

Substrate specificity, heat inactivation and inhibition of polyphenol oxidase from different aubergine cultivars

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Summary The effects of substrate specificity, heat inactivation, temperature, pH and inhibitors on polyphenol oxidase (PPO) activity obtained from three different aubergine cultivars were investigated to identify the most appropriate aubergine cultivar for dried preparations. PPO obtained from different aubergine cultivars (cultivars I, II and III) was partially purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation followed by dialysis. PPO showed activity with catechol and 4-methylcatechol but not with L-tyrosine. The best substrate for cultivar I (V_{max} : 3333.3 EU min^{-1} mL^{-1} , K_m : 8.7 mM and V_{max}/K_m : 384.9 min^{-1}) and cultivar III (V_{max} : 1000 EU min^{-1} mL^{-1} , K_m : 9.3 mM and V_{max}/K_m : 107.5 min^{-1}) was catechol, but 4-methylcatechol was the best substrate for cultivar II (V_{max} : 5000 EU min^{-1} mL^{-1} , K_m : 35.5 mM and V_{max}/K_m : 140.8 min^{-1}). The optimum pH for aubergine PPO was 7.0 with catechol as a substrate and 6.0 with 4-methylcatechol. Heat inactivation studies showed a decrease in enzyme activity at temperatures above 40 °C. For catechol and 4-methylcatechol substrates, the optimum temperature for maximum PPO activity was 30 °C for all aubergine cultivars except cultivar I using catechol which was 20 °C. The effects of compounds such as tropolone, D,L-dithiothreitol and glutathione as inhibitors of the reactions catalysed by PPO were tested. Generally, tropolone was the most effective inhibitor.

Keywords Aubergine, heat inactivation, inhibition, inhibitors, polyphenol oxidase.

Introduction

Aubergine (*Solanum melongena*) is a member of the Solanum genus of the Solanaceae family. It is an annual plant in temperate climates and a small tree in tropical climates (Davis, 1978). Aubergine is both consumed as a fresh vegetable and also dried and stored for future use in Turkey and in other countries.

The browning reaction catalysed by polyphenol oxidase (PPO) during the drying processes is a crucial problem. In most cases, the browning reaction is considered to be detrimental to the

quality of the product. It is caused primarily by the oxidation of phenolic substances to quinones catalysed by PPO (*o*-diphenol: O_2 oxidoreductase, E.C. 1.10.3.1). The quinones then condense to form dark pigments. Because the browning is considered unfavourable (Arslan *et al.*, 1997), there has been much interest in PPO among biochemists and food technologists (Rocha & Morais, 2001).

Mayer & Harel (1979) have published a detailed review on PPO from plants. PPO obtained from different plant tissues shows different substrate specificities and degrees of inhibition (Arslan *et al.*, 1997). Therefore, characterization of the enzyme could help to develop more effective methods for controlling browning of plants and products.

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PPO is a copper-containing enzyme which is widely distributed in fruits such as grape (Lamikanra *et al.*, 1992), apple (Oktay *et al.*, 1995; Yemencioğlu *et al.*, 1997), strawberry (Wesche-Ebeling & Montgomery, 1990), palmito (Lourenco *et al.*, 1990), bean (Paul & Gowda, 2000), raspberry (Gonzalez *et al.*, 1999), tea leaf (Halder *et al.*, 1998), pear (Wissemann & Montgomery, 1985; Gauillard & Richard-Forget, 1997), lettuce (Chazarra *et al.*, 1996), banana (Oba *et al.*, 1992) and apricot (Arslan *et al.*, 1998). In these studies, the effects of substrate specificity, heat inactivation, temperature, pH and inhibitors on PPO activity have been investigated.

There is little research comparing PPO activities obtained from different cultivars of aubergine. However, Perez-Gilabert & Garcia-Carmona (2000) studied characterization of catecholase and cresolase activities of eggplant PPO and found that one of the most effective inhibitors was tropolone. Almeida & Nogueira (1995) also investigated the control of PPO activity in aubergine. Rocha & Morais used controlled atmosphere storage to influence PPO activity in relation to colour changes of minimally processed 'Jonagored' apple and found controlled atmosphere storage inhibited the PPO activity of apple cubes during storage. Furthermore, they found that the higher the concentration of carbon dioxide in the storage atmosphere the higher the inhibition of PPO and the lower the browning (Rocha & Morais, 2001).

In this work, the effects of substrate specificity, heat inactivation, temperature, pH and inhibitors on PPO activity obtained from three different aubergine cultivars were studied to identify the most appropriate aubergine cultivar for dried preparations.

Materials and methods

Aubergine cultivars I (*Solanum melongena* var. *insanum*), II (*Solanum melongena* var. *falcatum*) and III (*Solanum melongena* var. *zhukovskiyi*) used in this study were harvested in winter from a field near Balıkesir in Turkey. All chemicals used in this study were the best grade available and were used without further purification as they were obtained from Sigma Chemical Co. (Deisenhofen, Germany). Enzyme assays were measured with the

aid of a Cary 1E UV-Visible Spectrophotometer (Varian, Australia).

Extraction of PPO

The extraction procedure was adopted from Wesche-Ebeling & Montgomery (1990). The aubergines, of commercial maturity, were freshly harvested and kept for 2 days in the refrigerator (+4 °C) before extracting PPO. After peeling, aubergine cultivars were washed with distilled water three times. To prepare the crude extract, 10 g of fruit tissue was cut quickly into thin slices and homogenized in a Waring blender for 2 min using 100 mL of 0.1 M phosphate buffer (pH 6.5) containing 5% poly(ethylene glycol) and 10 mM ascorbic acid. The crude extract was filtered, and the filtrate was centrifuged at 20 000 g for 30 min at 4 °C. The supernatant was brought to 80% (NH₄)₂SO₄ saturation with solid (NH₄)₂SO₄. Inactive proteins were partially removed by ammonium sulphate precipitation. The precipitated PPO was separated by centrifugation at 20 000 g for 30 min. The pellet then was dissolved in a small volume of 0.1 M phosphate buffer (pH 6.5) and dialysed against 0.05 M phosphate buffer (pH 7.0) for 2 days with three changes of buffer. The dialysed sample was used as the PPO enzyme source in the following experiments (Wesche-Ebeling & Montgomery, 1990).

Enzyme assay

Enzyme activity was determined by measuring the increase in absorbance with a Cary 1E UV-Visible Spectrophotometer (Varian) set 420 nm when using catechol and 4-methylcatechol as substrates (Oktay *et al.*, 1995). In each measurement, the volume of solution in a quartz cuvette was kept constant as 3 mL. About 0.1 mL of PPO was used with 0.6 mL of 0.1 M catechol and 2.3 mL of 0.1 M phosphate buffer (catechol substrate pH 6.5) and 0.2 mL of 0.1 M 4-methylcatechol and 2.7 mL of 0.1 M phosphate buffer (4-methylcatechol substrate pH 6.5). The 0.1 M concentration was chosen to avoid the influence of enzymatic extract ionic strength on PPO activity, described by Angleton & Flurkey (1984). One unit of PPO activity was defined as the amount of enzyme that causes an increase in absorbance of

0.001 min⁻¹ mL⁻¹. PPO activity was assayed at 20 °C in triplicate measurements (Oktay *et al.*, 1995).

Effect of pH

The activity of the enzyme was determined in the pH range of 4.0–9.0 by using 0.1 M acetate (pH 4–6) and 0.1 M phosphate (pH 6–9) buffer adjusted with 0.1 M NaOH or 0.1 M HCl. The optimum pH value of PPO for each cultivar was obtained using two different substrates (catechol and 4-methylcatechol) (Oktay *et al.*, 1995).

Enzyme kinetics and substrate specificity

Michaelis constants (K_m) and maximum velocities (V_{max}) for each aubergine cultivar were determined using two different substrates (catechol and 4-methylcatechol) at various concentrations. The absorbance of the oxidation products was measured as described previously. Data were plotted according to the method of Lineweaver and Burk (Oktay *et al.*, 1995).

Activation energy

The activation energy was calculated from experimental results for enzyme reactions by using the Arrhenius equation, which is

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

where A is the enzyme activity value, Z is the frequency factor, E_a is the activation energy and T is the temperature. The graph of $\ln A$ vs. $1/T$ was a straight line. The parameter Z is obtained from the intercept point at $1/T=0$ and the activation energies of reactions are calculated from the slopes of lines (Doğan *et al.*, 2000).

Effect of temperature

PPO activity was measured at temperature range from 20 to 60 °C by using different aubergine cultivars and different substrates. The effect of temperature on the activity of PPO was tested by heating the standard reaction solution to an appropriate temperature before introduction of the enzyme. Once temperature equilibrium was

reached, the enzyme was added and the reaction was followed spectrophotometrically at constant temperature at given time intervals (Arslan *et al.*, 1998).

Heat inactivation of PPO

The thermal denaturation of PPO was studied at 40, 50 and 60 °C, 1 mL of enzyme solution in a test-tube was incubated at the required temperature for fixed time intervals. At the end of a time interval, the test-tube was cooled in an ice bath. The activity of the enzyme was then determined at 20 °C (Arslan *et al.*, 1998).

Results and discussion

Effect of pH

For each substrate and each aubergine cultivar, pH optima have been determined. Figure 1a,b shows the changes of enzyme activity for various aubergine cultivars with different substrates. As is seen in Fig. 1a, each cultivar has a different pH curve and level of activity using different substrates, except that all of the aubergine PPO showed a clear pH optimum around 7.0 with catechol as a substrate. However, as seen from Fig. 1b, aubergine PPO showed a flatter pH profile with 4-methylcatechol as a substrate for each of the three aubergine cultivars. The maximum pH optima was 6.0 for cultivar I and around 6.5 for II and III when 4-methylcatechol was used as a substrate. Perez-Gilabert & Garcia-Carmona (2000) found that the enzyme showed a broad maximum at pH ~ 5.5 with 4-methylcatechol as a substrate. Although several studies reported a pronounced acidic pH optimum of 4.5–5.0 with 4-methylcatechol (Janovitz-Klapp *et al.*, 1989; Zhou *et al.*, 1993), a broader optimum of 5.0–6.5 was obtained for Granny Smith pulp (Marques *et al.*, 1995), and two pronounced pH optima at pH 6.0 and 9.0 were reported for Amasya apple (Oktay *et al.*, 1995). Generally, fruits show maximum PPO activity at or near neutral pH values (Betrosian *et al.*, 1960; Chan & Yang, 1971; Wong *et al.*, 1971; Cash *et al.*, 1976; Siddiq *et al.*, 1992) (e.g. 4.5 for green olive, Ben-Shalom *et al.*, 1977; 7.0 for banana, Palmer, 1963, and *Dioscorea bulbifera* enzyme, Anosike & Ayaebene, 1981; 4.2

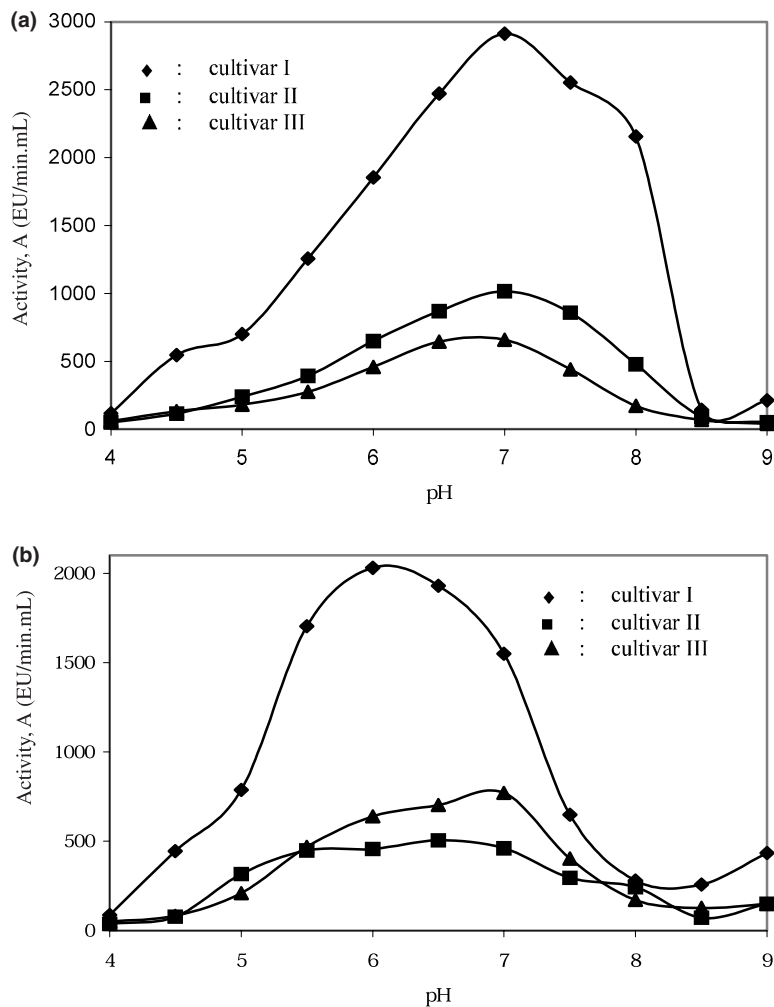


Figure 1 The changing of enzyme activities of different aubergine cultivars with different substrates as a function of pH: (a) catechol and (b) 4-methylcatechol.

for *Prunus avium*, Pifferi *et al.*, 1974; 6.2 for peach, Anosike & Ojimekwe, 1982). Thus, the optimum PPO activity has been found to vary with the source of enzyme and substrate in a relatively wide range of pH. Although, in most cases, pH optima has been reported between 4.0 and 7.0, it should be noted that the pH optima can also be affected by the type of buffer and the purity of enzyme (Lourenco *et al.*, 1990).

Heat inactivation of PPO

Tables 1 and 2 show that enzyme activity changes with different aubergine cultivars and different substrates as a function of temperature and time. When catechol (Table 1) and 4-methylcatechol (Table 2) were used as substrates, the enzyme

activity decreased with increasing temperature and inactivation time. The time required for 50% inactivation using catechol as a substrate at 40 °C was 8 min for Cultivar II and 13 min for Cultivar III. At 50 °C these times were found as 40, 7 and 9 min for Cultivars I, II and III, respectively. At 60 °C, it was 10 min for Cultivar I, 5.5 min for Cultivar II and 6.8 min for Cultivar III. The time required for 4-methylcatechol at 40 °C was 9.7, 5.8 and 9.8 min for Cultivars I, II and III, respectively, at 50 °C 9.0, 5.1 and 9.0 min for Cultivars I, II and III, respectively, and at 60 °C 8.0, 5.0 and 6.0 min for Cultivars I, II and III, respectively. It has been noted that heat stability of the enzyme may be related to ripeness of the plant and molecular forms of enzyme (Zhou & Feng, 1991).

Table 1 The experimental data relation to change of enzyme activities of different aubergine cultivars with catechol substrate as a function of temperature and time

Temperature (°C)	Time (min)	Substrate	Cultivars		
			I	II	III
40	0	Catechol	100.0	100.0	100.0
	10		86.4	64.0	53.0
	20		82.5	46.6	37.2
	30		75.5	44.5	35.1
	40		70.0	40.7	34.5
	50		68.3	37.5	33.7
	60		66.9	37.2	32.4
50	0	Catechol	100.0	100.0	100.0
	10		65.2	45.7	38.5
	20		59.5	42.9	33.5
	30		51.6	36.8	30.1
	40		50.7	33.7	29.6
	50		45.3	30.1	28.7
	60		45.0	25.2	26.6
60	0	Catechol	100.0	100.0	100.0
	10		50.8	35.3	27.2
	20		44.9	28.5	21.9
	30		38.4	25.4	18.0
	40		33.9	21.5	14.0
	50		30.0	17.2	13.5
	60		27.2	12.5	10.4

Table 2 The experimental data relation to change of enzyme activities of different aubergine cultivars with 4-methylcatechol substrate as a function of temperature and time

Temperature (°C)	Time (min)	Substrate	Cultivars		
			I	II	III
40	0	4-Methylcatechol	100.0	100.0	100.0
	10		51.6	39.9	28.0
	20		51.5	38.5	26.0
	30		50.8	37.7	25.5
	40		50.3	32.5	24.5
	50		48.2	31.8	23.0
	60		41.4	28.0	22.9
50	0	4-Methylcatechol	100.0	100.0	100.0
	10		44.6	41.3	22.1
	20		40.3	30.0	20.0
	30		36.0	26.0	17.6
	40		32.6	22.5	16.3
	50		31.8	17.5	15.2
	60		26.4	12.1	12.0
60	0	4-Methylcatechol	100.0	100.0	100.0
	10		40.7	25.5	14.8
	20		31.2	17.3	13.3
	30		25.0	11.3	9.7
	40		19.5	10.6	7.4
	50		16.4	6.8	6.7
	60		13.0	5.9	5.5

Enzyme kinetics and substrate specificity

In order to investigate of enzyme kinetics and substrate specificity, Lineweaver–Burk graphs were drawn to calculate the K_m and V_{max} values. The aubergine cultivars investigated showed different PPO activity with respect to substrates such as catechol and 4-methylcatechol. In the determination of substrate specificity, *o*-dihydroxyphenols and monohydroxyl phenolic compounds were tested. Catechol and 4-methylcatechol were oxidized by different aubergine PPO preparations but no reaction took place with L-tyrosine. According to these results, aubergine PPO could be a diphenol oxidase. This is similar to the findings for Yali PPO (Roudsari *et al.*, 1981), Bartlett pears (Rivas & Whitaker, 1973) and kiwi fruit (Park & Luh, 1985). Perez-Gilabert & Garcia-Carmona (2000) found that diphenol oxidation predominates over monophenol. Kinetic parameters, V_{max} and K_m , for cultivars I, II and III were found as 3333.3 EU min⁻¹ mL⁻¹ and 8.7 mM; 1428.0 EU min⁻¹ mL⁻¹ and 11.0 mM and 1000.0 EU min⁻¹ mL⁻¹ and 9.3 mM with catechol as a substrate, and 5000.0 EU min⁻¹ mL⁻¹ and 21.5 mM; 5000.0 EU min⁻¹ mL⁻¹ and 35.5 mM, 2000.0 EU min⁻¹ mL⁻¹ and 30.0 mM with 4-methylcatechol as a substrate, respectively. The V_{max}/K_m ratio is called as the 'catalytic power' and the size of this ratio gives the most effective substrate (Rocha *et al.*, 1998). It is found that the most effective substrate was catechol for cultivar I (V_{max} : 3333.3 EU min⁻¹ mL⁻¹, K_m : 8.7 mM and V_{max}/K_m : 384.9 min⁻¹) and cultivar III (V_{max} : 1000 EU min⁻¹ mL⁻¹, K_m : 9.3 mM and V_{max}/K_m : 107.5 min⁻¹) and 4-methylcatechol for cultivar II (V_{max} : 5000 EU min⁻¹ mL⁻¹, K_m : 35.5 mM and V_{max}/K_m : 140.8 min⁻¹). Although cultivar I has the highest PPO activity for each substrate, cultivar III shows the lowest PPO activity. Therefore, the most suitable aubergine cultivar for future use, when dried, because of the lowest PPO activity is cultivar III. Perez-Gilabert & Garcia-Carmona (2000) found that values of V_{max} , K_m and V_{max}/K_m were 14 EU min⁻¹ mL⁻¹, 2.7 mM and 5.2 min⁻¹, respectively. The K_m values for cultivar I (using catechol as substrate) are much lower than that for olive enzyme (Ben-Shalom *et al.*, 1977) and Yali PPO (Roudsari *et al.*, 1981). The calculated K_m value of cultivar I (8.7 mM) using catechol as a

substrate is close to the value reported for PPO from Amasya apple using L-dopa as the substrate (K_m : 6.5 mM) (Oktay *et al.*, 1995). It was found that the K_m for PPO varies with the source of the enzyme (Park & Luh, 1985; Arslan *et al.*, 1997).

Effect of temperature

Optimum temperatures for maximum PPO activity were 20, 30 and 30 °C for cultivars I, II and III, respectively, using catechol as a substrate and 20 °C for cultivars I, II and III with 4-methylcatechol. For catechol and 4-methylcatechol, activity decreased gradually with increasing temperature and showed very little activity at 60 °C.

Activation energy

For each substrate, plots were drawn by using temperature–activity values obtained from the optima temperature study. Activation energies for both substrates and aubergine cultivars were calculated from the slopes. It was found that activation energies using catechol as a substrate were -78.5, -89.2 and -80.5 cal mol⁻¹ for cultivars I, II and III, respectively, and -103.0, -129.0 and -104.0 cal mol⁻¹ for cultivars I, II and III, respectively, with 4-methylcatechol. In this study, activation energy values for all cultivars of aubergine have been calculated from the Arrhenius equation. The calculated E_a values fit well with the E_a (120 cal mol⁻¹) value obtained from Amasya apple (Oktay *et al.*, 1995).

Effect of inhibitors

Enzymatic browning of fruits may be delayed or eliminated by removing the reactants such as oxygen and phenolic compounds or by using PPO inhibitors. Complete elimination of oxygen from fruits during the drying process is difficult because oxygen is ubiquitous (Roudsari *et al.*, 1981). Different inhibitors such as sodium metabisulphite (Lee *et al.*, 1983; Augustin *et al.*, 1985), ascorbic acid (Lee *et al.*, 1983; Augustin *et al.*, 1985; Jiang *et al.*, 1999), D,L-dithiothreitol (Lee *et al.*, 1983), sodium cyanide (Park & Luh, 1985; Lee *et al.*, 1983), glutathione (Lee *et al.*, 1983; Park & Luh, 1985; Jiang *et al.*, 1999), tropolone (Khan & Andrawis, 1985; Perez-Gilabert & Garcia-Carmona,

2000), thiourea (Lee *et al.*, 1983; Zhou & Feng, 1991), sodium diethyldithiocarbamate (Lee *et al.*, 1983; Park & Luh, 1985; Zhou & Feng, 1991) have been used by researchers to prevent the enzymatic browning. Lee *et al.* (1983) studied the effect of inhibitors such as D,L-dithiothreitol and glutathione on PPO activity from DeChaunac grapes and found that these inhibitors were effective in decreasing the activity of PPO; Wesche-Ebeling & Montgomery (1990) studied the effect of D,L-dithiothreitol on PPO activity obtained from strawberry and found that it was an effective inhibitor of PPO; Oktay *et al.* (1995) studied the effect of glutathione on PPO activity obtained from Amasya apple and found that it decreased the PPO activity; Raymond *et al.* (1993) studied the effectiveness of D,L-dithiothreitol on PPO activity obtained from sunflower seeds and found that it decreased the PPO activity; and Cash *et al.* (1976) studied the effect of D,L-dithiothreitol on PPO activity obtained from grape and found that it decreased the PPO activity. Table 3 shows I_{50} values of D,L-dithiothreitol, tropolone and glutathione inhibitors using catechol and 4-methylcatechol as substrates. As seen from Table 3, the sensitivity of PPO to inhibitors was different from cultivar to cultivar. Calculated I_{50} values are summarized in Table 3. As can be seen from the table, the most effective inhibitor for Cultivar I was tropolone, D,L-dithiothreitol and tropolone

for Cultivar II and tropolone for Cultivar III. Tropolone seems to be a very effective inhibitor for aubergine PPO. Glutathione inhibits very little and this is in agreement with the result reported by Anosike & Ojimekwe (1982). Tropolone, in our study and in the study by Perez-Gilabert & Garcia-Carmona (2000), was the most effective inhibitor of eggplant PPO. However, Almeida & Nogueira (1995) demonstrated that the PPO of eggplant was most resistant to inhibition and indicated that the most adequate alternative method to substitute for the use of SO₂ in the control of PPO was a combination of ascorbic acid, citric acid and heat treatment. The enzymatic browning by a specific inhibitor may involve a single mechanism or be a result of an interplay of two or more mechanism of inhibitor action (Roudsari *et al.*, 1981). Tropolone, a competitor of Cu²⁺, is described as one of the most powerful specific PPO inhibitors (Khan & Andrawis, 1985). Inhibitors such as D,L-dithiothreitol, tropolone and glutathione at a concentration of 0.01 mM caused inhibition effects. These inhibitors will reduce quinones to phenols and will prevent enzymatic browning only as long as it is present in the reduced form.

Conclusions

It is found that aubergine PPO was a diphenol oxidase. The most effective substrate was catechol

Table 3 The effects of some inhibitors on the activity of polyphenol oxidase (PPO) obtained from different aubergine cultivars

PPO sources	Substrates	Inhibitors	$I_{50} \times 10^5$ M
Cultivar I	Catechol	Tropolone	3.67
Cultivar II	Catechol	Tropolone	1.74
Cultivar III	Catechol	Tropolone	0.94
Cultivar I	4-Methylcatechol	Tropolone	4.02
Cultivar II	4-Methylcatechol	Tropolone	4.48
Cultivar III	4-Methylcatechol	Tropolone	4.88
Cultivar I	Catechol	D,L-dithiothreitol	3.70
Cultivar II	Catechol	D,L-dithiothreitol	1.73
Cultivar III	Catechol	D,L-dithiothreitol	1.83
Cultivar I	4-Methylcatechol	D,L-dithiothreitol	9.57
Cultivar II	4-Methylcatechol	D,L-dithiothreitol	1.97
Cultivar III	4-Methylcatechol	D,L-dithiothreitol	3.27
Cultivar I	Catechol	Glutathione	8.69
Cultivar II	Catechol	Glutathione	2.08
Cultivar III	Catechol	Glutathione	2.44
Cultivar I	4-Methylcatechol	Glutathione	12.38
Cultivar II	4-Methylcatechol	Glutathione	8.37
Cultivar III	4-Methylcatechol	Glutathione	7.69

for cultivars I and III, and 4-methylcatechol for cultivar II. Although aubergine cultivar PPO showed a clear pH optimum with catechol as a substrate, it revealed a flatter pH profile with 4-methylcatechol as a substrate. Furthermore, it was found that the enzyme activity for all aubergine cultivars decreased with increasing temperature and inactivation time, and showed very little activity at about 60 °C. It was found that activation energies using catechol as a substrate were -78.5 , -89.2 and -80.5 cal mol⁻¹ for cultivars I, II and III, respectively, and -103.0 , -129.0 and -104.0 cal mol⁻¹ for cultivars I, II and III, respectively, with 4-methylcatechol. It was found that generally tropolone was the most effective inhibitor. The most suitable cultivar for future use in drying, because of the lowest PPO activity, is cultivar III.

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