Some Kinetic Properties of Polyphenol Oxidase Obtained from Various *Salvia* Species (*Salvia viridis* L., *Salvia virgata* Jacq. and *Salvia tomentosa* Miller)

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Polyphenol oxidase (PPO) was partially purified by $(NH_4)_2SO_4$ precipitation followed by dialysis from different organs of *Salvia* species (*Salvia virgata* Jacq., *Salvia viridis* L. and *Salvia tomentosa* Miller). Polyphenol oxidase activity was measured spectrophotometrically at 420 nm using catechol as a substrate. V_{max}, K_M and V_{max}/K_M values for polyphenol oxidase activity from different organs of *Salvia* species were determined. *S. tomentosa* Miller was the species with the highest PPO activity, followed by *S. virgata* Jacq and *S. viridis* L. *S. tomentosa* Miller was the most suitable *Salvia* species for dark-tea preparations because of the highest V_{max}/K_M values. The effects of various inhibitors on the reaction catalysed by the enzyme were tested and calculated I_{50} values, reduced the enzyme activity by 50%. The most effective inhibitor was L-cysteine followed by ascorbic acid. Activation energies, E_a, were determined from Arrhenius equation.

Key Words: polyphenol oxidase, *Salvia* species, inhibitors, activation energy

INTRODUCTION

Polyphenol oxidase (PPO) is an enzyme widely distributed in plants (Singh and Ravindranath, 1994). Tissue browning, a major cause of quality loss during harvesting, post-harvest handling/storage, and processing of fruits, plants and vegetables (Mathew and Parpia, 1971) is attributed to the reaction catalysed by the polyphenol oxidase (EC 1.14.18.1, PPO). PPO, which is ubiquitous in nature and widely distributed in higher plants, animals and microorganisms, is a binuclear copper containing enzyme that catalyses two ostensibly distinct reactions: (1) hydroxylation of monophenols to *o*-diphenols, the only specific reaction catalysed by this enzyme (cresolase or monophenolase); and (2) oxidation of the self-generated *o*-diphenols to the corresponding *o*-quinones (catecholase or diphenolase) (Gowda and Paul, 2002). These reactions, known as enzymatic browning are not generally desiderable for the food industry, but in some plants used for preparation of dark tea. The genus *Salvia*, with about 700 species, is one of the most widespread members of the Lamiaceae family (Kintzios et al., 1999). *Salvia* genus is used worldwide as antioxidant, food, culinary herbs and herbal tea. Furthermore, *Salvia* genus are known as garden sage (or island-tea) in Turkey.

There are many works related to antioxidant activities (Ollanketo et al., 2002), antifungal activities (Soković et al., 2002) and essential oil composition (Couladis et al., 2002) of *Salvia* genus but no one of these works is related to PPO activity. However, there are many works related to PPO from different plants such as wheat (Okot-Kotber et al., 2002), *Allium* sp. (Arslan et al., 1997), sorghum (Dicko et al., 2002), *Beta vulgaris* L. (Escribano et al., 2002), tea leaf (Halder et al., 1998), lettuce (Chazarra et al., 1996) and *Anethum graveolens* L. (Arslan and Tozlu, 1997).

This study searched the effects of pH, temperature and inhibitors on PPO activity obtained from stems, leaves and flowers of three different *Salvia* species such as *Salvia virgata* Jacq. (SVG), *Salvia viridis* L. (SVI) and *Salvia tomentosa* Miller (STO). Furthermore, the most appropriate *Salvia* species for dark-tea preparations was determined.

MATERIALS AND METHODS

Materials

Salvia species such as *Salvia virgata* Jacq. (SVG), *Salvia viridis* L. (SVI) and *Salvia tomentosa* Miller (STO) used in this study were freshly collected in spring from a field near Balikesir in Turkey, and kept for 2 days in a refrigerator at 4 °C before PPO extraction. All

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chemicals used in this study were of analytical grade and were used without further purification.

Methods

Enzyme Extraction

Salvia species were cleaned to remove visible soil and then washed with tap water and bidistilled water several times. *Salvia* was subsequently separated in stems, leaves and flowers, and washed again with bidistilled water. Extract was prepared with 10 g of *Salvia* plants placed in a Dewar flask under liquid nitrogen, which decomposed cell membrane, transferred to a stainless stell waring blender, and grounded to a powder under liquid nitrogen. Before using it, the powder was transferred to a small beaker. The frozen plant powder was added to the extraction solution (100 mL of 0.1 M phosphate buffer containing 5% polyethyleneglycol at pH = 6.5 and 10 mM ascorbic acid) and mixed with a magnetic stirrer for 4 min at 4 °C. The crude extract was filtered, and the filtrate was centrifuged at $20\,000 \times g$ 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 $20\,000 \times g$ for 30 min. The precipitate was dissolved in a small amount of 0.05 M phosphate buffer (pH 7.0) and dialysed at 4 °C in the same buffer for 2 days with three changes of buffer during dialysis. Dialysis tubing (16 mm average diameter Sigma) retains >90% of cytochromec (M.W. 22.400) in solution over a 10-h period. The dialysed sample was used as the PPO enzyme source in the following experiments (Wesche-Ebeling and Montgomery, 1990).

Assay for PPO Activity

Polyphenol oxidase activity was determined using a spectrophotometric method based on the initial rate of increase in absorbance at 420 nm (Dogan et al., 2002). Unless otherwise stated, 2.4 mL of 0.1 M phosphate buffer (pH 6.5), 0.5 mL of 0.1 M catechol as substrate, and 0.1 mL of the enzyme extract were pippeted and mixed in a quartz cuvette of 4 mL volume. In each measurement, the volume of solution in the cuvette was kept constant at 3 mL. The 0.1 M concentration was chosen to avoid the influence of enzymatic extract ionic strength on PPO activity. A portion of the mixture was rapidly transferred into a 1.0 cm path length cuvette. Absorbance was recorded immediately and at 10 s intervals, at 20 ± 1 °C with a Cary |1E|g UV–Visible Spectrophotometer (Varian). The instrument was zeroed using the same mixture without enzyme. The assay mixture was repeated twice using the same stock of the enzyme extract. Enzyme activity was calculated from the linear portion of the curve. One unit of PPO activity was defined as amount of enzyme that causes an increase in absorbance of 0.001 per min for 1 mL enzyme at 420 nm and 25 °C.

Effect of pH

The optimum pH for PPO activity was determined at pH values of 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0, respectively, 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 HNO_3. The optimum pH value for PPO activity obtained from different parts of plants was obtained using catechol as substrate. As mentioned above, each assay mixture was repeated twice using the same stock of the enzyme extract.

Effect of Substrate Concentration

Polyphenol oxidase activity was assayed in a quartz cuvette with 4 mL volume at 420 nm by mixing 0.1 mL of enzyme extract, and 0.1 M catechol substrate and 0.1 M phosphate solutions. In each measurement, the volume of solution in a quartz cuvette was kept constant as 3 mL. Each assay mixture was repeated twice and the data were plotted according to linear regression analysis (with the method of Lineweaver and Burk). Michaelis constants, K_M and V_{max}, for different parts of each *Salvia* species were calculated from the plots of 1/V versus 1/[S] (Dogan et al., 2002).

Effect of Temperature

The optimum temperature for PPO activity was determined at 20, 30, 40, 50, 60 and 70 °C, respectively, by using different parts of plants. The effect of temperature on the PPO activity was tested by heating the standard reaction solutions (substrate and buffer solutions) to the appropriate temperature with circulating water-bath before introduction of the enzyme. Once temperature equilibrium was reached, enzyme was added and the reaction was followed spectrophotometrically at constant temperature at given time intervals. Again, each assay mixture was repeated twice (Arslan et al., 1998).

Activation Energy

The activation energy is calculated from experimental results obtained for enzyme reactions by using Arrhenius equation, which is given as

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

where V is the enzyme activity value (EU/mL · min), Z is the frequency factor (EU/mL · min), E_a is the activation energy (cal/mol) and T is the temperature

(K). The graph of $\ln V$ versus $1/T$ will give a straight line. The parameter Z is obtained from intercept point at $1/T = 0$ and the activation energies of reactions are calculated from the slopes of lines (Dogan et al., 2000, 2002).

Effect of Inhibitors

To determine the effects of inhibitors, firstly, PPO activity was measured with the mixture of 0.4 mL of 0.1 M catechol, 0.2 mL of enzyme solution and 2.4 mL of 0.1 M phosphate buffer and inhibitor solution at various volumes. Inhibitors studied were ascorbic acid, sodium azide and L-cysteine. To determine the inhibitor concentration that reduced the enzyme activity by 50% (I_{50}), regression analysis graphs were drawn by using percent inhibition values. I_{50} values were determined from the graphs (Arslan et al., 1997).

RESULTS AND DISCUSSION

Enzyme Kinetics

Lineweaver-Burk graphs were drawn to calculate the K_M and V_{max} values for stems, leaves and flowers of *Salvia* species (Table 1). Phenolic compounds make up 25–35% of the dry matter content of plants. Flavanol compounds were 80% of the phenols while the remainder were proanthocyanidis, phenolic acids, flavonols and flavones. During tea fermentation the flavanols are oxidised enzymatically to compounds which are responsible for the colour and flavour of tea. Flavour intensity of tea is correlated with the total content of the phenolic compounds and polyphenol oxidase. The polyphenol oxidase, which is located mainly within the cells of plant epidermis, are of great importance for tea fermentation (Belitz and Grosch, 1999) because it causes the enzymatic browning, desiderable for development of dark-tea. The highest PPO activity can be determined according to K_M and V_{max}/K_M values. The lower K_M and the higher V_{max} the higher PPO activity. The V_{max}/K_M ratio is called the "catalytic powder" (Dogan et al., 2002). According to this value (Table 1), *Salvia tomentosa* Miller was the species with the highest PPO activity, followed by *Salvia virgata* Jacq. and *Salvia viridis* L. Leaves had the highest PPO activity followed by flowers and stems. As a result, it can be said that *Salvia tomentosa* Miller was the most suitable *Salvia* species for dark-tea preparations because of the highest V_{max}/K_M values. On the contrary, for green-tea, then *Salvia viridis* L. was the most appropriate *Salvia* species.

Table 1. V_{max}, K_M and V_{max}/K_M values calculated for PPO activity obtained from organs of different *Salvia* species using catechol as a substrate.

Salvia Species	V_{max} (EU/mL · min)	K_M (mM)	V_{max}/K_M (EU/mmol · min)
Salvia viridis L.			
Stems	3005	23.8	126.3
Leaves	7305	5.4	1352.8
Flowers	6691	22.8	293.5
Salvia virgata Jacq.			
Stems	1263	33.6	37.6
Leaves	2949	23.2	127.1
Flowers	4358	15.2	286.7
Salvia tomentosa Miller			
Stems	10361	58.0	178.6
Leaves	11198	6.7	1671.3
Flowers	8888	6.1	1457.0

K_M values for different organs of *Salvia* species varied from 5.4 to 58.0 mM. These values are smaller compared to other vegetables such as Chinese cabbage ($K_M = 682.5$ mM; Nagai and Suzuki, 2001), but higher than values obtained for *Anethum graveolens* ($K_M = 1.6$ mM; Arslan and Tozlu, 1997) and beet root ($K_M = 0.45$ mM; Escribano et al., 2002). V_{max} values of different organs of *Salvia* species studied in this study were from 1263 to 11198 EU/mL · min. K_M and V_{max} values for PPO activity varied with the type of substrate, buffer, food sources and purity of the enzyme extract as previously stated (Arslan et al., 1997).

Effect of pH

All organs of *Salvia* species showed a clear pH for maxima in PPO activity around 7.0 with catechol as substrate within the pH range studied (Figure 1(a)–(c)). In general, most plants, vegetables and fruits show maximum activity at or near neutral pH values (Siddiq et al., 1992). Furthermore, it was also found that the maximum PPO from various sources have different pH values: *Allium* sp. (pH 7.5; Arslan et al., 1997) and Malatya apricot (pH 8.5; Arslan et al., 1998); Stanley plum (pH 6.0; Siddiq et al., 1992), field bean seed (pH 4.0; Paul and Gowda, 2000), Jerusalem artichoke (pH 6.0; Zawistowski et al., 1988), Mango kernel (pH 4.9; Aragba et al., 1998), green olive (pH 4.5; Ben-Shalom et al., 1977) and potato (pH 5.0; Balasingam and Ferdinand, 1970). On the other hand, raspberry (Gonzalez et al., 1999) has two different maxima at pH 5.5 and 8.0. PPO activity varies with the source of enzyme and substrate within 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 optimum pH can also be affected by the type of buffer and the purity of enzyme (Dogan et al., 2002).

Effect of Temperature

The temperature for maximum PPO activity at pH 6.5 for all organs of *Salvia* species (SVG, SVI and STO) was found at 40 °C with catechol as substrate. Above 40 °C, the enzyme activity decreased and at 70 °C was very

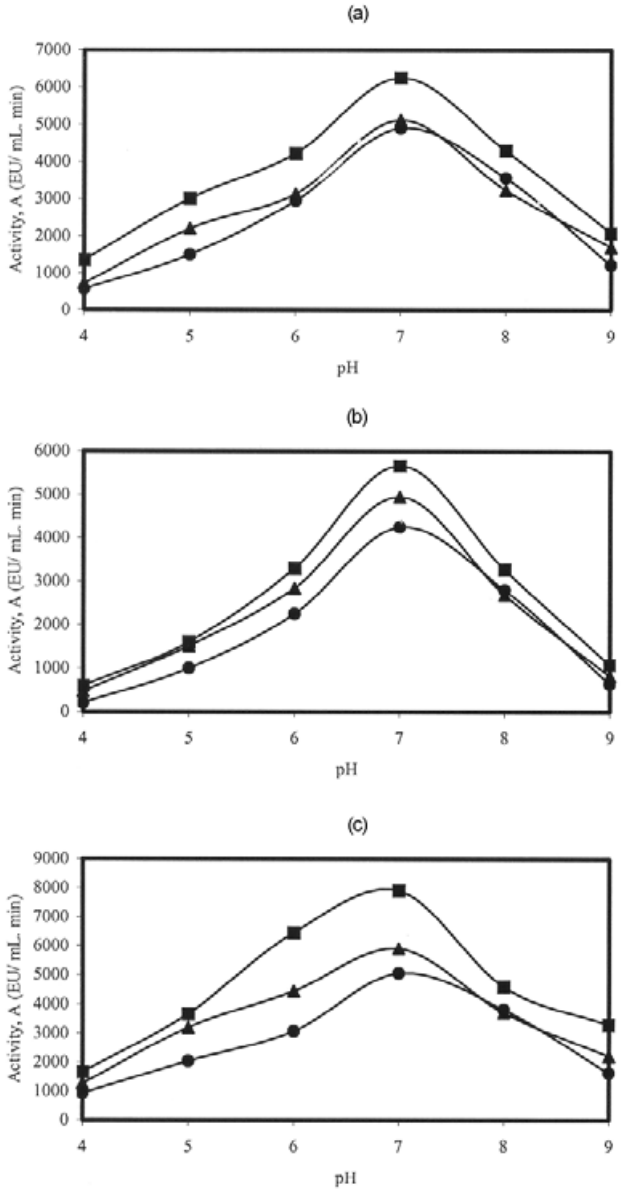

Figure 1. Changes in PPO activity of different *Salvia* species versus pH. (a) *Salvia virgata* Jacq. (b) *Salvia viridis* L. (c) *Salvia tomentosa* Miller. (▲) Leaves, (■) Flowers, (●) Stems.

little. This temperature was very different from those obtained for Amasya apple (18 °C; Oktay et al., 1995), grape (25 °C, Wissemann and Lee, 1981) and Stanley plums (20 °C, Siddiq et al., 1992).

Effect of Inhibitors

The inhibition of browning can be the result of: (i) inactivation of PPO, (ii) elimination of one of the substrates (O_2, polyphenols) for the reaction, and (iii) the action of inhibitors on reaction products of enzyme action to inhibit the information of coloured products in secondary reactions (Augustin et al., 1985). The prevention of enzyme browning of plants may be retarded or eliminated by removing the reactants such as oxygen and phenolic compounds or by the use of PPO inhibitors. Complete elimination of oxygen from plants during processing is difficult because oxygen is ubiquitous (Roudsari et al., 1981). Various 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 (Lee et al., 1983), glutathione (Lee et al., 1983; Jiang et al., 1999), tropolone (Perez-Gilabert and Garcia-Carmona, 2000), thiourea (Lee et al., 1983), sodium diethyldithiocarbamate (Lee et al., 1983) have been used to prevent the enzymatic browning. D,L- dithiothreitol was an effective inhibitor of PPO activity in strawberries (Wesche-Ebeling and Montgomery, 1990), sunflower seeds (Raymond et al., 1993) and in grapes (Cash et al., 1976; Lee et al., 1983). Oktay et al. (1995) studied the effect of glutathione on PPO activity obtained from Amasya apple and found that it decreased the PPO activity.

The substrate concentration was constant as 0.013 M for all inhibitor studies and concentrations of inhibitors were 1.66×10^{-3}, 3.33×10^{-3}, 5.00×10^{-3} and 6.66×10^{-3} M. I_{50} values (Table 2) were calculated from inhibition curves obtained with ascorbic acid

Table 2. Effects of some inhibitors on the activity of polyphenol oxidase (PPO) obtained from organs of different *Salvia* species.

PPO Source	Organs	Inhibitors	$I_{50} \times 10^3$ (M)	R^2
Salvia	Stems		1.64	0.9944
virgata Jacq	Leaves	Ascorbic acid	3.62	0.9897
	Flowers		2.53	0.9933
Salvia viridis L.	Stems		2.32	0.9638
	Leaves	Ascorbic acid	6.10	0.9914
	Flowers		3.10	0.9848
Salvia tomentosa	Stems		4.10	0.9922
Miller	Leaves	Ascorbic acid	7.44	0.9750
	Flowers		6.01	0.9946
Salvia	Stems		2.04	0.9754
virgata Jacq.	Leaves	Sodium azide	3.19	0.9943
	Flowers		2.76	0.9873
Salvia viridis L.	Stems		2.86	0.9973
	Leaves	Sodium azide	3.64	0.9971
	Flowers		3.13	0.9909
Salvia tomentosa	Stems		5.56	0.9885
Miller	Leaves	Sodium azide	7.85	0.9951
	Flowers		7.05	0.9946
Salvia	Stems		1.46	0.9984
virgata Jacq.	Leaves	L-Cysteine	2.51	0.9978
	Flowers		1.81	0.9918
Salvia viridis L.	Stems		1.88	0.9924
	Leaves	L-Cysteine	3.41	0.9938
	Flowers		2.64	0.9847
Salvia tomentosa	Stems		1.35	0.9985
Miller	Leaves	L-Cysteine	2.63	0.9915
	Flowers		2.28	0.9980

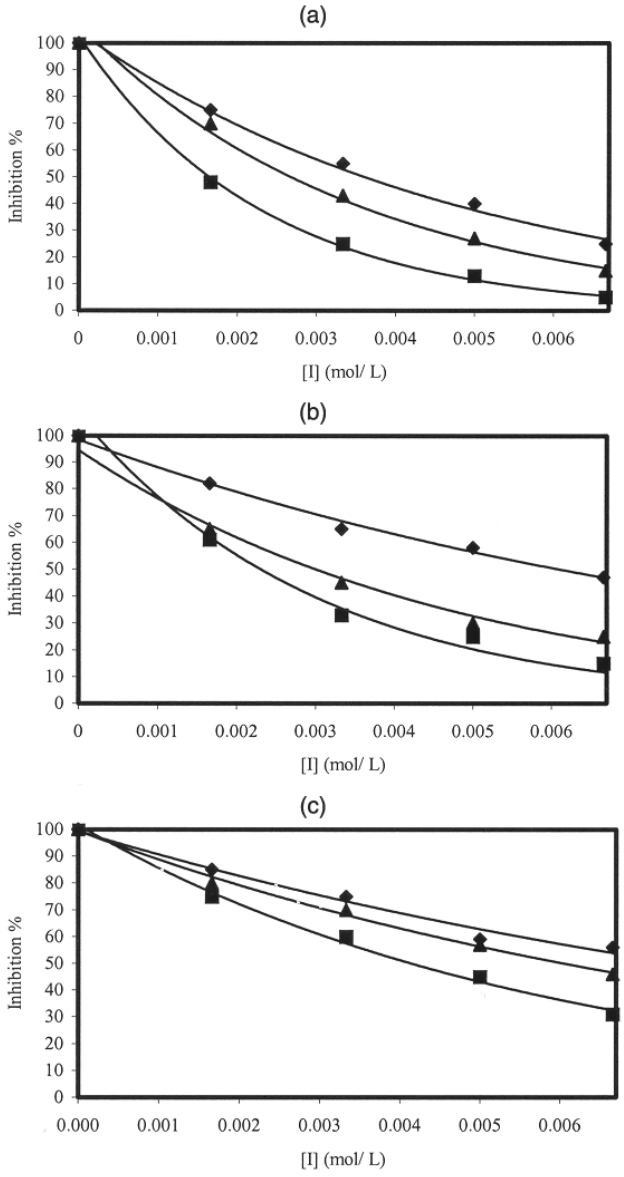

Figure 2. Inhibition of PPO extracted from different *Salvia* species with different ascorbic acid concentrations. (a) *Salvia virgata* Jacq. (b) *Salvia viridis* L. (c) *Salvia tomentosa* Miller. (◆) Leaves, (▲) Flowers, (■) Stems.

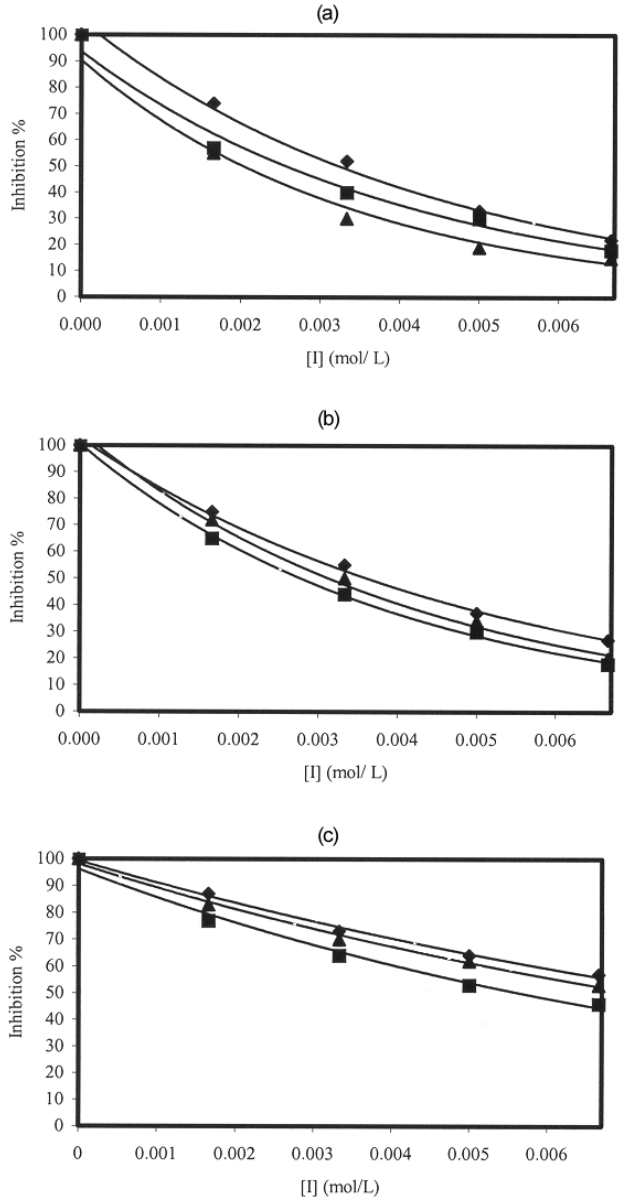

Figure 3. Inhibition of PPO extracted from different *Salvia* species with different sodium azide concentrations. (a) *Salvia virgata* Jacq. (b) *Salvia viridis* L. (c) *Salvia tomentosa* Miller. (◆) Leaves, (▲) Flowers, (■) Stems.

(Figure 2), sodium azide (Figure 3) and L-cysteine (Figure 4) using catechol as substrate.

According to I_{50} values, L-cysteine was the most effective inhibitor for both *Salvia* species and their organs, followed by ascorbic acid and sodium azide, respectively. L-cysteine can easily form complexes with quinons and, therefore, inhibit secondary oxidation and polymerisation reactions thus consuming the substrate present (Davis and Pierpoint, 1975). L-cysteine can also act as a reducing agent (Wesche-Ebeling and Montgomery, 1990). Ascorbic acid reduces quinones to phenols and does not directly inhibit PPO (Anderson, 1968). It will prevent enzymatic browning only as long as it is present in the reduced form. Natrium azide's toxicity to a metal enzyme, especially a copper enzyme, is mainly due to its strong coordination ability with the metal in the active site, which provokes changes in the coordination number and conformation of the active site, and depredates the active centre metal. In the reaction between the copper amine oxidase and azide, azide probably hinders the bond of the precursor tyrosine to the copper. This prevents the formation of this key intermediate and inhibits the activity of the oxidase (Schwartz et al., 2001).

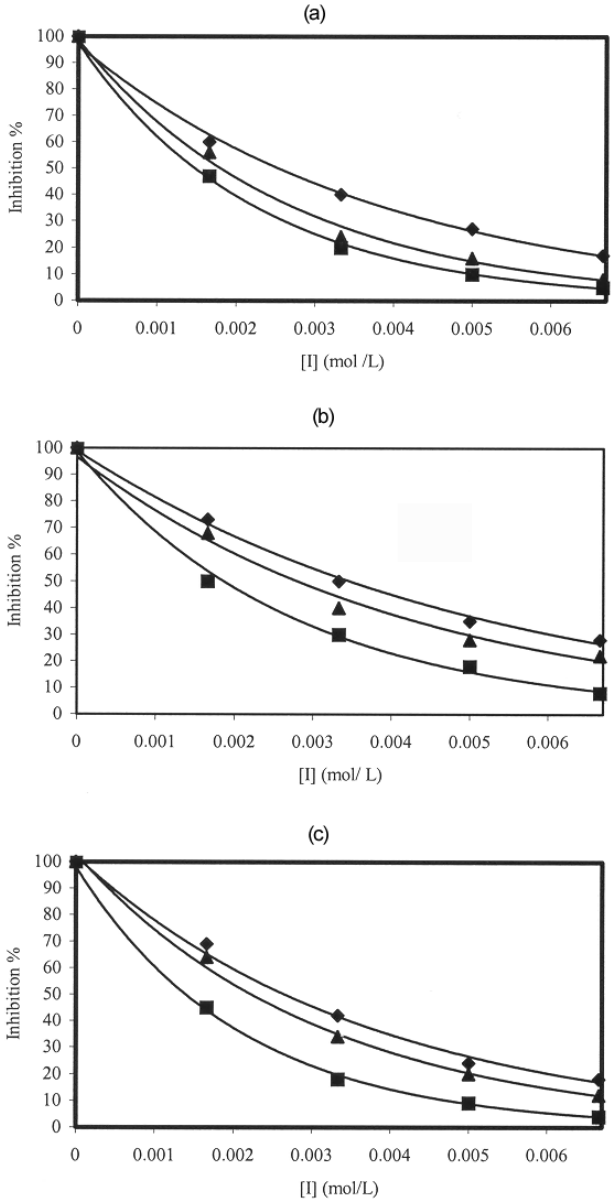

Figure 4. Inhibition of PPO extracted from different *Salvia* species with different L-cysteine concentrations. (a) *Salvia virgata* Jacq. (b) *Salvia viridis* L. (c) *Salvia tomentosa* Miller. (◆) Leaves, (▲) Flowers, (■) Stems.

Table 3. Activation energy values calculated for PPO activity obtained from organs of different *Salvia* species.

PPO Source	Organs	E_a (kcal/mol)	Z (EU/mL min)	R^2
Salvia virgata Jacq.	Stems	−11.1	8.8×10^{-7}	0.9920
	Leaves	−11.9	4.8×10^{-7}	0.9974
	Flowers	−11.4	10.0×10^{-7}	0.9725
Salvia viridis L.	Stems	−14.2	1.1×10^{-12}	0.9760
	Leaves	−22.1	6.9×10^{-8}	0.9717
	Flowers	−20.0	1.2×10^{-11}	0.9904
Salvia tomentosa Miller	Stems	−6.9	7.8×10^{-2}	0.9944
	Leaves	−8.9	5.4×10^{-3}	0.9786
	Flowers	−7.2	5.5×10^{-2}	0.9904

Activation Energy

Activation energy values, E_a, were calculated from Arrhenius equation at different temperatures (40, 50, 60 and 70 °C). Activation energy values varied from −6.9 to −22.1 kcal/mol (Table 3). Furthermore, Z values have also given in Table 3.

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