-Canovas, 1997).

The kinetic data were plotted as reciprocals of activities versus DHPPA concentrations. The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) were determined as the reciprocal absolute values of the intercepts on the x- and y-axis, respectively, of the linear regression curve (Lineweaver & Burk, 1934). Substrate specificity (V_{max}/K_m) was calculated by using the data obtained on a Lineweaver–Burk plot.

2.5.4. Effect of inhibitors

Sodium azide, sodium metabisulfite, ascorbic acid and EDTA were used as PPO inhibitors at 1 mM final concentrations and the effect of inhibitors on cherry laurel PPO activity was determined using DHPPA as a substrate. 1 ml reaction mixture contained 0.1 ml DHPPA at 10 mM final concentration in 50 mM phosphate buffer at pH 5.0, and 0.05 ml enzyme solution and 0.1 ml of inhibitor solutions at 10 mM concentrations.

2.5.5. Native polyacrylamide gel electrophoresis

Native polyacrylamide gel electrophoresis was performed on a Hoeffer SE 600 Series Electrophoresis dual slab cell unit (California, USA) using preparative 12% polyacrylamide gels (Laemmli, 1970) under native conditions, as reported previously (Dincer et al., 2002). After electrophoresis, the gels were stained for PPO activity in 24 mM L-DOPA in 50 mM sodium phosphate buffer (pH 7.5) at room temperature for 2 h, then in 1 mM ascorbic acid solution, until appearance of isoenzyme bands.

3. Results and discussion

3.1. General

The polyphenoloxidase (PPO) activities were characterized on crude enzyme preparations extracted from cherry laurel fruits of *L. officinalis* 'Globigemmis' and *L. officinalis* 'Oxygemmis' to confirm enzyme activity at the early stage of fruit development. The fruits were fully green and unripe.

Native electrophoresis showed isoforms of cherry laurel PPO having R_f values of 0.50 (minor band) and 0.57 (major band), respectively, indicating the presence of at least two isoforms of PPO in the cherry laurel fruits from each cultivar at the early stage stage of maturation (Fig. 1). The presence of 2–4 PPO isoenzymes has also been reported for fruits of the Rosaceae family (Costantinides & Bedford, 1967; Dincer et al., 2002; Fraignier, Marques, Fleuriert, & Macheix, 1995; Haruta, Murata, Kadokura, & Homma, 1999; Pifferi & Cultrera, 1974; Şakiroglu, Küfrevioglu, Kocacaliskan, Oktay, & Onganer, 1996). Moreover, immunological and molecular comparison of PPOs in Rosaceae fruits indicated that these enzymes shared similar structural and functional properties (Haruta et al., 1999).

4-Methylcatechol and DHPPA, as diphenolic substrates, were both oxidized by the crude enzymes from the two cultivars. A high level of activities was observed in the presence of DHPPA. It seems, from the assays, that the enzymes from cherry laurel fruits were unable to oxidize the monophenolic compounds, either *L* -tyrosine or PHPPA. This result indicates that crude enzymes from both cultivars contain a diphenolase responsible for the oxidation of either 4-methylcatechol or DHPPA which is consistent with previous reports on the PPOs from other plant sources (Cash, Sistrunk, & Stutte, 1976; Dincer et al., 2002; Ding, Chachin, Ueda, & Imahori, 1998; Perez-Gilabert & Garcia-Carmona, 2000; Siddiq, Sinha, & Cash, 1992). Catalytic efficiencies, using the V_{max}/K_m ratio (Table 1), indicate that the

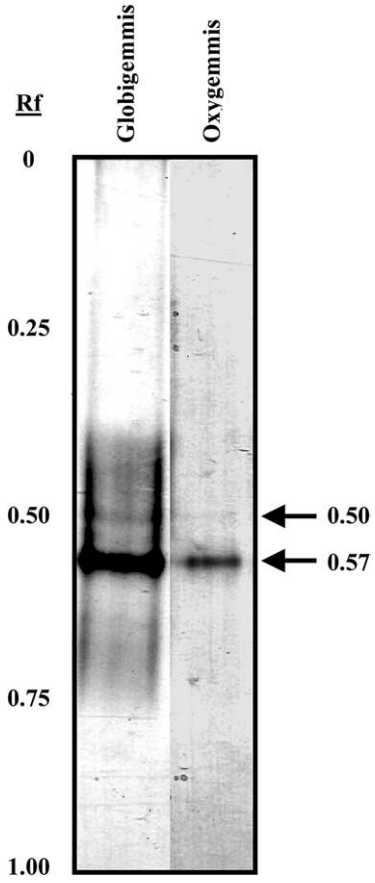

Fig. 1. *L* -DOPA staining for PPO of cherry laurel fruits from: (a) *L. officinalis* 'Globigemmis'; (b) *L. officinalis* 'Oxygemmis'.

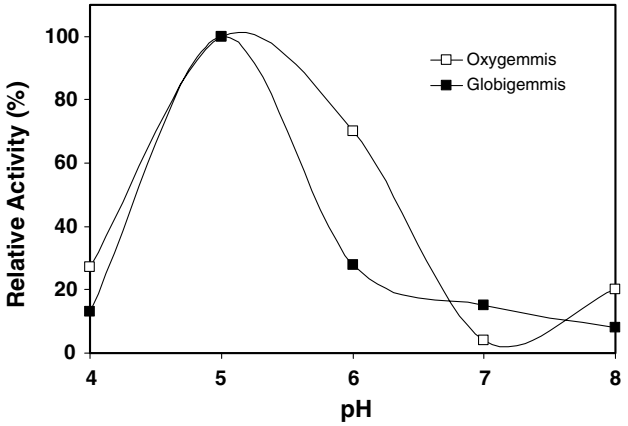

Fig. 2. pH-activity profiles for cherry laurel diphenolases in 50 mM acetate buffer (pH 4.0–5.0), in 50 mM phosphate buffer (pH 6.0–7.0) and in 50 mM Tris-HCl buffer (pH 8.0–9.0).

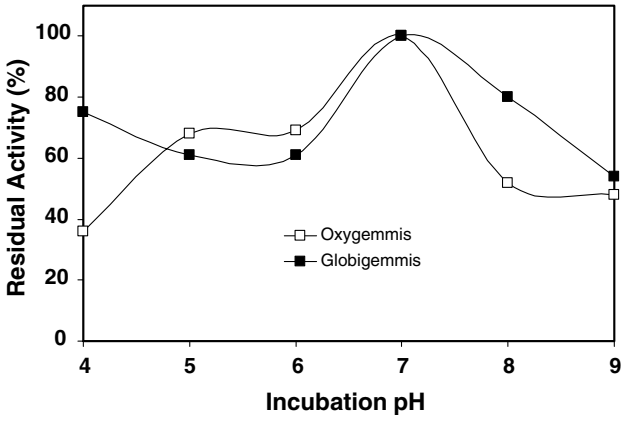

Fig. 3. pH stabilities of cherry laurel diphenolases from *L. officinalis* 'Globigemmis' and 'Oxygemmis' cultivars.

two diphenolases had similar activities against DHPPA as a diphenolic substrate.

3.2. pH optimum and stability

The pH-activity profiles for both enzymes of the cherry laurel fruits from each cultivar gave a pH-optimum of pH 5.0 in the presence of DHPPA as a substrate (Fig. 2). It seems that, although each diphenolase from both cultivars catalyze oxidation of DHPPA at acidic values, the enzyme from *L. officinalis* 'Oxygemmis' is more active than that from *L. officinalis* 'Globigemmis' over a wider range of pH values. Similar pH behaviour was also reported for other fruits of the Rosaceae family (Fraignier et al., 1995; Haruta et al., 1999; Özen et al., 2004; Yemenicioğlu & Cemeroğlu, 2003). It is clear, from previous reports, that the pH-optimum for PPOs is highly dependent on the enzyme source and the nature of substrate used (Duangmal & Owusu Apenten, 1999). pH-stability profiles have shown that both enzymes were fully stable at pH 7.0 and retained over 50% of their original activities at physiological pH values after 24 h of incubation at 4 °C (Fig. 3). Although the diphenolase from cultivar 'Oxygemmis' loses its activity by 50% when kept at pH 8.0 for 24 h, the enzyme from 'Globigemmis' still possesses its original activity (on at least

Table 1
Optimization of cherry laurel (*L. officinalis*) diphenolase-catalyzed oxidation of DHPPA and sensitivities of diphenolase activities toward some common PPO inhibitors at 1 mM final concentrations

Cultivar	pH optimum	Temperature optimum (°C)	V_{max}/K_m (min_{-1})	Inhibition (%) (mean ± SD)			
				Ascorbic acid	Metabisulfite	Sodium azide	EDTA
L. officinalis 'Globigemmis'	5.0	50	1.7 ± 1.1	7.1 ± 1.8	10.1 ± 1.8	49.1 ± 2.0	103 ± 5.2
L. officinalis 'Oxygemmis'	5.0	40	3.3 ± 1.6	2.3 ± 0.8	16.3 ± 2.0	50.6 ± 4.1	101 ± 4.4

80%). The stabilities of the two diphenolases significantly decrease at pH values higher than physiological pHs.

3.3. Thermal activity and stability

Temperature–activity profiles of the diphenolases from the two cherry laurel fruits are shown in Fig. 4. It is clear that both enzymes possess very high diphenolase activities at temperatures ranging from 30 to 65 °C. The enzyme from *L. officinalis* 'Oxygemmis' showed a temperature optimum at 40 °C whereas that from *L. officinalis* 'Globigemmis' at 50 °C. Similar temperature optima were also reported for apricot (Yemenicioğlu & Cemeroğlu, 2003) and dog-rose fruits (Şakiroglu et al., 1996). Although the diphenolase activity of cherry laurel fruit from 'Oxygemmis' decreased to less than 50% upon temperature increases to 80 °C, the enzyme from 'Globigemmis' was 75% active even at this temperature. This indicates that the diphenolase activities from two cultivars with similar characteristics may possess different temperature optima.

The thermostabilities of the diphenolases from cherry laurel fruits were determined at 40 and 70 °C, and are presented in the form of the residual percentage activity (Fig. 5). The non-linear decay of the profiles could be regarded as biphasic which may support the existence of isoenzymes in cherry laurel fruits with different thermal behaviours (Weemaes, Ludikhuyze, van den Broeck, Hendrickx, & Tobback, 1998). Although the enzyme from *L. officinalis* 'Globigemmis' was quite stable at 40 °C for 30 min, the diphenolase from 'Oxygemmis' lost its original activity (about 33% and 56%) in the first 10 min of incubation at 40 and 70 °C, respectively. The residual activities for the 'Oxygemmis' diphenolase dropped to 13% at the end of 30 min of incubation at 70 °C. The enzyme fully retained its activity at 40 °C

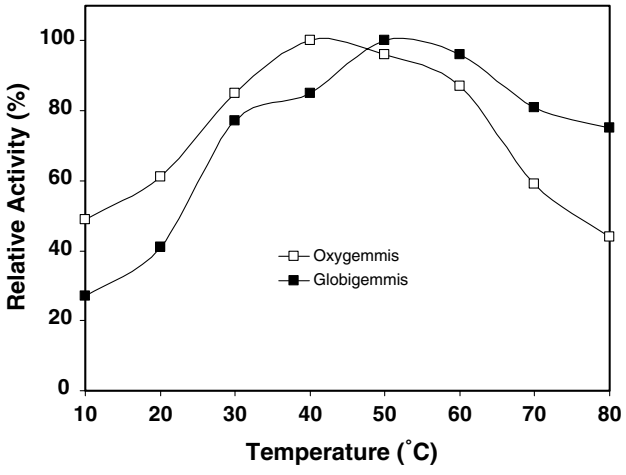

Fig. 4. Temperature–activity profiles for cherry laurel diphenolases *L. officinalis* 'Globigemmis' and 'Oxygemmis' cultivars in 50 mM acetate buffer (pH 5.0).

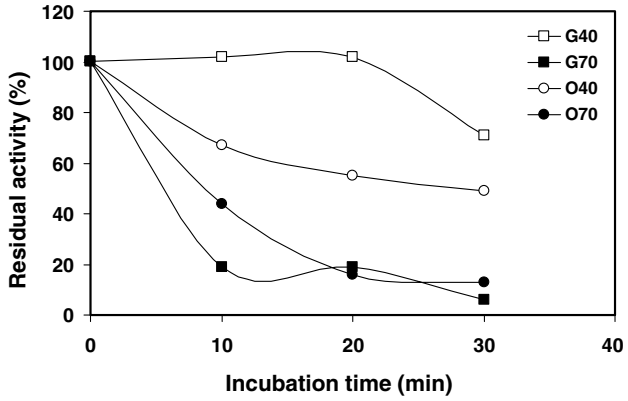

Fig. 5. Thermal stabilities of the cherry laurel diphenolases from *L. officinalis* 'Globigemmis' (G) and 'Oxygemmis' (O) cultivars determined at either 40 °C (open symbols) or 70 °C (closed symbols) in 50 mM acetate buffer at pH 5 in the presence of DHPPA as substrate.

for 20 min of incubation and over 70% of original activity was retained for 30 min at the same temperature. However, a 30-min incubation at 70 °C almost completely destroyed the diphenolase activity from *L. officinalis* 'Globigemmis'. It is clear that the diphenolase activities are reduced at higher inactivation temperatures because of heat-denaturation of the enzyme or some conformational changes in the tertiary structure due to the increasing temperatures (Duangmal & Owusu Apenten, 1999; Yemenicioğlu & Cemeroğlu, 2003).

E_a values for diphenolases from cultivars *L. officinalis* 'Globigemmis' and 'Oxygemmis' were calculated to be 37.8 and 49.2 kJ mol^{-1}, respectively, from the plot of $1/T$ vs ln k and used for the calculation of $\Delta H^{\#}$ (Table 2). The average values for $\Delta H^{\#}$ and $\Delta S^{\#}$ were 35.3 ($\pm$0.5) kJ mol^{-1} and $-$944.3 ($\pm$17.3) kJ mol^{-1} K^{-1} for 'Globigemmis', and 46.3 ($\pm$0.5) kJ mol^{-1} and $-$909 ($\pm$23.8) kJ mol^{-1} K^{-1} for 'Oxygemmis' diphenolase heat-inactivation, respectively. It is obvious from these data that the cherry laurel diphenolases from both cultivars share similar thermodynamic properties and heat-sensitivities. It has been previously reported that heat-sensitivities of PPOs may depend on ripeness of the plant or different molecular forms of enzyme from the same plant source (Park & Luh, 1985).

3.4. Effect of inhibitors

The effects of general PPO inhibitors, namely ascorbic acid, sodium azide, EDTA and sodium metabisulfite, at 1 mM fixed final concentrations, on crude diphenolases of cherry laurel fruits from the two cultivars were examined. Their potentials for the inhibiton of the diphenolases from the two cultivars are shown by the percentage inhibiton of DHPPA oxidation (Table 1). Ascorbic acid was the most effective inhibitor, followed by metabisulfite, and it caused nearly complete inhibition of DHPPA oxidation by diphenolases from both

Table 2
Thermodynamic parameters for thermal inactivation of cherry laurel diphenolases from two cultivars

Temperature (°C)	*L. officinalis* 'Globigemmis'			*L. officinalis* 'Oxygemmis'		
	$\Delta G^{\#}$ (kJ mol^{-1})	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$(kJ mol^{-1}K^{-1})	$\Delta G^{\#}$(kJ mol^{-1})	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$(kJ mol^{-1} K^{-1})
20	304	36	−914	307	47	−887
30	318	36	−931	317	47	−891
40	330	35	−942	329	46	−901
50	341	35	−946	332	46	−886
60	353	35	−953	353	46	−920
70	365	35	−962	366	46	−933
80	375	35	−962	380	46	−945
Mean (±SD)	340.8 (±25.4)	35.3 (±0.5)	944.3 (±17.3)	340.6 (±26.6)	46.3 (±0.5)	909.0 (±23.8)

*E_a values for cultivars *L. officinalis* 'Globigemmis' and 'Oxygemmis' were calculated to be 37.8 and 49.2 kJ mol^{-1} K^{-1}, respectively, from the plot of $1/T$ vs $\ln k$ and used for the calculation of $\Delta H^{\#}$.

cultivars. The mechanism of inhibition by ascorbate may involve reduction of quinonoid compounds produced by the diphenolases, and chelation or reduction of copper centres at the active site of the enzyme (Martinez & Whitaker, 1995; Sapers, 1993; Zawistowski et al., 1991). The inhibition of cherry laurel diphenolases by metabisulfite was also very strong, which is consistent with the earlier reports indicating that the thiol compounds are potent inhibitors of PPOs (Ding et al., 1998; Duangmal & Owusu Apenten, 1999; Friedman & Bautista, 1995; Yang, Fujita, Ashrafuzzaman, Nakamura, & Hayashi, 2000). Azide caused almost 50% inhibition of DHPPA oxidation by diphenolases of cherry laurel fruits. EDTA, at 1 mM concentration, had no effect on crude diphenolase from the two cultivars. It seems that EDTA was not a good inhibitor for these enzymes. This could be a result of the acidic pH (5.0) of the reaction mixture which may prevent binding of EDTA to the metal centre at the active site of the enzyme (Luh & Phitakpol, 1972). It has been reported that the nature of the inhibitor may affect its action on PPO (Duangmal & Owusu Apenten, 1999; Martinez & Whitaker, 1995; Rescignio, Sollai, Pisu, Rinaldi, & Sanjust, 2002; Sapers, 1993).

It can be concluded from the present study that the crude enzymes prepared from the cherry laurel fruits of two cultivars at an early stage of development possess diphenolase activities sharing similar functional properties. The enzymes from both 'Globigemmis' and 'Oxygemmis' cultivars are very active against DHPPA at acidic pH values with temperature optima of 50 and 40 °C, respectively. Although the pH-stability profiles have shown that both enzymes were fully stable at pH 7.0, their stabilities dramatically decrease at alkaline pH values, especially above pH 9.0. Thermal-stabilities of the cherry laurel diphenolases indicate that the enzymes from the two cultivars share similar thermodynamic properties and heat-sensitivities. In addition, the diphenolases were highly sensitive toward ascorbate and metabisulfite.

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