Characterisation of polyphenol oxidase from *Melissa officinalis* L. subsp. *officinalis* (lemon balm)

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Abstract

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Polyphenol oxidase (PPO) from *Melissa officinalis* L. subsp. *officinalis* (lemon balm) was partially purified by ammonium sulphate precipitation and dialysis; and then it was characterised in detail in terms of pH and temperature optima, thermal stability, kinetic parameters, and inhibition properties. Based on experimental results, it was found out that (*i*) the optimum pH and temperature values of PPO were 6.5, 4.0, and 8.5 and 40, 50, and 60°C for catechol, 4-methylcatechol and pyrogallol substrates, respectively; (*ii*) the best substrate was pyrogallol due to the highest V_{max}/K_m value, followed by catechol and 4-methylcatechol; (*iii*) enzyme activity decreased due to heat denaturation of the enzyme with increasing temperature and inactivation time for all substrates; (*vi*) gallic acid and L-glutamic acid did not inhibit PPO; and (*v*) the most effective inhibitor was glutathione. Furthermore, the phenolic and protein contents of lemon balm extract were also determined according to the Folin-Ciocalteu and Bradford methods, respectively.

Keywords: lemon balm; protein; enzyme kinetics; inhibition

Lemon balm (*Melissa officinalis* subsp. *officinalis*), a member of the *Lamiaceae* family (formerly *Labiatae*), is one of the important medicinal plant species. It has been traditionally used for different medicinal purposes as tonic, antispasmodic, carminative, diaphoretic, surgical dressing for wounds, sedative-hypnotic strengthening of the memory, and relief of stress induced headache, but in modern pharmacology its value is in the management of mild to moderate Alzheimer's, against migraine and rheumatism, antitumel and antioxidant activities (Moradkhani *et al*. 2010). In addition lemon balm essential oils have been shown to possess antiviral and antimicrobial activity (Allahverdiyev *et al*. 2004; Dikbaş *et al*. 2010). Furthermore, the lemon-scented leaves add flavour to jellies, liqueurs, fruit salads, and cold drinks (Bahtiyarca & Cosge 2006).

Another important property of lemon balm is that it contains an enzyme called polyphenol oxidase (PPO), widely distributed in plants. The presence of PPO in plant tissues is of concern to processors and researchers. PPO catalyses the formation of highly active quinones that react with amino or sulfhydryl groups in proteins or enzymes. These reactions lead to changes in physical, chemical, or nutritional characteristics of proteins and, in many cases, to inactivation of enzymes including PPO (Mayer & Harel 1979). Quinones also lead to polymerisation and condensation reactions between proteins and polyphenols, because they are very reactive compounds that strongly interact with other molecules, leading to a large variety of dark-coloured compounds (Dogan *et al*. 2009). Because of the importance of this reaction in the food industry, PPO has been intensively studied in several plant tissues such as aubergine (Dogan *et al*. 2002), *Origanum* (Dogan *et al*. 2005a), apricot (Arslan *et al*. 1998), *Thymus* species (Dogan *et al*. 2003a,b), *Salvia* types (Gundogmaz *et*

al., 2003), *Ocimum basillicum* L. (Dogan *et al*. 2005b), Thymbra (Dogan *et al*. 2006), *Cynara scolymus* L. (Dogan *et al*. 2005c), DeChaunac grapes (Lee *et al*. 1983), *Allium* sp. (Arslan *et al*. 1997), Amasya apple (Oktay *et al*. 1995), *Ferula* sp. (Erat *et al*. 2006).

One of the most important physiological disorders of lemon balm is russet spotting, a postharvest disorder in which soluble phenolic compounds are accumulated and oxidised by PPO. Therefore, prevention of enzymatic browning requires a detailed study of the lemon balm PPO. We did not find any study related to lemon balm PPO in literature. The aim of this paper was (*i*) to partially purify and kinetically characterise the lemon balm PPO and (*ii*) to determine its protein and phenolic contents. Therefore, the characterisation of PPO from lemon balm was studied in terms of substrate specificity, optimum pH, and temperature, thermal inactivation and inhibition effects of various inhibitors in order to help to predict the behaviour of lemon balm PPO.

MATERIAL AND METHODS

Material. Lemon balm was collected from the campus area of Necatibey Education Faculty of Balikesir University in Balikesir (Turkey) and stored at –80 °C until used as enzyme source. All chemicals used in this study were analytical grade and were used without further purification.

Extraction of PPO. Lemon balm samples were washed with tap water and bidistilled water several times before extraction procedure. Lemon balm (20 g) was ground in liquid N_2 for 10 min to rupture cell membranes. The frozen plant powder was added to the extraction solution (100 ml of 0.1M phosphate buffer containing 5% poly(ethylene glycol) at pH 6.5 and 10mM ascorbic acid and mixed with a Waring blender for 2 min at 4°C. 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% saturation with solid $(NH_4)_2SO_4$. The precipitated PPO was separated by centrifugation at 15 000 *g* for 60 min at 4°C. The precipitate was dissolved in a small amount of 0.05M phosphate buffer (pH 7.0) and dialysed at 4°C in the same buffer for 2 days with four changes of buffer during dialysis (Sigma, St. Louis, USA; avg. flat width 25 mm (1.0 in)). The dialysed extract was used as the PPO enzyme source in the following experiments (Dogan & Dogan 2004).

Assay of enzyme activity. Enzyme activity was determined by measuring the increase in absorbance at 420 nm for catechol and 4-methylcatechol substrates and at 320 nm for pyrogallol substrate with a Cary |1E| g UV-visible spectrophotometer (Varian, Mulgrave, Australia). Enzyme activity was calculated from the linear portion of the curve. One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001 per min for 1 ml of the enzyme at 25°C. The reaction mixture contained 2.9 ml of substrates in various concentrations prepared in the homogenisation buffer and 0.1 ml of the enzyme. Reference cuvette contained all the components except the substrate, with a final volume of 3 ml. All measurements were done in duplicate at least, and error bars are shown as mean ± SD in related figures (Dogan *et al*. 2007).

pH pptima. The pH optima of PPO activity were determined for catechol, 4-methylcatechol and pyrogallol substrates at pH values of 4.0–9.0 using 0.1M acetate (pH = 4–6) and 0.1M phosphate (pH = 6–9) buffer adjusted with 0.1M NaOH and HNO_3.

Temperature optima. The temperature optima of PPO activity were determined in the temperature ranges of 10–70°C. The effect of temperature on PPO activity was assayed by heating the standard reaction solutions (substrate and buffer solutions) to the appropriate temperature with circulating water bath before the introduction of PPO. The temperature was maintained by a Beckmann Peltier temperature controller attached to the cell-holder of the spectrophotometer. Once temperature equilibrium was reached, PPO was added and the reaction was followed spectrophotometrically at a constant temperature at given time intervals. The reaction mixture contained 0.6 ml of 0.1M substrate, 2.3 ml of 0.1M buffer solution and 0.1 ml of lemon balm PPO solution (Dogan *et al*. 2002).

Enzyme kinetics and substrate specificity. The catalytic activity of PPO enzyme was measured using L-tyrosine, catechol, 4-methylcatechol and pyrogallol as substrates, and the rate of PPO reaction was determined at various substrate concentrations in the standard reaction mixture in terms of the increase in absorbance at the wavelength of maximum absorption for the corresponding chromophore. Graphical evaluation of the results was obtained by inserting the data into the Michaelis-Menten equation. To obtain the equation

of a straight line and more reliable determination of V_{max} and K_m, the Michaelis-Menten equation was transformed into the double reciprocal form, called the Lineweaver-Burk plot. Substrate specificity (V_{max}/K_m) was calculated using the data from the Lineweaver-Burk plot (Dogan *et al*. 2009).

Heat inactivation. In order to determine the heat inactivation of PPO, the enzyme solution in 100mM phosphate buffer, pH 6.5, within eppendorf tubes, was incubated in an incubator in the range of 35–75°C for 1 h at various heating times. After the mixture was cooled in an ice bath and brought to room temperature, residual PPO activity was determined spectrophotometrically. The percentage residual PPO activity was calculated by comparison with the unheated enzyme activity (Dogan & Salman 2007).

Effects of inhibitors. Various inhibitors such as glutathione, ascorbic acid, benzoic acid, sodium azide, gallic acid, and glutamic acid for inhibiting the PPO activity were tested using catechol, 4-methylcatechol and pyrogallol as substrates. Furthermore, to determine the inhibitor concentration that reduced the enzyme activity by 50% (IC_{50}), regression analysis graphs were drawn using percent inhibition values. IC_{50} values were determined from the graphs (Dogan *et al*. 2007).

Protein determination. Protein contents of the enzyme extracts were determined according to Bradford method using bovine serum albumin as a standard (Bradford 1976).

Determination of total phenolic content. Total phenolics were determined using the Folin-Ciocalteu reagent. Samples (2 g) were homogenised in 80% aqueous ethanol at room temperature and centrifuged in the cold at 10 000 *g* for 15 min, and the supernatant was saved. The residue was re-extracted twice with 80% ethanol, and the supernatants were pooled, put into evaporating dishes, and evaporated to dryness at room temperature. The residue was dissolved in 5 ml of distilled water. One hundred microliter of this extract was diluted to 3 ml of the water, and then, 0.5 ml of Folin-Ciocalteu reagent was added. After 3 min, 2 ml of 20% sodium carbonate was added, and the contents were mixed thoroughly. The colour was developed and the absorbance was measured at 650 nm in a Carry |1E|g UV-visible spectrophotometer after 60 min using catechol as a standard. The result was expressed as milligrams of catechol per 100 g of fresh weight material (Singleton & Rossi 1965).

RESULTS AND DISCUSSION

Total phenolic content

Phenolic compounds are widely distributed in the plant kingdom and are considered to be secondary metabolites. Structurally they contain an aromatic ring bearing one or more hydroxyl groups, together with a number of other substituents. The polyphenolic composition of fruits and vegetables varies in accordance with species, cultivar, degree of ripening and environmental conditions of growth and storage. Browning, and particularly rapidly occurring types, are caused by enzymatic oxidation of phenolic compounds (Dogan *et al*. 2009). This relationship has been demonstrated in a number of plants, and therefore the total phenolic content has been used as a measure of browning potential in several crops such as apples and grapes (Dogan *et al*. 2005b).

We found that the level of total phenolic content in the lemon balm extracts was 280 mg per 100 g of fresh weight. As seen in Table 1, the total phenolic content of some vegetables such as turmeric (176 mg), broccoli (88 mg), tomatoes (68 mg),

Table 1. Total phenolic contents of some vegetables

Vegetables	Phenolic content (mg/100 g)	References
Melissa officinalis L. subsp. *officinalis*	280	In this study
Turmeric	176	Kaur and Kapoor (2002)
Broccoli	88	Kaur and Kapoor (2002)
Tomatoes	68	Kaur and Kapoor (2002)
Sweet potato	92	Kaur and Kapoor (2002)
Malta plum	199.4	Lee and Withaker (1995)
Green pepper	206.0	Lee and Withaker (1995)
Yellow pepper	191.2	Lee and Withaker (1995)
Red pepper	180.3	Lee and Withaker (1995)
Spinach	269.0	Lee and Withaker (1995)
White onion	216	Lee and Withaker (1995)
Bitter melon	257.2	Lee and Withaker (1995)
Cynara scolymus L.	425	Dogan *et al.* (2005c)
Beetroot	323	Kaur and Kapoor (2002)
Black carrots	350	Kaur and Kapoor (2002)
Lettuce	304	Dogan and Salman (2007)
Onion	349	Djeridane *et al.* (2006)
Mint	400	Djeridane *et al.* (2006)

sweet potato (92 mg), Malta plum (199.4 mg), green pepper (206.0 mg), yellow pepper (191.2 mg), red pepper (180.3 mg), spinach (269.0 mg), white onion (216 mg), bitter melon (257.2 mg) is lower than that obtained for lemon balm. On the other hand, the total phenolic content of vegetables such as *Cynara scolymus* L. (425 mg), beetroot (323 mg), black carrots (350 mg), lettuce (304 mg), onion (349 mg), mint (400 mg) is higher than that obtained for lemon balm. It can be said that lemon balm has a rich phenolic compound content.

The protein content of lemon balm was found to be 230 μg/ml. The protein content of banana, apple, and gum tree in the literature was found to be 1.5–2.1, 0.19, and 0.0526 mg/ml, respectively (Dogan *et al*. 2005c). Also, in our previous study, we found that the protein content in the leaves of *O. onites* and *O. vulgare* ssp. *hirtum* plants in vegetative and generative stages varied in the range of 5.89–4.34 and 7.96–55.59 mg/g, respectively (Dogan 2003).

Optimum pH

The pH value of the medium affects the enzyme activity significantly. The enzyme activity with increasing pH values reaches a maximum value and then drops to zero in the alkaline region. Optimum pH values for lemon balm PPO were determined in pH ranges of 4.5–9.0. As seen in Figure 1, it was found that optimum pH values for lemon balm PPO were 6.5, 4.0, and 8.5 for catechol, 4-methylcatechol and pyrogallol as substrates, respectively. The following optimum pH values for PPO enzymes from different sources have been reported: 6.5 for potato, 7.0 for banana, 7.2 for guava, 7.0 for marula, 5.5 and 7.0 for Anamur banana, 7.0 for *Ferula* sp., 7.5 for *Allium* sp., 7.3 for kiwi and 4.0 for Jerusalem artichoke using catechol as a substrate; 6.5 for artichoke heads, 4.5 for green olive, 5.0 for potato, 7.2 for guava, 5.0 for Chinese cabbage, 7.0 for dog rose, 8.0 for artichoke, 9.0 for sweet basil, and 7.5 for sago log using pyrogallol as a substrate; and 6.0 for artichoke heads, 4.5 for sago log, 4.0 sweet potato, 6.0 for *Ferula* sp., 6.0 for aubergine, and 4.5 for strawberry using 4-methylcatechol as a substrate (Park & Luh 1985; Arslan *et al*. 1997; Erat *et al*. 2006; Dogan & Salman 2007; Aydemir 2010). We found that (*i*) optimum pH value of lemon balm PPO for catechol substrate was lower than that in the literature, but higher than that of *C. scolymus* L., (*ii*) for 4-methylcatechol substrate it was lower than that in the literature, and (*iii*) for pyrogallol substrate it was similar to the values in the literature. Alyward and Haisman (1969) reported that the optimum pH for maximum PPO activity in plants varies depending on the extraction method, the substrates used for assay, and the localisation of the enzyme in the plant cell.

Optimum temperature

Figure 2 shows the changes in lemon balm PPO activity with temperature for three different substrates. Optimum temperatures were 40, 50, and 60°C for catechol, 4-methylcatechol and pyrogallol substrates, respectively. Similar results were found for *Thymus* types (Dogan *et al*. 2003a; Dogan & Dogan *et al*. 2004), *Salvia* types (Gundogmaz *et al*., 2003) and *C. scolymus* L. (Dogan *et al*. 2005c) PPOs using catechol as a substrate. Furthermore,

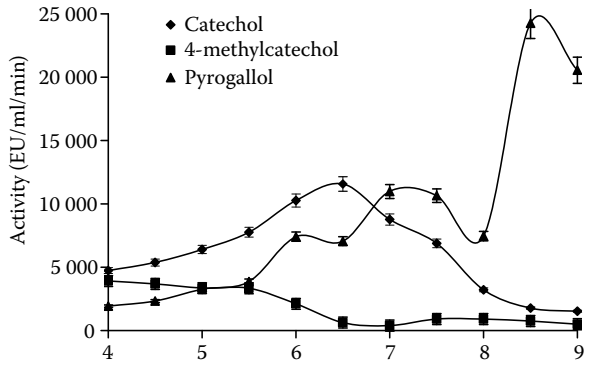

Figure 1. Changes in enzyme activities of lemon balm PPO with different substrates as a function of pH

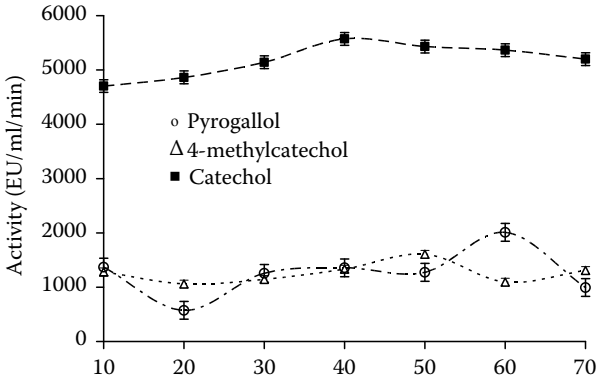

Figure 2. Effect of temperature on lemon balm PPO activity

it was determined that the optimum temperatures for coffee leaf and endosperm PPOs (Mazzafera & Robinson 2000) and *H. tuberosus* PPO (Ziyan & Pekyardimci 2003) using 4-methylcatechol and pyrogallol as substrates was 30°C; for dog rose PPO 20 and 15°C (Dogan *et al*. 2005c); medlar fruit PPO 55 and 35°C (Dogan *et al*. 2005c), respectively. These results show that the optimum temperature of lemon balm PPO varies depending on substrates.

Substrate specificity

To determine the substrate specificity of lemon balm PPO, mono-, di- and triphenolic substrates were tested. The enzyme showed activity to catechol, 4-methylcatechol and pyrogallol. No activity was detected toward L-tyrosine. Some plant polyphenol oxidases catalyse both the hydroxylation of monophenols and the oxidation of *o*-diphenols. However, many polyphenol oxidases are lacking monophenol activity (Benjamin & Montgomery 1973; Rivas & Whitaker 1973). Literature results have shown that tomato seeds and *Solanum tuberosum* (Benjamin & Montgomer 1973; Rivas & Whitaker 1973), *Sorghum* grains (Dicko *et al*. 2002), *Averrhoa carambola* L. (During *et al*. 2006), DeChaunac grapes (Lee *et al*. 1983), artichoke (Dogan *et al*. 2005c), and *O. basillicum* L. (Dogan *et al*. 2005b) PPOs showed diphenolase activity and that strawberry (Wesche-Ebeling & Montgomery 1990), gum arabic (Billaud *et al*. 1996) and apple (Javovitz-Klap *et al*. 1989) PPOs showed triphenolase activity.

Substrate saturation curves for each diphenolic and triphenolic substrate indicated that lemon balm PPO follows simple Michaelis-Menten kinetics. Lineweaver-Burk plots for the kinetic analysis of the reaction rates, at a series of concentrations for each substrate, resulted in individual V_{max} and K_m values. The V_{max}, K_m and V_{max}/K_m values of lemon balm PPO enzyme were determined according to the method of Lineweaver-Burk and found to be 10 000 EU/ml/min, 60mM and 167 EU/ml/min/mM for catechol; 1110 EU ml/min, 55.5mM and 20 EU/ml/min/mM for 4-methylcatechol; and 2500 EU/ml/min, 12.5mM and 200 EU/ml/min/mM for pyrogallol as substrates. V_{max}/K_m ratio, called the catalytic power and a better parameter to find the most effective substrate, was calculated, and it was found that pyrogallol is the most suitable substrate for lemon balm PPO activity, followed by catechol and 4-methylcatechol.

Thermal inactivation

In the inactivation experiments, the residual activity of lemon balm PPO was determined after cooling in an ice bath at different temperatures such as 35, 55, and 75 °C by the times of 10, 20, 30, 40, 50, and 60 min using catechol, 4-methylcatechol and pyrogallol as substrates. The thermal inactivation profiles of partially purified lemon balm PPO are shown in Figure 3. The enzyme activity decreased due to heat denaturation of the enzyme with increasing temperature and inactivation time for catechol, 4-methylcatechol, and pyrogallol substrates. It was previously reported that heat

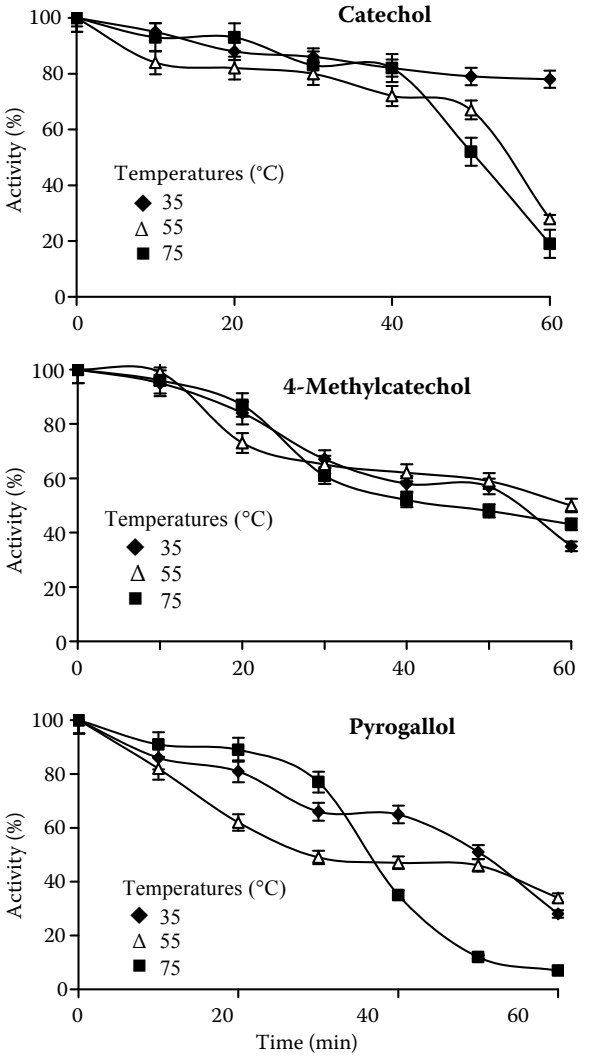

Figure 3. Change in lemon balm PPO activity as a function of temperature and time

sensitivity of PPOs may depend on the ripeness of the plant or on different molecular forms of the enzyme from the same plant source (Park & Luh 1985; Dogan *et al*. 2005b).

Effects of inhibitors

Effects of various inhibitors such as ascorbic acid, sodium azide, glutathione, benzoic acid, gallic acid and L-glutamic acid on lemon balm PPO activity were studied at various inhibitor concentrations using catechol, 4-methylcatechol, and pyrogallol as substrates. According to the obtained experimental data, L-glutamic acid and gallic acid did not inhibit lemon balm PPO. The prevention of enzymatic browning by a specific inhibitor may involve a single mechanism or may be the result of interplay of two or more mechanisms of inhibitory action. There are various mechanisms through which enzyme inhibitors can act (Dogan & Salman 2007).

The Lineweaver-Burk equation for competitive inhibition can be given as follows:

$$\frac{1}{V_0} = \left(\frac{aK_m}{V_{\max}}\right)\frac{1}{[S]} + \frac{1}{V_{\max}} \quad (1)$$

where

$$a = \left(1 + \frac{[I]}{K_i}\right) \quad (2)$$

S – substrate concentration (M)
I – inhibitor concentration (M)
K_i – dissociation constant of the enzyme-inhibitor complex
$V_{\max}$ – maximum velocity at saturating concentration of substrate (EU ml/min)
v_0 – enzyme activity value (EU ml/min)
K_m – Michaelis constant (M)

The Lineweaver-Burk equation for competitive inhibition is linear with the slope $aK_m/V_{\max}$ and intercepts $1/V_{\max}$ (Voet & Voet 2003; Dogan *et al*. 2009). As seen in Table 2, the inhibition type was found to be competitive inhibition for benzoic acid, sodium azide, and glutathione inhibitors using catechol as a substrate; and for glutathione and sodium azide inhibitors using 4-methylcatechol as a substrate; and for sodium azide and ascorbic acid inhibitors using pyrogallol as substrate. Figure 4 shows the effect of ascorbic acid inhibitor on lemon balm PPO using pyrogallol as a substrate (other figures not shown). A substance that competes directly with the normal substrate for an enzymatic binding site is known as competitive inhibitor. A competitive inhibitor shows chemical similarity to the substrate. In such inhibition, the inhibitor does not affect the turnover number of the enzyme.

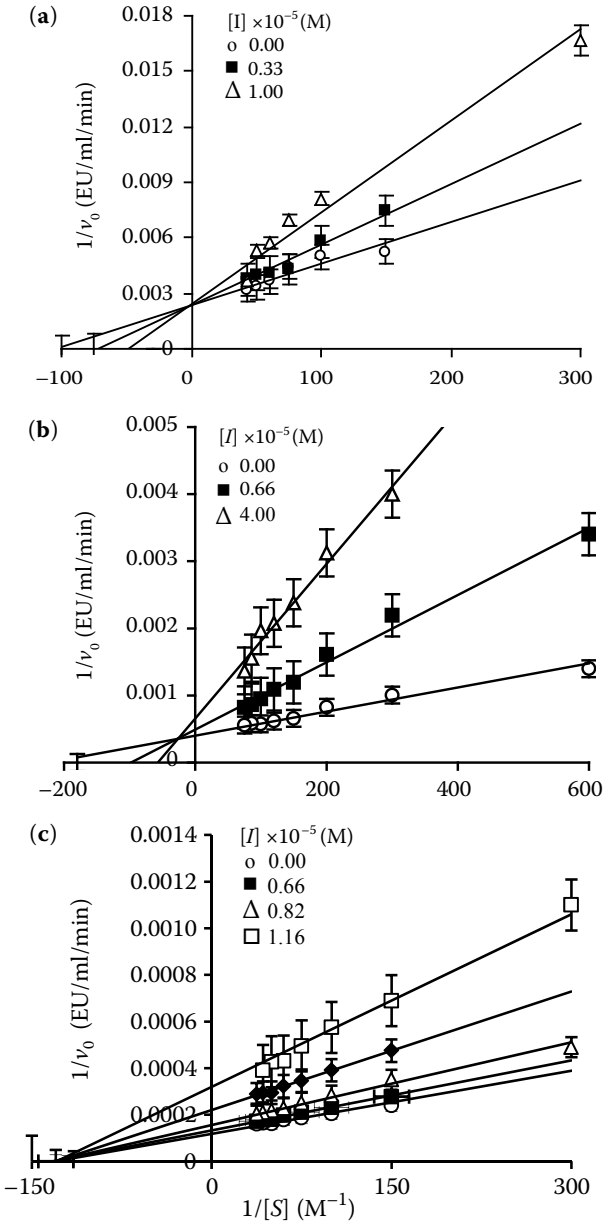

Figure 4. Lineweaver-Burk double reciprocal plots showing the inhibition of lemon balm PPO: inhibitor – ascorbic acid and substrate (**a**) pyrogallol, (**b**) 4-methylcatechol, and (**c**) catechol

The inhibition mechanism of each inhibitor in enzymatic browning may be different depending on the molecular structures of inhibitor, substrate, and enzyme source used. For example, sodium

azide toxicity toward a metal enzyme, especially in the case of a copper enzyme, is mainly due to its strong coordination ability with the metal within the active site, which provokes changes in the coordination number and conformation of the active site and depredates the active central metal. The reaction between the copper amine oxidase and azide probably hinders the bond of the precursor tyrosine to copper. This prevents the formation of this key intermediate and inhibits the activity of the oxidase. Glutathione does not affect the enzyme directly and oxygen uptake may be stimulated or inhibited depending upon the particular phenol being oxidised. Ascorbic acid has been widely used as an antibrowning agent for processing fruits and vegetables. Ascorbic acid is a moderately strong reducing compound, which is acidic in nature, forms neutral salts with bases, and is highly water-soluble. Ascorbic acid also acts as an oxygen scavenger for the removal of molecular oxygen in polyphenol oxidase reactions. Polyphenol oxidase inhibition by ascorbic acid has been attributed to the reduction of enzymatically formed *o*-quinones to their precursor diphenols. Ascorbic acid is however irreversibly oxidised to dehydroascorbic acid during the reduction process, thus allowing browning to occur upon its depletion (Dogan *et al*. 2009). Differences in the type and degree of inhibition of various PPOs were reported. Guanata *et al*. (1987) observed a competitive-type inhibition for grape PPO with cinnamic and benzoic acid inhibitors using 4-methylcatechol as a substrate; Dogan and Dogan (2004) for *Thymus* PPO with glutathione inhibitor using 4-methylcatechol, pyrogallol and catechol as substrates; Paul and Gowda (2000) for field bean PPO with tropolone, ascorbic acid, and cysteine-HCl inhibitors using catechol as a substrate; and Robert *et al*. (1997) for palmito PPO with benzoic acid inhibitor using 4-methylcatechol as a substrate, and Aydemir (2010) for rosemary PPO with ascorbic acid and L-cysteine inhibitor using catechol as a substrate.

In mixed inhibition, the inhibitor binds to both the enzyme itself and the ES complex yielding two K_i constants. Mixed inhibition affects enzyme kinetics as a combination of competitive and uncompetitive inhibition. K_m is increased and V_{max} is reduced. The mixed inhibitors presumably bind to sites that are involved in both substrate binding and catalysis, and act like a combination of competitive and uncompetitive inhibition (Voet & Voet 2003; Dogan *et al*. 2009). The Lineweaver-Burk equation for mixed-type inhibition is given as follows:

$$\frac{1}{V_0} = \left(\frac{aK_m}{V_{max}}\right)\frac{1}{[S]} + \frac{a'}{V_{max}} \quad (3)$$

where

$$a' = \left(1 + \frac{[I]}{K_i}\right) \quad (4)$$

Double reciprocal plots for mixed-type inhibition consist of lines that have the slope aK_m/V_{max} with the $1/v_0$ intercept of a'/V_{max} and $1/[S]$ intercept of $-a'/aK_m$. The lines for different values of $[I]$ intersect to the left of the $1/v_0$ axis. The Lineweaver-Burk equation indicated that lemon balm PPO inhibition by ascorbic acid using 4-methylcatechol as a substrate is a mixed type. Figure 4 shows the mixed-type inhibition for lemon balm PPO. The results lead to a series of lines which intersect to the left of the vertical axis and above the horizontal axis, with a decrease in V_{max} and, conversely, an increase in K_m. In the literature, the mixed-type inhibition was found for potato PPO with cinnamic acid inhibitor (Macrae & Duggleby 1968), for soluble potato PPO (Sanchez-Ferrer *et al*. 1993) and mushroom PPO (Kahn & Andrawis 1985) with tropolone inhibitor, for lettuce PPO with tropolone and 4-aminobenzoic acid inhibitors using 4-methylcatechol as a substrate (Dogan & Salman 2007), and for lettuce PPO with glutathione and ascorbic acid inhibitors using pyrogallol as a substrate (Dogan & Salman 2007). As seen above, the type of inhibition depends not only on the origin of the PPO studied but also on the substrate used.

For the special case that K_i and K_i' ($a = a'$), this intersection is, in addition, on the $1/[S]$ axis, a situation that, in an ambiguity of nomenclature, is sometimes described as non-competitive inhibition. The double reciprocal plot for this model shows that the inhibitor decreases V_{max}. In a classic example of pure non-competitive inhibition, the uninhibited reaction and the enzyme in the presence of inhibitor will yield the same K_m value. Non-competitive inhibitors decrease V_{max} but have no effect on K_m. A non-competitive inhibitor does not compete with substrate and the $[S]$ has no influence on the degree of inhibition of the enzyme catalytic rate. In this case where the non-competitive inhibitor reacts with the enzyme at a site other than the active site, both the free enzyme (E) and the enzyme-substrate complex (E-

Table 2. Inhibition types, K_i and IC_{50} values of polyphenol oxidase from lemon balm using catechol, 4-methylcatechol, and pyrogallol as substrates

Inhibitors	Substrates	[I] (M)	K_i (M)	K_i' (M)	[IC_{50}] (M)	Type of inhibition
Ascorbic acid	catechol	0.6×10^{-3} 0.8×10^{-3} 1.0×10^{-3} 1.1×10^{-3}	5.9×10^{-3} 7.4×10^{-3} 0.8×10^{-3} 0.9×10^{-3}	–	2×10^{-3}	non-competitive
Glutathione		0.6×10^{-4} 1.3×10^{-4} 1.6×10^{-4}	2.9×10^{-5} 9.9×10^{-5} 2.9×10^{-5}	–	1.2×10^{-4}	competitive
Sodium azide		0.6×10^{-2} 1.3×10^{-2} 1.6×10^{-2}	4.4×10^{-3} 1.7×10^{-3} 1.4×10^{-3}	–	7.9×10^{-6}	competitive
Benzoic acid		0.3×10^{-2} 1.0×10^{-2} 2.0×10^{-2}	2.2×10^{-3} 2.5×10^{-3} 1.7×10^{-3}	–	1.4×10^{-2}	competitive
Ascorbic acid	4-methylcatechol	0.6×10^{-4} 4.0×10^{-4}	0.4×10^{-4} 1.0×10^{-4}	2.64×10^{-4} 5.33×10^{-4}	1.5×10^{-4}	mixed
Glutathione		0.6×10^{-4} 1.3×10^{-4}	1.1×10^{-5} 7.0×10^{-6}	–	7.5×10^{-5}	competitive
Sodium azide		1.6×10^{-3} 5.0×10^{-3} 15.0×10^{-3}	0.6×10^{-3} 0.5×10^{-3} 0.3×10^{-3}	–	7.9×10^{-6}	competitive
Benzoic acid		1.3×10^{-4} 2.3×10^{-4}	0.9×10^{-4} 1.1×10^{-4}	–	9.6×10^{-5}	non-competitive
Ascorbic acid	pyrogallol	0.3×10^{-5} 1.0×10^{-5}	6.6×10^{-6} 6.6×10^{-6}	–	3×10^{-6}	competitive
Glutathione		1.0×10^{-5} 1.3×10^{-5}	2.8×10^{-6} 6.6×10^{-6}	–	5.3×10^{-5}	non-competitive
Sodium azide		0.6×10^{-2} 2.0×10^{-2}	1.8×10^{-2} 1.9×10^{-2}	–	1.2×10^{-2}	competitive

S) react with the inhibitor. The actual mechanism of action of the inhibitor varies with each kind of molecule. Figure 4 shows the effect of ascorbic acid on lemon balm PPO using catechol as a substrate. The inhibition constants were obtained by fitting the experimental data to Equation (3). When values of K_i and K_i' were of the same order of magnitude, the mixed-type system could be considered to be a non-competitive inhibition. As seen in Table 2, we found that the inhibition type was non-competitive inhibition for ascorbic acid using catechol, for benzoic acid using 4-methylcatechol, and for glutathione using pyrogallol as a substrate. A non-competitive inhibition with glutathione was reported for rosemary PPO (Aydemir 2010).

Percent activity graphs were drawn to find IC_{50} values at five constant inhibitor concentrations, which show about 50% inhibition effect. Table 2 shows the percent inhibition of lemon balm PPO with glutathione, benzoic acid, sodium azide and ascorbic acid inhibitors using catechol, 4-methylcatechol and pyrogallol as substrates. As seen from the table, the sensitivity of PPO to inhibitors changed from substrate to substrate.

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