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# Inhibition of polyphenol oxidase obtained from various sources by 2,3-diaminopropionic acid

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**Abstract: This paper reports for the first time the inhibition of the catecholase activities of mushroom, artichoke (*Cynara scolymus* L) and *Ocimum basilicum* L polyphenol oxidase by 2,3-diaminopropionic acid. Polyphenol oxidases from artichoke and *O basilicum* L were purified by ammonium sulfate precipitation, dialysis and a Sepharose 4B-L-tyrosine-*p*-aminobenzoic acid-affinity column. In inhibition studies, 2,3-diaminopropionic acid showed uncompetitive inhibition for mushroom PPO using catechol and pyrogallol as substrates, competitive inhibition for *O basilicum* L PPO using catechol as a substrate, and uncompetitive inhibition for artichoke PPO using catechol as a substrate. Furthermore, sodium azide, which is an inhibitor of PPO, was used as an inhibitor for comparison with the inhibition potency of 2,3-diaminopropionic acid. The highest 2,3-diaminopropionic acid inhibition observed with *O basilicum* L ($K_i$ =0.89 mM), followed by artichoke ($K_i$ =1.42 mM) and mushroom ($K_i$ =2.47 mM), respectively.**
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**Keywords:** inhibition; polyphenol oxidase; 2,3-diaminopropionic acid; artichoke; *Ocimum basilicum* L

## INTRODUCTION

Enzymatic browning of fruits and vegetables is caused mainly by the conversion of native phenolic compounds to quinones, which are polymerized, in turn, to brown, red or black pigments. The enzymes responsible for the sequence of reactions are usually referred to as polyphenol oxidases [EC 1.14.18.1; diphenol: oxygen oxidoreductase; polyphenoloxidase (PPO)], but are also known as tyrosinases, catecholases, cresolases and phenolases.[1] Browning occurs when the phenolic substrates, the PPO enzymes and oxygen are brought together under appropriate conditions of pH, temperature and water activity. Bruising, cutting, peeling or otherwise disrupting cells promotes enzymatic browning of many fruits and vegetables. Senescence or disease also can lead to enzymatic browning. For fresh or processed fruits and vegetables, the enzymatic browning produces undesirable colours and off-flavours. In addition to the loss of aesthetic quality of fruits and vegetables, enzymatic browning also reduces nutritional quality through the destruction of nutrients such as ascorbic acid.[2] Because of this decrease in market value and the concomitant economic losses, control of enzymatic browning is very important to food manufacturing industries.

Because of the deleterious effect of enzymatic browning on fruits and vegetables, much work has been devoted to the development of methods for eliminating, or at least retarding, the process. Several methods have so far been used to attain the inhibition of PPO activity in fruits and vegetables. Thermal treatments, exclusion of oxygen, the use of antibrowning agents and addition of chemicals are among the most effective methods.[2] In the food industry, enzymatic browning can be avoided by using thermal treatment of PPO, but traditional heat treatments cause adverse effects such as colour alteration, flavour damage, and vitamin and nutritional losses.[3] Oxygen can be excluded from the reaction by immersing the fruits and vegetables in water, syrup or brine or by exposure to vacuum or modified atmospheric packing. However, such treatment is not definitive because, when the packages are opened, oxygen is reintroduced and browning will restart.[4] A common approach for enzymatic browning prevention is the use of antibrowning agents, which act primarily on the enzyme or react with substrates and/or products of enzymatic catalysis so that pigment formation is inhibited. The use of these antibrowning agents in the food industry is, however, constrained by such considerations as toxicity, effects on taste, flavour, colour, and texture and cost.[5]

Chemical additives can be used to prevent enzymatic browning: bisulfite, ascorbic acid and its analogs, and cysteine as a reducing agent. The most common method for controlling browning is to use sulfites in any of their forms (sulfur dioxide, sodium or potassium metabisulfite, sodium or potassium

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bisulfite).[6] The chemical action of the bisulfites is to react with the *o*-quinones forming colourless complex compounds.[7] Sulfites are unique and multifaceted compounds because they are economical and can control both enzymatic and non-enzymatic browning while functioning as antimicrobials, bleaching agents, reducing agents, and antioxidants.[8] However, because of adverse health effects, the FDA has prohibited their use in fruits and vegetables served or sold raw to consumers.[9] Other chemical compounds, such as acidifiers (citric, malic and phosphoric acids), chelators (EDTA) and reducing agents (ascorbic acid, alone or in combination) have been used.[10] However, recent changes in lifestyle and an awareness of the relationship between food and health have led to a demand for safer and fewer added chemicals without toxic effects in processed food.

The 2,3-diamino acid family constitutes a key structural component in a variety of antibiotics[11] and antifungal dipeptides[12] as well as in other biologically active molecules.[13] It has been stated that 2,3-diaminopropanoic acid, a non-protein amino acid, does not have any toxic activity.[14] 2,3-Diaminopropionic acid is also a common constituent of the amino acid pools of seeds from various species of *Mimosa* and *Acacia*.[15,16] The purpose of this work was to study the inhibition effect of 2,3-diaminopropionic acid on PPO activity obtained from three different sources, mushroom, artichoke (*Cynara scolymus* L) and *Ocimum basilicum* L and to compare the effects with the inhibition potency of sodium azide, which is a inhibitor of PPO. This effect has not been previously reported in literature.

## MATERIALS AND METHODS

### Materials

Artichoke (*Cynara scolymus* L) and *Ocimum basilicum* L used in this study were obtained from a local garden in İzmir and Aydin cities (Turkey), respectively. Mushroom PPO was purchased from Sigma Chem Co. The chemicals used in this study were the best grade available, were used without further purification and were obtained from Sigma Chem Co and Merck (Germany). Enzyme assays were measured with the aid of a Cary 1E g UV-visible spectrophotometer (Varian, Australia).

### Extraction of PPO

The extraction procedure was adopted from Wesche-Ebeling and Montgomery.[17] Artichoke and *O basilicum* L were kept for 2 days in the refrigerator (+4 °C) before extracting PPO. A sample (10 g) was placed in a Dewar flask under liquid nitrogen for 10 min in order to decompose cell membranes. The 10-g sample was homogenised using a Waring blender for 2 min in 100 ml of 0.1 M phosphate buffer (pH 6.5) containing 10 mM ascorbic acid and 5% poly(ethylene glycol). The crude extract was filtered, and the filtrate was centrifuged at 15 000 × *g* for 30 min at 4 °C. The supernatant was brought to 80% ammonium sulfate saturation with solid ammonium sulfate. Inactive proteins were partially removed by ammonium sulfate precipitation. The precipitated PPO was separated by centrifugation at 15 000 × *g* for 30 min at 4 °C. The pellet then was dissolved in a small volume of 0.1 M phosphate buffer (pH: 7.0) and dialyzed at 4 °C in the same buffer for 2 days with three changes of buffer. After dialysis, the active fraction was purified by affinity chromatography. The gel used was synthesized according to the method of Arslan and coworkers.[18,19] The purified sample was used as the PPO enzyme source in the following experiments. The purification procedures are summarized in Table 1 for artichoke and in Table 2 for *O basilicum* L. As can be seen in Tables 1 and 2, the PPO was finally purified up to 43-fold for artichoke and 11.5-fold for *O basilicum* L.

### Assay of PPO Activity

Enzyme activity was determined by measuring the increase in absorbance at 420 nm and 320 nm for

**Table 1.** Purification of artichoke PPO

| Type of extract | Volume of extract (ml) | Protein concentration ($\mu g\,ml^{-1}$) | Activity ($EU\,ml^{-1}$) | Specific activity ($EU\,\mu g^{-1}$ protein) | Purification (-fold) |
|---|---|---|---|---|---|
| Crude extract | 75 | 576 | 3215 | 5.6 | — |
| $(NH_4)_2SO_4$ precipitation | 12 | 1171 | 6588 | 5.6 | 1.00 |
| Dialysis | 12 | 926 | 8506 | 9.2 | 1.63 |
| Affinity | 2 | 4.2 | 1659 | 394.4 | 42.95 |

**Table 2.** Purification of *Ocimum basilicum* L PPO

| Type of extract | Volume of extract (ml) | Protein concentration ($\mu g\,ml^{-1}$) | Activity ($EU\,ml^{-1}$) | Specific activity ($EU\,\mu g^{-1}$ protein) | Purification (-fold) |
|---|---|---|---|---|---|
| Crude extract | 75 | 693 | 3695 | 5.33 | — |
| $(NH_4)_2SO_4$ preciptation | 12.2 | 2265 | 7619 | 3.36 | 0.63 |
| Dialysis | 12.1 | 1831 | 9438 | 5.15 | 1.53 |
| Affinity | 2.4 | 8.9 | 530 | 59.46 | 11.54 |

catechol and pyrogallol substrates, respectively with a spectrophotometer. Enzyme activity was calculated from the linear portion of the curve. One unit of PPO activity (EU) was defined as the amount of enzyme that caused an increase in absorbance of 0.001 $min^{-1}$ for 1 ml of enzyme at 25 °C.[20]

### Inhibition of PPO activity by 2,3-diaminopropionic acid

2,3-Diaminopropionic acid was evaluated for its effectiveness as an inhibitor of mushroom, *C scolymus* L and *O basilicum* L PPOs using catechol and pyrogallol as substrates. Furthermore, sodium azide, which is known to be an inhibitor of PPO, was also used to compare the inhibition potency of 2,3-diaminopropionic acid. Three millilitres reaction mixture contained substrates at various concentrations in 0.1 M phosphate buffer (pH 6.5), 0.1 ml of enzyme solution and inhibitor solutions at fixed concentrations. Inhibition constants ($K_i$) were deduced from the Dixon plots for each inhibitor.[21]

### Determination of protein content

Protein content was determined according to the Bradford method using bovine serum albumin as a standard.[22]

## RESULTS AND DISCUSSION

The inhibition effect of 2,3-diaminopropionic acid on PPO activity obtained from three different sources, mushroom, artichoke and *Ocimum basilicum* L, using catechol and pyrogallol as substrates was investigated in this study. These three enzyme sources were chosen because they exhibited different properties. Many substances alter the activity of an enzyme by combining with it in a way that influences the binding of substrate and/or its turnover number. Substances that reduce an enzyme's activity in this way are known as inhibitors. Many inhibitors are substances that structurally resemble their enzyme's substrate but either do not react, or react very slowly, compared with the substrate. Such inhibitors are commonly used to probe the chemical and conformational nature of a substrate-binding site as part of an effort to elucidate the enzyme's catalytic mechanism.[23] The inhibiton of browning can be the result of (1) inactivation of PPO, (2) elimination of one of the substrates (oxygen, polyphenols) for the reaction and (3) the action of inhibitors on reaction products of enzyme action to inhibit the formation of coloured products in secondary reactions.[24] The prevention of enzymatic browning by a specific inhibitor may involve a single mechanisms or be the result of an interplay of two or more mechanisms of inhibitor action. There are various mechanism through which enzyme inhibitors can act.

(i) *Competitive inhibition*. In competitive inhibition, inhibitor, I, competes with substrate, S, for binding to the active site of the enzyme. The Lineweaver–Burk equation for competitive inhibition is:

$$\frac{1}{v_0} = \left(1 + \frac{[I]}{K_i}\right)\frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \qquad (1)$$

In this equation, [S] is substrate concentration ($mol\,L^{-1}$); [I] is inhibitor concentration ($mol\,L^{-1}$); $K_i$ is the dissociation constant of the enzyme-inhibitor complex; $V_{max}$ is the maximum velocity at saturating concentration of substrate ($EU\,ml^{-1}\,min^{-1}$); $v_0$ is the enzyme activity value ($EU\,ml^{-1}\,min^{-1}$); and $K_m$ is the Michaelis constant (M). In competitive inhibition, $V_{max}$ value does not change when the $K_m$ value increases. $K_i$ values were obtained by plotting $1/v_0$ versus 1/[S]. Lineweaver–Burk plots for *O basilicum* L PPO using five or six concentrations of catechol in the absence and presence of three different concentrations of 2,3-diaminopropionic acid were typical of competitive inhibition, as illustrated in Fig 1, in which $V_{max}$ values have not changed when $K_M$ values have increased. This result was shown to be the competitive inhibition of reaction between inhibitor and substrate catalyzed by *O basilicum* L PPO. The dependencies obtained justified this type of inhibition. The inhibition constants given for *O basilicum* L PPO in Table 3 were obtained by fitting of the experimental data with eqn (1). A similar result was obtained with agaritine on mushroom PPO when L-tyrosine was used as a substrate.[25] Jonavitz-Klapp *et al* found that the inhibition effect of some carboxylic acids on PPO using 4-methylcatechol as a substrate was competitive.[26]

(ii) *Non-competitive inhibition*. In reversible, non-competitive inhibition, both inhibitor and substrate can bind simultaneously to the enzyme molecule. Clearly two molecules must bind to different sites on the enzyme. The presence of inhibitor does not affect substrate binding but does interfere with the catalytic functioning of the enzyme. The actual mechanism of action of the

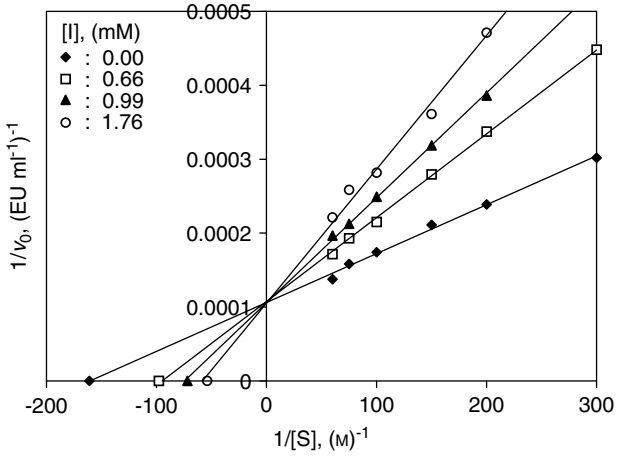


**Figure 1.** Lineweaver–Burk double reciprocal plots showing inhibition of *Ocimum basilicum* L PPO by 2,3-diaminopropionic acid using catechol as a substrate at pH 6.5 and 25 °C.

**Table 3.** Inhibition types and $K_i$ values of polyphenol oxidase obtained from different sources using catechol and pyrogallol as substrates

| PPO sources | Inhibitors | Substrates | [I], (mM) | $K_i$, (mM) | Type of inhibition | $R^2$ |
|---|---|---|---|---|---|---|
| *Ocimum basilicum* L | 2,3-Diaminopropionic acid | Catechol | 0.66 | 0.41 | Competitive | 0.9943 |
| | | | 0.99 | 1.03 | | 0.9993 |
| | | | 1.76 | 1.22 | | 0.9993 |
| Mushroom | Sodium azide | Catechol | 1.00 | 1.61 | Non-competitive | 0.9953 |
| | | | 1.67 | 1.70 | | 0.9964 |
| | | | 2.33 | 4.06 | | 0.9976 |
| Artichoke | Sodium azide | Catechol | 0.67 | 4.60 | Non-competitive | 0.9997 |
| | | | 1.33 | 3.60 | | 0.9985 |
| | | | 1.66 | 2.80 | | 0.9983 |
| | | | 2.50 | 2.70 | | 0.9975 |
| *Ocimum basilicum* L | Sodium azide | Catechol | 0.03 | 0.08 | Non-competitive | 0.9959 |
| | | | 0.07 | 0.05 | | 0.9826 |
| | | | 0.10 | 0.04 | | 0.9971 |
| Mushroom | 2,3-Diaminopropionic acid | Catechol | 1.33 | 2.68 | Uncompetitive | 0.9994 |
| | | | 2.67 | 2.42 | | 0.9971 |
| | | | 3.33 | 2.32 | | 0.9974 |
| Mushroom | 2,3-Diaminopropionic acid | Pyrogallol | 1.67 | 12.70 | Uncompetitive | 0.9997 |
| | | | 5.00 | 14.50 | | 0.9985 |
| Artichoke | 2,3-Diaminopropionic acid | Catechol | 0.44 | 0.31 | Uncompetitive | 0.999 |
| | | | 0.88 | 2.06 | | 0.9982 |
| | | | 1.32 | 1.90 | | 0.9973 |

inhibitor varies with each kind of molecule. The Lineweaver–Burk equation for non-competitive inhibition is

$$\frac{1}{v_0} = (1 + \frac{[I]}{K_i})(\frac{K_m}{V_{max}})\frac{1}{[S]} + (1 + \frac{[I]}{K_i})\frac{1}{V_{max}} \quad (2)$$

The plot of this equation consists of lines that have the slope $(1 + ([I]/K_i))(K_m/V_{max})$ and intercept $(1 + ([I]/K_i))(1/V_{max})$. As is seen from Figs 2, 3 and 4, it was found that the type of inhibition for mushroom, artichoke and *O basilicum* L PPOs were non-competitive inhibition using catechol as a substrate. Again, $K_i$ values obtained are given in Table 3.

(iii) *Uncompetitive inhibition*. In uncompetitive inhibition, the inhibitor binds directly to the enzyme–substrate complex but not to the free

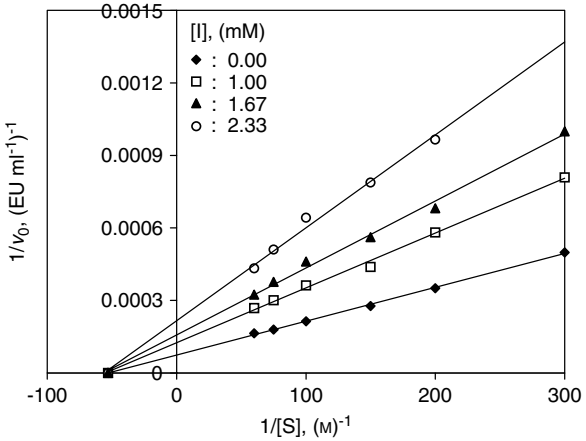


**Figure 2.** Lineweaver–Burk double reciprocal plots showing inhibition of mushroom PPO by sodium azide using catechol as a substrate at pH 6.5 and 25 °C.

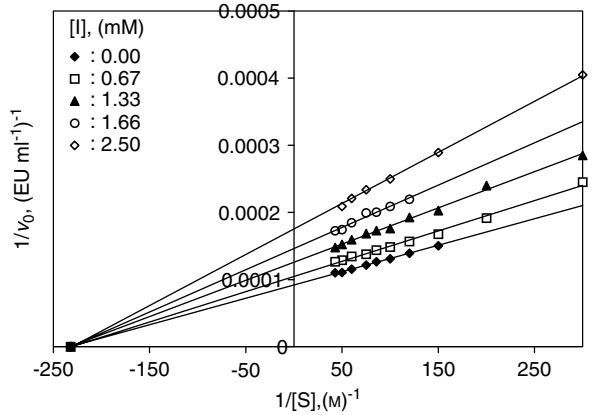


**Figure 3.** Lineweaver–Burk double reciprocal plots showing inhibition of artichoke PPO by sodium azide using catechol as a substrate at pH 6.5 and 25 °C.

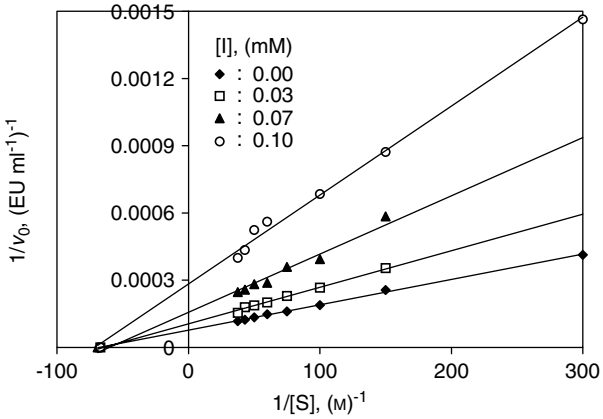


**Figure 4.** Lineweaver–Burk double reciprocal plots showing inhibition of *Ocimum basilicum* L PPO by sodium azide using catechol as a substrate at pH 6.5 and 25 °C.

enzyme. The Lineweaver–Burk equation for uncompetitive inhibition can be given as:

$$\frac{1}{v_0} = \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]} + \left(1 + \frac{[I]}{K_i}\right)\frac{1}{V_{max}} \qquad (3)$$

The Lineweaver–Burk equation for uncompetitive inhibition is linear with slope $K_m/V_{max}$ and intercept $(1 + [I]/K_i)/V_{max}$.

The inhibitory effect of 2,3-diaminobenzoic acid on the PPO was determined from Lineweaver–Burk double reciprocal plots. 2,3-Diaminopropionic acid for mushroom PPO showed a simple linear uncompetitive inhibition when catechol and pyrogallol were used as substrates. The dependencies obtained (Figs 5 and 6) justified assigning this type of inhibition. Furthermore, it was found that the type of inhibition was competitive of the inhibition of the artichoke PPO with sodium azide inhibitor using catechol as a substrate (Fig 7). The binding of the uncompetitive inhibitor, which needs to resemble the substrate, is envisaged to cause structural distortion of the active site, thereby

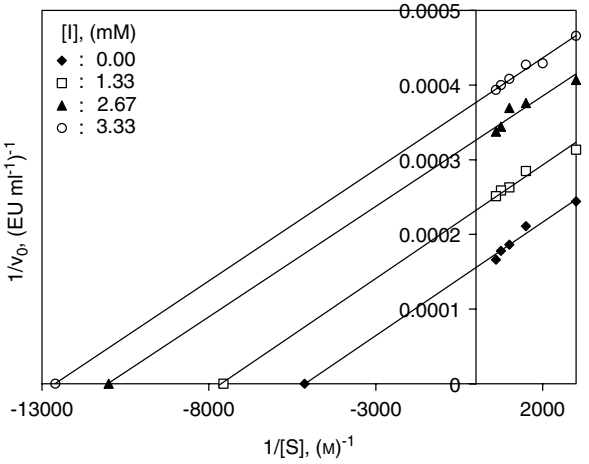


**Figure 5.** Lineweaver–Burk double reciprocal plots showing inhibition of mushroom PPO by 2,3-diaminopropionic acid using catechol as a substrate at pH 6.5 and 25 °C.

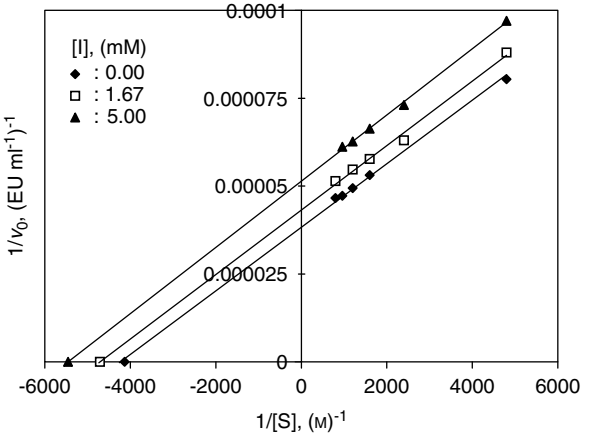


**Figure 6.** Lineweaver–Burk double reciprocal plots showing inhibition of mushroom PPO by 2,3-diaminopropionic acid using pyrogallol as a substrate at pH 6.5 and 25 °C.

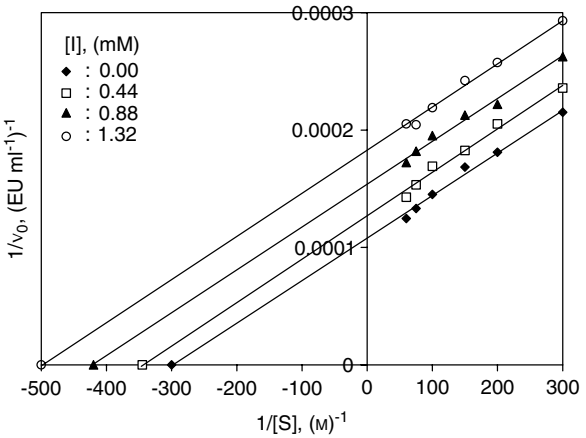


**Figure 7.** Lineweaver–Burk double reciprocal plots showing inhibition of artichoke PPO by 2,3-diaminopropionic acid using catechol as a substrate at pH 6.5 and 25 °C.

rendering the enzyme catalytically inactive. Uncompetitive inhibition requires that the inhibition affect the catalytic function of the enzyme but not its substrate binding.[23] The inhibition constant ($K_i$) values obtained were 2.47 and 13.51 mM for mushroom PPO at pH 6.5 and 25 °C with catechol and pyrogallol as substrates, respectively.

As described above, the type of inhibiton observed depended on the substrate and enzyme source used. Hence, no general rule can easily be established with regard to the type of inhibition observed. It has been reported that agaritine for mushroom PPO showed a simple linear uncompetitive inhibition when L-dopa was used as a substrate, but it showed a competitive inhibition when L-tyrosine was used as a substrate.[25] Walker and Wilson[27] suggested the existence of two distinct sites on the enzyme: one for the binding of the substrate and another, adjacent, site for binding the inhibitor. The type of inhibition also depends on the origin of the PPO studied. 2,3-Diaminopropionic acid exhibited the highest inhibition value with *O basilicum* L PPO ($K_i = 0.89$ mM), followed by artichoke PPO ($K_i = 1.42$ mM) and mushroom PPO ($K_i = 2.47$ mM), respectively, using catechol as a substrate. Again, as seen in Table 3, it was found that $K_i$ values for mushroom, artichoke and *O basilicum* L PPOs were 2.46, 3.43 and 0.06 mM, respectively, using sodium azide as a inhibitor and catechol as a substrate. Therefore, it can be said that 2,3-diaminopropionic acid has shown approximately the same extent of inhibition when compared with sodium azide. As a result, 2,3-diaminopropionic acid can be used as an inhibitor of PPO when comparing with sodium azide, which is also an inhibitor of PPO.

A linear regression method was used to determine whether the experimental data fitted with the inhibition equations. Linear regression coefficients are given in Table 3. As can be seen from this Table, the fact that regression coefficient values are in the range 0.98–0.99 shows that the experimental data fit better with related inhibition equations.

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