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Food Chemistry 88 (2004) 69–77
Food Chemistry
www.elsevier.com/locate/foodchem

Determination of kinetic properties of polyphenol oxidase from *Thymus* (*Thymus longicaulis* subsp. *chaubardii* var. *chaubardii*)

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Received 25 April 2003; received in revised form 20 December 2003; accepted 20 December 2003

Abstract

A partial characterization of polyphenol oxidase (PPO) activity of *Thymus longicaulis* subsp. *chaubardii* var. *chaubardii* is described. Polyphenol oxidase of *Thymus* was isolated by $(NH_4)_2SO_4$ precipitation and dialysis. The effects of substrate specificity, pH, temperature, heat-inactivation and glutathione inhibitor on polyphenol oxidase activity obtained from *T. longicaulis* subsp. *chaubardii* var. *chaubardii* were investigated. Polyphenol oxidase showed activity toward catechol, 4-methylcatechol and pyrogallol. Pyrogallol was the most suitable substrate, due to the lowest K_M (5.5 mM) and the biggest V_{max}/K_M (1260/min) values. It was found that the optimum pH values did not change with temperature, and were 6.5 for catechol and pyrogallol and 5.5 for 4-methycatechol at all temperatures. Optimum temperatures were 25 °C for catechol and 4-methylcatechol, and 35 °C for pyrogallol. Again, it was found that optimum temperature did not change with pH. Activation energy values were calculated from the Arrhenius equation and found to be in the range −1.72 and −7.48 kcal/mol for catechol, −3.56 and −9.17 kcal/mol for 4-methylcatechol, and −1.60 and −3.98 kcal/mol for pyrogallol as substrates, respectively. From heat-inactivation studies, the required times for 50% inactivation, using catechol, 4-methylcatechol and pyrogallol substrates, were 68.9, 66.4 and 96.3 min at 45 °C, 19.9, 17.9 and 34.3 min at 65 °C, and 4.1, 2.1 and 11.9 min at 85 °C, respectively. I_{50} and K_i values for glutathione inhibitor, using catechol, 4-methylcatechol and pyrogallol substrates, were calculated, and it was found that the type of inhibition was competitive.

Keywords: *Thymus longicaulis* subsp. *chaubardii* var. *chaubardii*; Polyphenol oxidase; Inhibitor; Activation energy; Inhibition; pH; Temperature

1. Introduction

Polyphenol oxidase (PPO) (EC. 1.14.18.1) is a copper-containing monooxygenase, widely distributed in nature, which is responsible for melanization in animals and browning in plants. The enzyme catalyzse two distinct reactions involving molecular oxygen: (a) the o-hydroxylation of monophenol (cresolase activity) and (b) the oxidation of *o*-diphenol to *o*-quinones (catecholase activity). Enzymatic browning, catalyzed by polyphenol oxidase (PPO), occurs when plant tissues are damaged and is an economic problem for processors and consumers. The main step in enzymatic browning is the oxidation of phenolic compounds to corresponding quinones by PPO in the presence of oxygen. The quinones then condense to form darkened pigments (Matheis, 1983).

An important food product in the east Anatolian part of Turkey is herb cheese. A number of herbs, such as *Thymus* sp., *Allium* sp. and *Ferule* sp. are used in making herb cheese. However, the most widely used one is *Thymus* sp. Thyme is an aromatic plant containing 1.0–2.5% essential oil, with thymol and carvacrol as main components (Wang et al., 1999). The extracts of thyme were reported to show antioxidant activity (Economou,

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doi:10.1016/j.foodchem.2003.12.025

Oreopoulou, & Thomopoulos, 1991), estrogen and progestin bioactivity (Zava, Dollbaum, & Blen, 1998), antimutagenic activity (Vukovic-Gacic & Simic, 1993), antifungal activity (Wilson, Solar, El-Ghaouth, & Wisniewshi, 1997) and antimicrobial activity (Farag, Daw, Hewedi, & El-Baroty, 1989). Members of this genus are called "kekik" in Turkish and are used as herbal tea and condiments (Vardar-Ünlü et al., 2002). The genus *Thymus* has importance through having essential oil. All of these properties make thyme very important as a medicine of the future. Herbs are placed into vats to achieve the desired flavor for the cheese. The herbs are collected from plateau in the spring season. Producers can use either single or mixed herbs to prepare pickle. After washing well, they are cut into slices and placed in plastic containers. Brine (16%) is poured into the container. For a period of 15 or 20 days, the pickled herbs are stored in a cool place. Producers mostly use whey brine instead of tap water brine. After that, the pickled herbs are ready to be added to the cheese. Approximately 2% of herbs, depending on the producer, are used for the vat-cheese milk (Akyüz, Coskun, Andic, & Altun, 1996). The pickled herbs are also sold in markets, so they can be found throughout the year.

Polyphenol oxidase has been investigated in plants, such as apples (Murata, Kurokami, & Homma, 1992; Murata, Kurokami, Homma, & Matsuhashi, 1993; Murata, Tsurutani, Tomita, Homma, & Kaneko, 1995; Oktay, Küfrevioglu, Kocacaliskan, & Sakiroglu, 1995), bananas (Galeazzi, Sgarbieri, & Constantinides, 1981; Kahn & Andrawis, 1985; Yang, Fujita, Ashrafuzzaman, Nakamura, & Hayashi, 2000), peaches (Flurkey & Jen, 1980), grapes (Lamikanra, Sharon, & Mitwe, 1992; Wissemann & Lee, 1981), kiwis (Park & Luh, 1985), pears (Rivas & Whitaker, 1973; Wisseman & Montgomery, 1985), raspberries (González, DeAncos, & Cano, 1999), strawberries (Wesche-Ebeling & Montgomery, 1990), plums (Siddiq, Sinha, & Cash, 1992), herbs (Arslan, Temur, & Tozlu, 1997), spinach (Golbeck & Cammarata, 1981), broad beans (Flurkey, 1989; Hutcheson & Buchanan, 1980), field beans (Paul & Gowda, 2000), wild potatoes (Kowalski, Eannetta, Hirzei, & Steffens, 1992), Jerusalem artichokes (Zawistowski, Biliaderis, & Murray, 1988a, 1988b), cabbages (Fujita, Saari, Maegawa, Tetsuka, Hayashi, & Tono, 1995) and tea leaves (Takeo & Baker, 1972). However, we have not encountered any report of PPO of *Thymus*, even through it is necessary to characterize the thyme-PPO for controlling enzymatic browning of plant products. Therefore, the characterization of the enzyme could help to develop more effective methods for controlling browning of plant cheese and products. In this study, we investigate the effects of substrate specificity, heat-inactivation, temperature, pH and inhibitor on polyphenol oxidase activity obtained from *Thymus longicaulis* subsp. *chaubardii* var. *chaubardii*.

2. Materials and methods

2.1. Plant material

Thymus longicaulis subsp. *chaubardii* var. *chaubardii* has been used as research material in this study. *T. longicaulis* subsp. *chaubardii* var. *chaubardii* was collected in spring from a field near Balıkesir in Turkey and stored at 4 °C until used in the study. 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|g UV–Vis Spectrophotometer (Varian, Australia).

2.2. Enzyme extraction and purification

For preparing the crude extract, 10 g of the plant was homogenized in 100 ml of 0.1 M phosphate buffer (pH 6.5) containing 5% polyethylene glycol and 10 mM ascorbic acid by using a Waring blender for 2 min. 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% $(NH_4)_2SO_4$ saturation with solid $(NH_4)_2SO_4$. The precipitated PPO was separated by centrifugation at 20,000*g* for 30 min. The precipitate was dissolved in a small amount of 5 mM phosphate buffer (pH 6.5) and dialyzed at 4 °C in the same buffer for 24 h with three changes of the buffer during dialysis. The dialyzed sample was used as the PPO enzyme source in the following experiments (Wesche-Ebeling & Montgomery, 1990).

2.3. Determination of PPO activity

PPO activity was determined by measuring the increase in absorbance at 420 nm for catechol and 4-methylcatechol and 320 nm for pyrogallol with a Cary |1E|g UV–Vis Spectrophotometer (Varian, Australia). The sample cuvette contained 0.1 ml of the enzyme, 2.3 ml of 0.1 M buffer solution and 0.6 ml of 0.1 M substrate solution. The blank sample contained only 0.6 ml of 0.1 M substrate and 2.4 ml of 0.1 M buffer solution. The 0.1 M concentration was chosen to avoid the influence of enzymatic extract ionic strength on PPO activity, described by Angleton and Flurkey (1984). The reaction was carried out in a 1-cm light path quartz cuvette. In each measurement, the volume of solution in a quartz cuvette was kept constant at 3 ml. The temperature was kept constant at 25 °C using a Beckmann Peltier temperature controller attached to the cell-holder of the spectrophotometer. The reaction was carried out at various temperatures and pH values with the substrates mentioned above. PPO activity was calculated from the linear portion of the curve (Wong, Luh, & Whitaker, 1971). One unit of PPO activity was defined as the

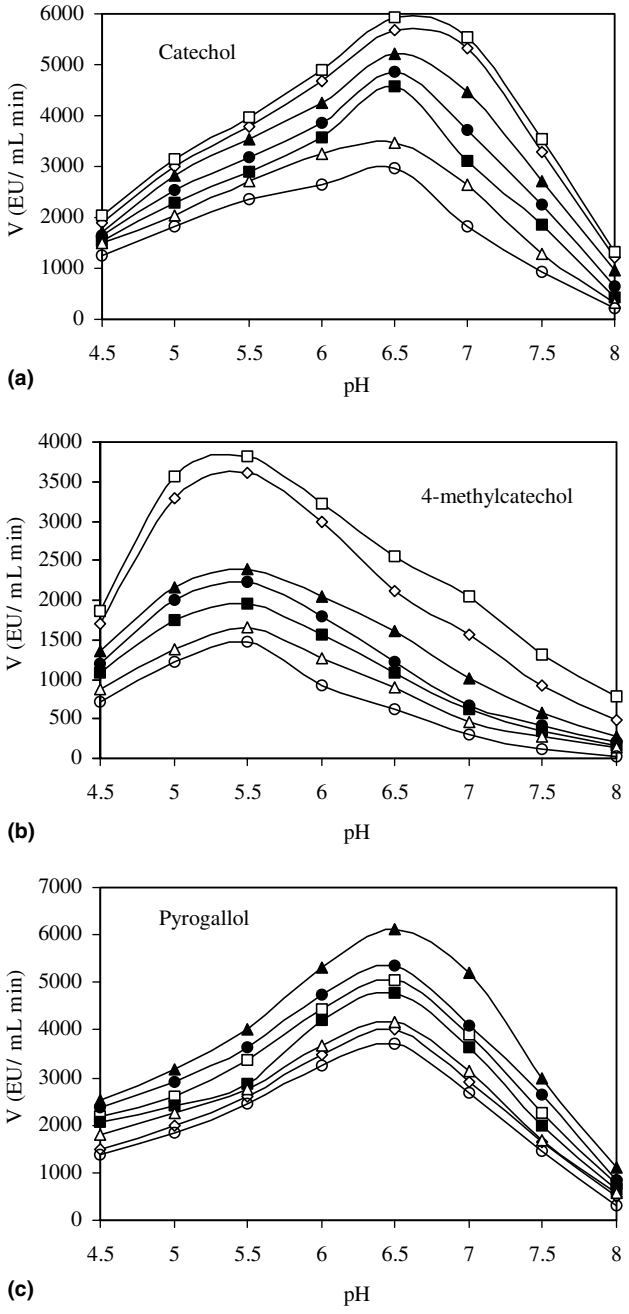

Fig. 1. The changing of enzyme activity with different substrates as a function of pH. ◊, 15 °C; □, 25 °C; ▲, 35 °C; ●, 45 °C; ■, 55 °C; △, 65 °C and ○, 75 °C.

amount of enzyme that caused an increase in absorbance of 0.001/min at 25 °C. PPO activity was repeated twice.

2.4. Enzyme kinetics

For determination of Michaelis constant (K_M) and maximum velocity (V_{max}) values of the enzyme, PPO activities were measured with the three substrates at various concentrations. K_M and V_{max} values of PPO, for each substrate, were calculated from a plot of $1/V$ vs $1/[S]$ by the method of Lineweaver and Burk.

2.5. Effect of pH

Optimum pH for *Thymus*-PPO activity was determined in the pH range 4.5–8.0 by using 0.1 M acetate (pH 4.5–6.0) and 0.1 M phosphate (pH 6.0–8.0) buffer adjusted with 0.1 M NaOH or 0.1 M HCl at different temperatures in the range 15–75 °C (Fig. 1). The optimum pH value of PPO was obtained by using three

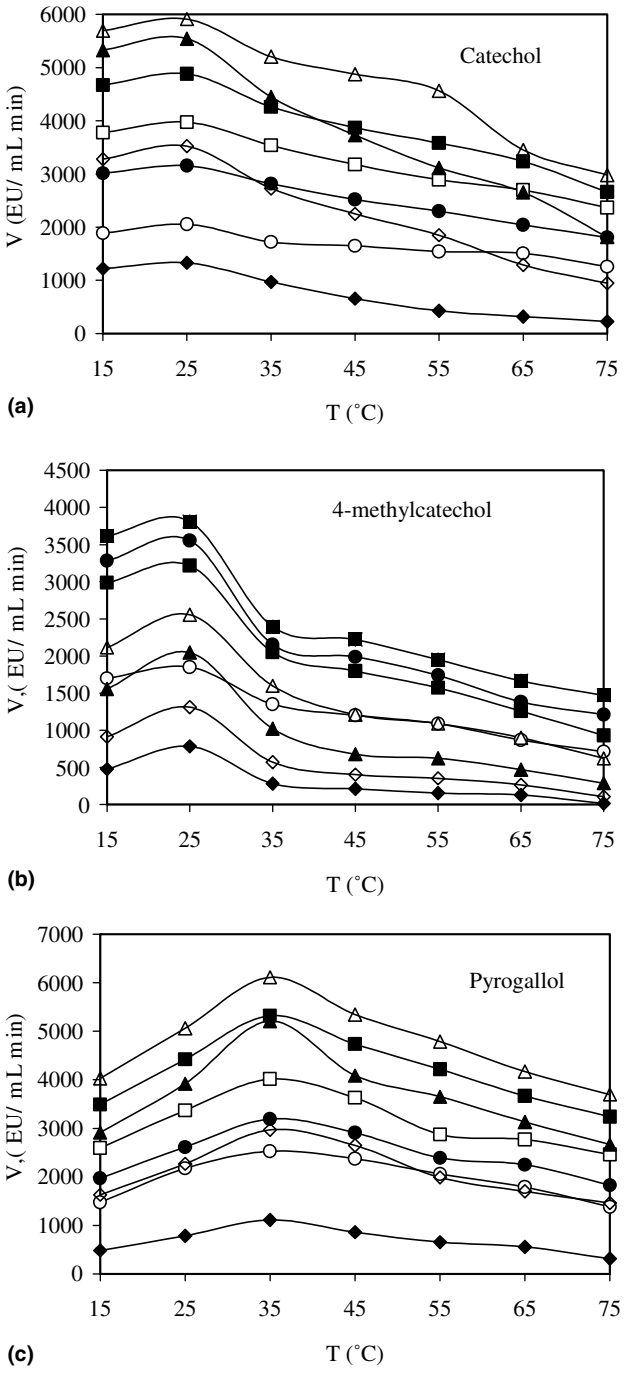

Fig. 2. The changing of enzyme activity with different substrates as a function of temperature. ○, 4.5; ●, 5.0; □, 5.5; ■, 6.0; △, 6.5; ▲, 7.0; ◊, 7.5; ◆, 8.0.

different substrates (catechol, 4-methylcatechol and pyrogallol). The reaction mixture contained 0.6 ml of 0.1 M substrate, 2.3 ml of 0.1 M buffer solution and 0.1 ml of enzyme solution. As mentioned, each assay mixture was repeated twice using the same stock of enzyme extract.

2.6. Effect of temperature

For determining the optimum temperature values of the enzyme, PPO activity was measured at different temperatures, in the range 15–75 °C, and different pH values, in the range 4.5–8.0, using three different substrates as indicated above (Fig. 2). The effect of temperature on the activity of PPO was tested by heating the standard reaction solutions (buffer and substrate) to the appropriate temperatures before introduction of the enzyme. The desired temperatures were provided by using a Beckmann Peltier temperature controller attached to the cell-holder of the spectrophotometer. Once temperature equilibrium was reached, enzyme was added and the reaction was followed spectrophotometrically at constant temperature at given time intervals. The reaction mixture contained 0.6 ml of 0.1 M substrate, 2.3 ml of 0.1 M buffer solution and 0.1 ml of enzyme solution. As mentioned, each assay mixture was repeated twice using the same stock of enzyme extract.

2.7. Activation energy

Activation energy studies of the PPO enzyme action were run at 25, 35, 45, 55, 65 and 75 °C for catechol and 4-methylcatechol, at 35, 45, 55, 65 and 75 °C, for pyrogallol, at different pH values, in the range 4.5–8.0, using three different substrates (catechol, 4-methycatechol and pyrogallol). The activation energies were calculated from experimental results for enzyme reactions by using the Arrhenius equation, which is written as

$$\ln V = \ln Z - \frac{E_a}{RT}, \quad (1)$$

where Z is the frequency factor, E_a is the activation energy and T is the temperature. The $\ln V$ values were plotted versus the reciprocal of absolute temperature, and the activation energy of a particular reaction was calculated from the slope. The parameter Z is obtained from intercept point at $1/T = 0$ (Dogan, Alkan, & Onganer, 2000; Dogan, Arslan, & Dogan, 2002).

2.8. Heat-inactivation

The thermal denaturation of PPO was studied at 45, 65 and 85 °C. For the study, 1 ml of enzyme solution in a test tube was incubated at the required temperature for fixed time intervals. At the end of the required time interval, the test tube was cooled in an ice bath. The activity of enzyme was then determined at 25 °C (Arslan et al., 1998; Dogan et al., 2002).

2.9. Effect of inhibitors

Inhibition of PPO by glutathione, as a specific inhibitor of enzyme, was measured at 420 nm for catechol and 4-methylcatechol, and 320 nm for pyrogallol at four different concentrations of inhibitor at pH 6.5 and 25 °C. Percent activity graphs were drawn from these results to find I_{50} values at four constant inhibitor concentrations, which shows about 50% inhibition effect. Later, using five different concentrations of the substrate solution, PPO activities were measured at these four constant inhibitor concentrations with the glutathion inhibitor. $1/V$ and $1/[S]$ values, obtained from these activity measurements, were used for drawing Lineweaver–Burk graphs. Finally, K_i constant values were found from the graphs.

3. Results and discussion

3.1. Enzyme kinetics and substrate specificity

There are a number of compounds such as dopamine (Nagai & Suzuki, 2001; Sakiroglu, Küfrevioglu, Kocacaliskan, Oktay, & Onganer, 1996), catechol (Arogba, Ajiboye, Ugboko, Essienette, & Afolabi, 1998; Dogan et al., 2002; Lee, Smith, & Pennesi, 1983; Nagai & Suzuki, 2001; Oktay et al., 1995; Sakiroglu et al., 1996; Wesche-Ebeling & Montgomery, 1990), D-catechin (Lee et al., 1983; Wesche-Ebeling & Montgomery, 1990), chlorogenic acid (Lee et al., 1983; Wesche-Ebeling & Montgomery, 1990), DL-dopa (Nagai & Suzuki, 2001; Oktay et al., 1995; Sakiroglu et al., 1996; Wesche-Ebeling & Montgomery, 1990), pyrogallol (Nagai & Suzuki, 2001; Lee et al., 1983; Oktay et al., 1995; Sakiroglu et al., 1996; Wesche-Ebeling & Montgomery, 1990), caffeic acid (Lee et al., 1983; Wesche-Ebeling & Montgomery, 1990), *p*-cresol (Lee

Table 1
K_M, V_{max}, V_{max}/K_M, optimum pH and temperature values for PPO

Substrates	V_{max} (EU/ml/min)	K_M (mM)	V_{max}/K_M (min^{-1})	pH	Temperature (°C)
Catechol	4830	18.0	269	6.5	25
4-Methylcatechol	4070	9.8	415	5.5	25
Pyrogallol	6990	5.5	1260	6.5	35

et al., 1983; Sakiroglu et al., 1996; Wesche-Ebeling & Montgomery, 1990), tyrosine (Lee et al., 1983; Sakiroglu et al., 1996), 4-methylcatechol (Dogan et al., 2002; Oktay et al., 1995; Sakiroglu et al., 1996; Wesche-Ebeling & Montgomery, 1990) used as substrates for polyphenol oxidase in the literature. In this study, we generally selected the most used three substrates (catechol, 4-methylcatechol and pyrogallol). V_{max}, K_M and V_{max}/K_M values of the PPO investigated with various substrates are shown in Table 1. In Table 1 the V_{max}/K_M ratio is called the "catalytic power" (Rocha, Pilar Cano, Galeazzi, & Morais, 1998) and is a good parameter for finding the most effective substrate (Baritaux, Amiot, Richard, & Nicolas, 1991). The maximum absorption of oxidation products was at 420 nm for catechol and 4-methylcatechol, and 320 nm for the pyrogallol. Michaelis constants (K_M) and maximum reaction velocities (V_{max}) were determined using these substrates at various concentrations (pH 6.5 and 25 °C). The Lineweaver–Burk plot of this enzyme showed K_M values of 18.0 mM for catechol, 9.8 mM for 4-methylcatechol and 5.5 mM for pyrogallol (Table 1). As seen from V_{max}/K_M and K_M values in Table 1, the enzyme has a relatively high affinity for pyrogallol, which was the best substrate of those tested, followed by 4-methylcatechol and catechol. The lowest activity for *Thymus* PPO was obtained with catechol as substrate. K_M values obtained for catechol in the literature were 12.52 mM for tea leaf (Halder, Tamuli, & Bhaduri, 1998), 3.13 mM for spinach (Golbeck & Cammarata, 1981), 10.5 mM for field bean seed (Paul & Gowda, 2000) and 4.0 mM for Jerusalem artichoke (Zawistowski et al., 1988b). *Thymus* PPO showed a lower affinity for 4-methylcatechol. Again, K_M values obtained for 4-methylcatechol in the literature were 10 mM for strawberry (Wesche-Ebeling & Montgomery, 1990), 3.1 mM for Amasya apple (Oktay et al., 1995) and 94.3 mM for field bean (Gowda & Paul, 2002). It has been reported that K_M values for pyrogallol in the literature are 27 mM for Amasya apple (Oktay et al., 1995), 0.2 mM for peach (Flurkey & Jen, 1980), 15.7 mM spinach (Golbeck & Cammarata, 1981) and 17.8 mM for tea leaf (Halder et al., 1998).

A similar result to our own was found for Chinese cabbage (Nagai & Suzuki, 2001). Catechol, pyrogallol and dopamine were tested as substrates for Chinese cabbage and K_M values were found to be 682.2, 15.4 and 62.0 mM, respectively. The most appropriate substrate for Chinese cabbage was pyrogallol. It has been found that the K_M for PPO varies with the source of the enzyme (Arslan et al., 1997; Park & Luh, 1985).

3.2. Effect of pH

The activity of PPO was measured at different pHs and temperatures, using catechol, 4-methylcatechol and pyrogallol as substrates. As seen in Fig. 1, the optimum pHs of the enzyme were found to be 6.5 for catechol and pyrogallol, and 5.5 for 4-methylcatechol. In our study, we found similar results for catechol and pyrogallol, but the optimum pH for 4-methylcatechol was different. In general, most plants show maximum PPO activity at or near neutral pH values (Betrosian, Steinburg, & Nelson, 1960; Cash, Sistrunk, & Stutte, 1976; Chan & Yang, 1971; Siddiq et al., 1992; Wong et al., 1971). Different optimum pHs for PPO obtained from various sources are reported in the literature. For example, it is reported that optimum pH values are 5.5 for strawberry (Wesche-Ebeling & Montgomery, 1990), 6.0 for DeChaunac grape (Lee et al., 1983), 7.0 for Amasya apple (Oktay et al., 1995), aubergine (Dogan et al., 2002), Yali pear (Zhou & Feng, 1991), raspberry (González et al., 1999), *Anethum graveolens* L. (Arslan & Tozlu, 1997), 7.2 for guava (Augustin, Ghazali, & Hashim, 1985), 7.5 for *Allium* sp. (Arslan et al., 1997) and 8.5 for Dog rose (Sakiroglu et al., 1996), using catechol as a substrate, 4.5 for strawberry (Wesche-Ebeling & Montgomery, 1990), 6.0 for aubergine (Dogan et al., 2002), 8.5 for Dog rose (Sakiroglu et al., 1996) and 9.0 for Amasya apple (Oktay et al., 1995), using 4-methylcatechol as a substrate, and 7.0 for Dog rose (Sakiroglu et al., 1996) and 8.6 for Amasya apple (Oktay et al., 1995) using pyrogallol as a substrate. Alyward and Haisman (1969) reported that the optimum pH for maximum PPO activity in plants varied from about 4 to 7, depending on the extracting methods, the purity of enzyme, the type of buffer used, the substrates used for assay and the localization of the enzyme in the plant cell.

3.3. Effect of temperature

The effects of temperatures between 15 and 75 °C at different pHs were assayed for each substrate and the results are shown in Fig. 2. As seen in Fig. 2, optimum temperatures are substrate-dependent. It is found that the optimum temperatures are 25 °C for catechol and 4-methylcatechol, and 35 °C for pyrogallol. After optimum temperature, PPO activity decreased with increasing temperature and showed very little activity at 75 °C. It is reported that optimum temperature values are 15 °C for Amasya apple (Oktay et al., 1995), 20 °C for DeChaunac grape (Lee et al., 1983), 25 °C for Dog rose (Sakiroglu et al., 1996), 30 °C for aubergine (Dogan et al., 2002) and 40 °C for Chinese cabbage (Nagai & Suzuki, 2001), using catechol as a substrate, 20 °C for Dog rose (Sakiroglu et al., 1996), 30 °C for aubergine (Dogan et al., 2002) and 56 °C for Amasya apple (Oktay et al., 1995), using 4-methylcatechol as a substrate, and 15 °C for Dog rose (Sakiroglu et al., 1996) and 70 °C for Amasya apple (Oktay et al., 1995), using pyrogallol as a substrate.

Table 2
E_a values (kcal/mol) calculated with different substrates for PPO

pH	Catechol	4-Methylcatechol	Pyrogallol
4.5	−1.72	−3.66	−1.98
5.0	−2.26	−4.06	−1.47
5.5	−2.06	−3.56	−1.69
6.0	−2.32	−4.60	−1.60
6.5	−2.27	−5.27	−1.63
7.0	−4.29	−5.06	−2.05
7.5	−5.29	−6.16	−2.39
8.0	−7.48	−9.17	−3.98

3.4. Activation energy

Activation energies were calculated in the temperature range 25–75 °C for catechol and 4-methylcatechol, and 35–75 °C for pyrogallol. For each substrate, Arrhenius plots were constructed by using temperature-activity values at different pHs, obtained from the optimum temperature studies. Activation energies (E_a) were calculated from slopes and given in Table 2. As seen in Table 2, activation energy values have changed in the range −1.72 and −7.48 kcal/mol for catechol, −3.56 and −9.17 kcal/mol for 4-methylcatechol and −1.60 and −3.98 kcal/mol for pyrogallol as substrates. Activation energies in the temperature range studied are negative. This may be explained by inactivation of the PPO by high temperature. Furthermore, it is clear that the activation energy does not change with change of pH. Activation energy values are generally not published; therefore, comparisons are not possible. There are only few data related to activation energy. Activation energy values are 7 kcal/mol for kiwifruit PPO, with catechin substrate (Park & Luh, 1985) and 21.4 kcal/mol for Mango kernel, with catechol substrate (Arogba et al., 1998).

3.5. Effect of inhibitor

Enzymatic browning of plants 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 plants during drying is difficult because oxygen is ubiquitous (Roudsari, Signoset, & Crovzet, 1981). There are a number of inhibitors, such as sodium metabisulphite (Augustin et al., 1985; Lee et al., 1983; Sakiroglu et al., 1996), ascorbic acid (Augustin et al., 1985; Lee et al., 1983; Sakiroglu et al., 1996; Yang et al., 2001), D,L-dithiothreitol (Dogan et al., 2002; Lee et al., 1983), sodium cyanide (Lee et al., 1983; Park & Luh, 1985), glutathione (Jiang, Fu, Zauberman, & Fuchs, 1999; Lee et al., 1983; Dogan et al., 2002; Park & Luh, 1985), tropolone (Kahn and Andrawis, 1985; Dogan et al., 2002; Perez-Gilabert & Garcia-Carmona, 2000), thiourea (Lee et al., 1983; Sakiroglu et al., 1996; Zhou & Feng, 1991), sodium diethyldithiocarbamate (Lee et al., 1983; Park & Luh, 1985; Yang et al., 2000; Zhou & Feng, 1991), myricetin (Jimenez & Garcia-Carmona, 1999), citric acid and acetic acid (Yang et al., 2000), L-cysteine, sodium azide, tannic acid, benzoic acid and β-mercaptoethanol (Sakiroglu et al., 1996) used by researchers to prevent enzymatic browning. In this study, glutathione was selected as an inhibitor to prevent the enzymatic browning of *Thymus* PPO. Lee et al. (1983) studied the effects of inhibitors, such as D,L-dithiothreitol and glutathione, on PPO activity obtained from DeChaunac grapes and found that these inhibitors were the most effective in

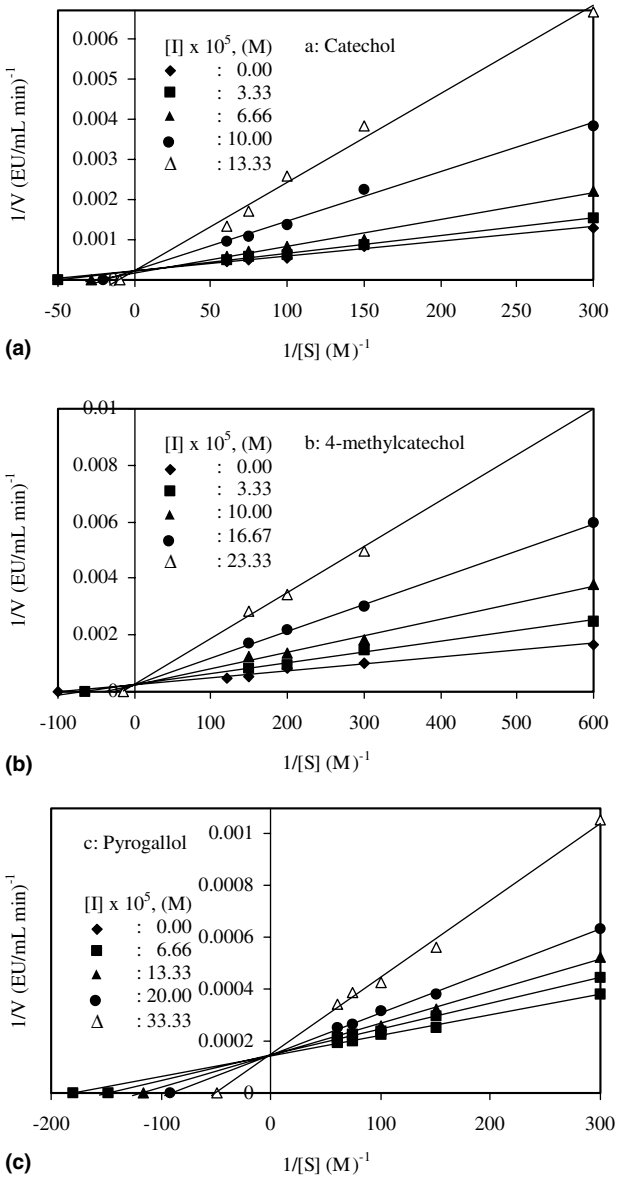

Fig. 3. The effect of glutathione inhibitor on PPO activity.

Table 3
K_i and I_{50} values for glutathione inhibitor

Substrates	$[I] \times 10^5$ (M)	I_{50} (M)	% Inhibitor	V_{max} (EU/ml/min)	$K_i \times 10^5$ (M)	K_M (mM)
	0.00		0.0	4830	–	18.0
	3.33		8.3	4504	2.72	19.5
Catechol	6.66	9.53×10^{-5}	25.3	4524	4.4	25.7
	10.0		43.3	4347	12.7	53.0
	13.3		66.6	4545	20.6	100
	0.00		0.0	4070	–	9.8
	3.33		42.9	4030	1.1	15.3
4-Methylcatechol	10.00	8.68×10^{-5}	62.8	4050	7.1	23.5
	16.7		71.9	4000	29.0	37.9
	23.33		83.3	4070	4628	66.3
	0.00		0.0	6990	–	5.5
	6.67		10.3	6800	6.3	6.8
Pyrogallol	13.33	43.5×10^{-5}	17.4	6900	22.6	8.6
	20.00		22.8	6760	76.5	10.9
	33.33		43.2	6850	4256	20.4

decreasing the activity 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; Jiang et al. (1999) investigated the browning control of litchi fruit by various inhibitors and found that glutathione was the best inhibitor for decreasing the enzymatic browning. Lineweaver–Burk plots of $1/V$ versus $1/[S]$, at four inhibitor concentrations using three different substrates, determined the type of inhibition (Fig. 3(a)–(c)). The inhibition constant K_i was calculated from these plots. Percent activity graphs were drawn from these results to find I_{50} values, which show about 50% inhibition effect, at four inhibitor concentrations, using three different substrates. Table 3 shows the inhibition results with different substrates. Percent inhibition and K_i values obtained for glutathione inhibitor with different substrates (Table 3) indicated that glutathione shows competitive inhibition. Sakiroglu et al. (1996) found a similar result. They studied K_i constants and percent inhibition values of the Dog rose PPO with different inhibitors, and found that glutathione showed competitive inhibition. Furthermore, Jiang et al. (1999) found that the most effective inhibitor, within inhibitors studied, was glutathione.

3.6. Heat-inactivation of PPO

The enzyme activity decreased with increasing temperature and inactivation time for three substrates. The required times for 50% inactivations, using catechol, 4-methylcatechol and pyrogallol as substrates, were 68.9, 66.4 and 96.3 min at 45 °C, 19.9, 17.9 and 34.3 min at 65 °C, and 4.1, 2.1 and 11.9 min at 85 °C, respectively. It has been noted that heat stability of the enzyme may be related to ripeness of the plant and molecular forms of the enzyme (Zhou & Feng, 1991).

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