# Characterization of Monophenolase Activity of Table Beet Polyphenol Oxidase. Determination of Kinetic Parameters on the Tyramine/Dopamine Pair

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Polyphenol oxidase from table beet leaves was extracted in both soluble and membrane-bound forms. In both cases, the enzyme obtained was in its latent state. The latent form was activated by sodium dodecyl sulfate. The enzyme showed monophenolase and diphenolase activity. The monophenolase activity showed a lag period before the steady state rate ($V_{ss}$) was reached. This lag period depended on the pH, the enzyme and substrate concentrations, and the presence of catalytic amounts of *o*-diphenol. The experimental results correspond with the mechanism previously described for PPO from other sources. The kinetic parameters for both soluble and membrane-bound forms on the tyramine/dopamine pair were determined.

**Keywords:** *Plant polyphenol oxidase; table beet; monophenols; enzyme kinetics; tyramine; dopamine*

## INTRODUCTION

Several vegetables suffer the phenomenon of color modification during their storage and transport, which decreases the market value of the product. Red beet suffers browning and discoloration during the industrial processing. Color alterations are due to either formation or degradation of pigmented compounds usually present in the vegetable and are mediated by endogenous enzymatic activities such as polyphenol oxidase. In canned beet root, the slices show a discoloration or "black ring" and it has been demonstrated that polyphenol oxidase (PPO)[1] is responsible for mediating this discoloration (Lee and Smith, 1979; Im et al., 1990).

In plants, polyphenol oxidase (EC 1.14.18.1) is predominantly located in the chloroplast thylakoid membranes; however, Mayer and Friend (1960) described the presence of phenolase activity in chloroplast, mitochondrial, and soluble fractions in homogenates from leaves of sugar beet.

In living tissues, the phenolic substrate and the enzyme are separated within the cells, but upon extraction or other cell-damaging treatment, the enzyme and substrate may come in contact, permitting rapid oxidation of *o*-diphenols. The *o*-quinones formed during oxidation are highly reactive substances which normally react further with other quinones, amino acids, peptides, and proteins, thus altering not only the structural and functional properties of the protein but also its nutritive value (Matheis and Whitaker, 1984; García-Carmona et al., 1988).

One unusual and intriguing characteristic of this enzyme is its ability to exist in an inactive or latent state (Mayer and Harel, 1979). PPO can be activated by a variety of treatments or agents including acid and base shock (Kenten, 1957), urea (Swain et al., 1966), polyamines (Jimenez-Atienzar et al., 1991), anionic detergent, such as SDS (Kenten, 1958; Flurkey, 1986; Golbeck and Camarata, 1981; Sanchez-Ferrer et al., 1993; Chazarra et al., 1996), proteases (Golbeck and Camma-

rata, 1981; King and Flurkey, 1987; Soderhal and Soderhal, 1989; Sanchez-Ferrer et al., 1989), and fatty acids (Golbeck and Camarata, 1981; Hutcheson et al., 1980). SDS is particularly interesting as an activating agent because few enzymes are known to be activated by it, in contrast to the many enzymes that SDS inactivates. Thus, PPO in some species, such as broad bean, is active at high SDS concentrations that would cause the loss of catalytic activity in many other enzymes (Moore and Flurkey, 1990). Activation of PPO by denaturants was first reported by Kenten (1958). Robb et al. (1964) and Swain et al. (1966) suggested that activation was related to a limited conformational change in the latent enzyme. One of the more recent and thorough studies examining SDS activation of broad bean PPO has been carried out by Moore and Flurkey (1990), who demonstrated by means of intrinsic fluorescence experiments that SDS produced a conformational change.

Mayer (1966) demonstrated that the polyphenol oxidase present in sugar beet is linked to some membrane structure in the chloroplast and that it can be removed from this structure by cleavage with proteolytic enzymes, causing both activation and solubilization of the enzyme. However, the latent form of polyphenol oxidase in table beet was studied for the first time by us (Escribano et al., 1997).

To extract the enzyme in its latent form, the extraction method must be very mild to prevent its activation or modification, as occurs with acetone powders and ammonium sulfate fractionation (Golbeck and Cammarata, 1981). The purification method based on the temperature-induced phase partitioning of Triton X-114 (TX-114) has been previously described (Escribano et al., 1997).

PPO is a copper enzyme which catalyzes two different reactions, using molecular oxygen: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of the *o*-diphenols to *o*-quinones (diphenolase activity).

There are only few studies on sugar-beet monophenolase activity (Mayer and Friend, 1960; Mayer, 1964). The monophenolase activity of polyphenol oxidase shows

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a characteristic lag period before the maximum velocity of the hydroxylation step is reached (Cabanes et al., 1987). This activity can be followed by observing either the color of the *o*-quinone or consumption of oxygen. The time required to reach the steady state rate depends on several factors: the enzyme source (Valero et al., 1988); the concentration of monophenol (the lag period being longer when monophenol concentration is increased) (García-Carmona et al., 1988; Sanchez-Ferrer et al., 1988); the enzyme concentration (with the lag period diminishing, but never totally disappearing when the enzyme concentration is increased) (Cabanes et al., 1987; Ros et al., 1994), and finally, the presence of catalytic amounts of *o*-diphenol that completely abolish the lag period (Rodriguez-Lopez et al., 1992).

Numerous reports on the polyphenol oxidase action mechanism have appeared over the last three decades to explain the characteristics of monophenolase and diphenolase activities. A comprehensive review of this enzyme mechanism, in agreement with kinetic and structural studies, has been published recently (Sanchez-Ferrer et al., 1995). The model assumed could be applied in general for tyrosinase obtained from different biological sources as well as for different substrates. This mechanism perfectly explains the properties of diphenolase activity as well as the peculiar characteristics of monophenolase activity such as the existence of a lag period. According to the results of this work, this mechanism is discussed broadly in the Results and Discussion section.

In the present paper, polyphenol oxidase extracted from table beet leaves in its latent state and activated with SDS is used as an enzyme source and a study on its activities on pairs of substrates tyramine/dopamine is performed.

## MATERIALS AND METHODS

**Plant Material.** Table beet (variety cylindre) plants were grown in vermiculite for five weeks at 23 °C and 70% humidity. They were watered twice a week with Cron medium (Bond, 1951).

**Enzyme Extraction.** Polyphenol oxidase of beet leaf cells in its latent state was partially purified from osmotically shocked chloroplast prepared by using the method described by Sanchez-Ferrer et al. (1990) with some modifications (Escribano et al., 1997). A 10 g sample of beet leaves was added to 50 mL of extraction buffer that was 100 mM sodium phosphate buffer (pH 7.3) containing serine protease inhibitors which were added immediately before use (1 mM phenyl-methylsulfonyl fluoride and 1 mM *p*-aminobenzamidine hydrochloride). The mixture was homogenized in a mortar with 10 g of sand, filtered through eight layers of gauze, and centrifuged at 100*g* for 15 min at 4 °C. The precipitate was discarded, and the supernatant was centrifuged at 120000*g* for 30 min at 4 °C. The supernatant was considered the soluble fraction (PPO-S), and the pellet containing the membrane fraction was resuspended with 5 mL of 1.5% (w/v) TX-114 in extraction buffer and digested overnight at 4 °C under continuous agitation. After high-speed centrifugation (120000*g* for 30 min) at 4 °C, the supernatant with PPO activity was subjected to temperature phase partitioning by adding TX-114 at 4 °C so that the final detergent concentration was 8% (w/v). The mixture was kept at 4 °C for 15 min and then warmed to 37 °C. After 15 min, the solution became spontaneously turbid due to the formation, aggregation, and precipitation of large micelles composed of detergent, hydrophobic proteins, and the remaining chlorophylls and phenolic compounds. This solution was centrifuged at 5000*g* for 15 min at 25 °C. The supernatant was used as a membrane-bound enzyme source in its latent state (PPO-B).

**Spectrophotometric Measurement of Enzymatic Activity.** The spectrophotometric determination of both tyrosi-
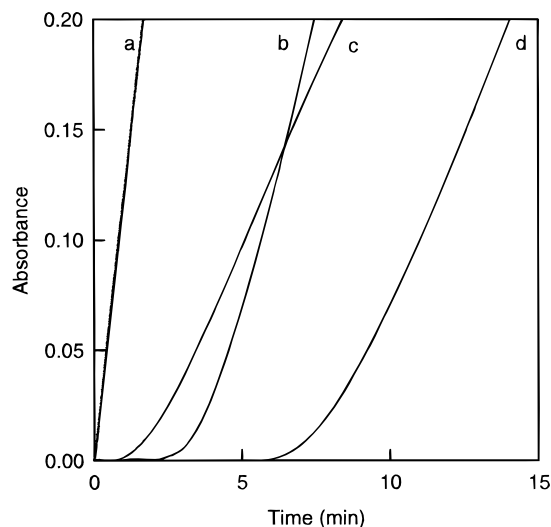


**Figure 1.** Enzymatic activities of PPO-B: (a) diphenolase activity (the reaction medium included 0.0075 mg/mL PPO, 0.69 mM SDS and 1 mM Dopamine in 50 mM sodium phosphate buffer, pH 6.8); (b−d) monophenolase activity [the reaction medium contained (b) 0.075 mg/mL PPO, 0.69 mM SDS, and 2 mM tyramine in 50 mM sodium phosphate buffer pH 6.8, (c,d) same as (b) except that in (c) the substrate concentration was 0.2 mM tyramine and in (d) the enzyme concentration was 0.038 mg/mL].

nase activities (monophenolase and diphenolase) on the substrates tyramine/dopamine by measuring the appearance of dopaminechrome at 480 nm ($\epsilon = 3300$ M$^{-1}$ cm$^{-1}$) (Rodriguez-Lopez et al., 1994) has been described in the literature . In this work, the international unit of enzyme (IU) was defined as the quantity of enzyme that produces 1 $\mu$mol of dopaminechrome/min at pH 6.8 and 25 °C.

Unless otherwise stated, the reaction medium (1.0 mL), at 25 °C, contained 50 mM sodium phosphate buffer (pH 6.8), 0.69 mM SDS, and L-tyramine or L-dopamine as substrate for monophenolase and diphenolase activities, respectively. The activity measurements were carried out at pH 6.8 in order to avoid the reaction of H$_2$O addition to the *o*-quinone that can occur at more acidic pH (Jimenez et al., 1984).

Protein concentration was determined by the Bradford Bio-Rad protein assay using serum albumin as a standard (Bradford, 1976).

**Data Analysis.** Kinetic data analysis was carried out by using linear and nonlinear regression fitting (Marquardt, 1963), using the Sigma Plot 2.01 for Windows program (Jandel Scientific, 1994).

## RESULTS AND DISCUSSION

PPO from table beet leaves was extracted as a soluble enzyme as well as a partially purified form from the membrane fraction. In both cases, the enzyme obtained was in its latent state and it was activated by SDS. The maximum of the activation was obtained using 0.69 mM SDS in the reaction medium. The enzyme had both diphenolase (Figure 1, curve a) and monophenolase activities (Figure 1, curves b−d).

**Characteristics of Monophenolase Activity.** Monophenolase activity was followed by measuring the action of PPO on L-tyramine. A marked lag period was observed when the appearance of the first stable product, dopaminechrome, was recorded. This lag is calculated as the intercept on the abscissa obtained by extrapolation of the linear part of the product accumulation curve. The steady state rate ($V_{ss}$) was defined as the slope of the linear part of the product accumulation curve. Both the lag period and the steady state rate were affected by the pH and the enzyme and
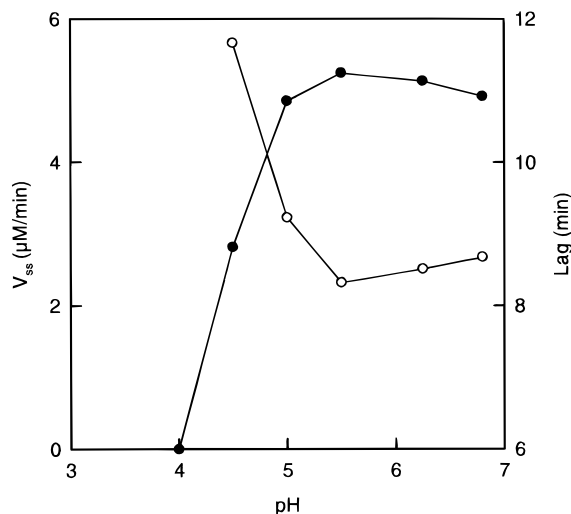
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**Figure 2.** Effect of pH on monophenolase activity (●) and on its lag period (○). The reaction medium included 0.014 mg/mL PPO-B, 0.69 mM SDS, and 0.5 mM tyramine in 50 mM sodium acetate (pH 4−5) or phosphate (pH 5.5−6.8) buffer.
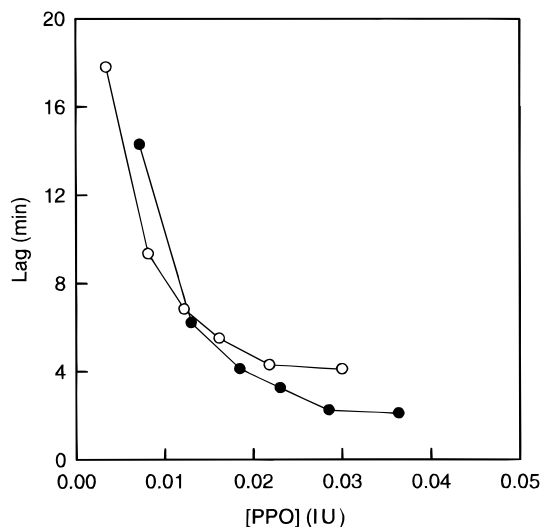


**Figure 3.** Dependence of the lag period on enzyme concentration. The reaction medium at 25 °C included 2 mM L-tyramine, 0.69 mM SDS in 50 mM phosphate buffer pH 6.8 with various PPO-B (●) or PPO-S (○) concentrations.



**Figure 4.** Effect of L-tyramine concentration on monophenolase activity of PPO (●) and on its lag period (○). The reaction medium at 25 °C contained 0.69 mM SDS, in 50 mM phosphate buffer, pH 6.8, and various tyramine concentrations, with 0.075 mg/mL (PPO-B) (A) or 0.1 mg/mL (PPO-S) (B).

substrate concentrations, as well as the presence of catalytic concentrations of *o*-diphenol.

*Effect of pH.* Monophenolase activity toward tyramine increased as the pH was increased from pH 4.0 and showed a maximum value at pH 5.5, even though it exhibited a broad plateau over the pH range of 5.0−7.0 (Figure 2). Similar results were obtained with the same activated enzyme using 4-*tert*-butylcatechol as a substrate (Escribano et al., 1997). The pH affected not only the enzyme activity but also the lag period (Figure 2), where the shortest lag period occurred at pH 5.5, coinciding with the highest steady state rate.

*Lag Period Dependence on Enzyme Concentration.* An increase of the enzyme concentration produced a decrease in the lag period. This effect was observed using the membrane-bound enzyme (PPO-B) and the soluble enzyme (PPO-S) (Figure 3). These results can be justified according to the proposed mechanism for tyrosinase (Ros et al., 1994; Sanchez-Ferrer et al. 1995). This behavior has been widely described for several PPO sources (Cabanes et al., 1987; Ros et al., 1994; Espin et al. 1995).
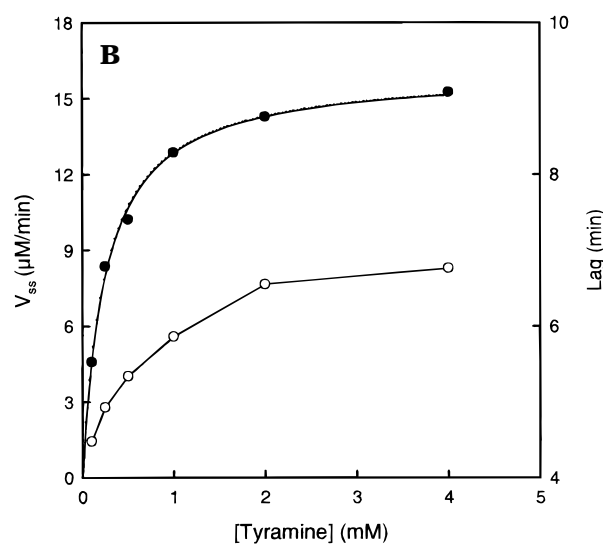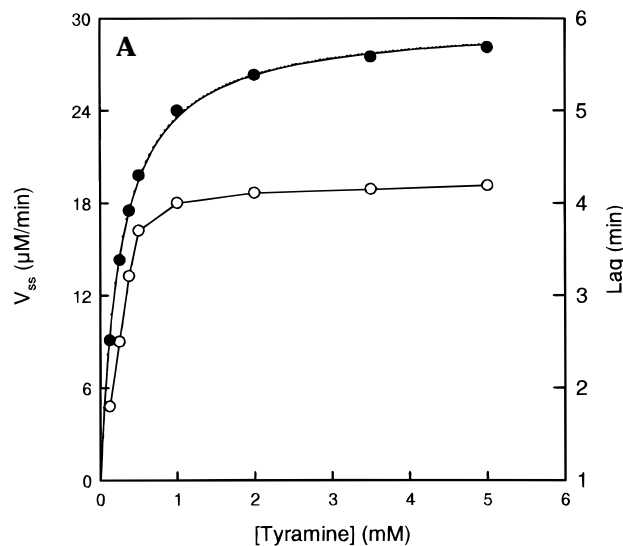
*Lag Period Control by Substrate Concentration.* As Figure 4 shows, both the lag period and the steady state rate also depended on the level of tyramine present in the reaction medium. As can be observed, the substrate-dependent lag is a parameter tending toward a maximum value, similar to that described for other plant PPOs (García Carmona et al. 1988; Sanchez-Ferrer et al. 1988; Ros et al. 1994; Espin et al. 1995).

The kinetic parameters ($K_m$ and $V_m$) were evaluated from the data in Figure 4 by using nonlinear regression fitting (Marquardt, 1963) (Table 1). As can be observed, $K_m$ values were similar for the membrane-bound enzyme and the soluble enzyme. However, the catalytic efficiency ($V_m^M/K_m^M$) of the monophenolase activity was 2 times higher for PPO-B than for PPO-S.

*Effect of o-Diphenol Addition.* The lag period observed in the monophenolase activity can be modified by the addition of different concentrations of diphenol (Figure 5). Since the lag period is the time required to reach the *o*-diphenol concentration in steady state, the addition of *o*-diphenol to the enzyme assay shortens the lag. An increase in the dopamine concentration shortened the lag period, although above a certain concentration a burst in activity resulted, so that the shortening

**Table 1. Kinetic Parameters That Characterize the Action of Polyphenol Oxidase from Table Beet Leaves on the Pair Monophenol/Diphenol:Tyramine/Dopamine**

| kinetic parameters[a] | tyramine at pH 6.8 and SDS | dopamine at pH 6.8 and SDS | dopamine at pH 5.5 and SDS | dopamine at pH 5.5 |
|---|---|---|---|---|
| | | PPO-S | | |
| $V_m$ (mM/min) | 0.0016 | 0.050 | 0.09 | 0.015 |
| $K_m$ (mM) | 0.253 | 0.42 | 0.36 | 0.38 |
| $V_m/K_m$ (min$^{-1}$) | 0.0064 | 0.12 | 0.25 | 0.039 |
| | | PPO-B | | |
| $V_m$ (mM/min) | 0.0030 | 0.08 | 0.102 | 0.027 |
| $K_m$ (mM) | 0.265 | 0.45 | 0.40 | 0.44 |
| $V_m/K_m$ (min$^{-1}$) | 0.011 | 0.18 | 0.26 | 0.06 |

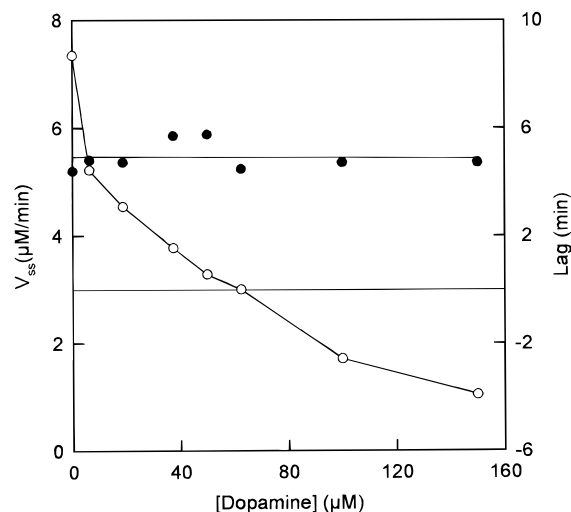[a] The $V_m$ values are normalized with the same protein concentration (mg/mL).



**Figure 5.** Effect of catalytic amounts of dopamine on the steady state rate of monophenolase activity (●) and on the lag period (○). The reaction medium at 25 °C contained 0.69 mM SDS, 0.5 mM tyramine, and 0.014 mg/mL PPO-B in 50 mM sodium phosphate buffer, pH 6.8, with various dopamine concentrations (0−150 mM).
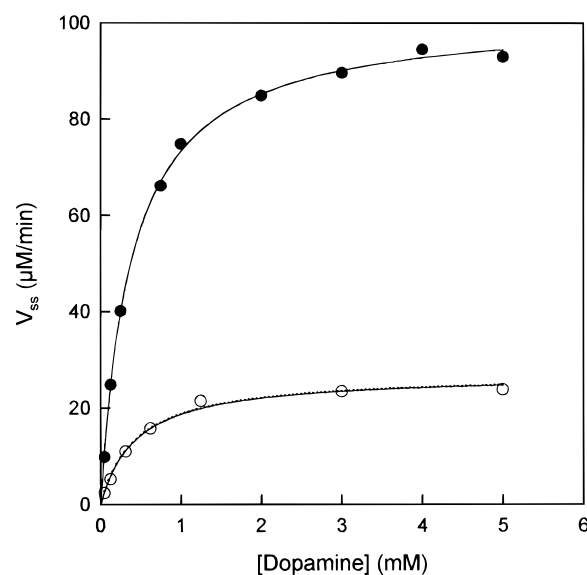


**Figure 6.** Effect of dopamine concentration on the initial rate in the presence and absence of SDS. The reaction medium at 25 °C contained 0.011 mg/mL (PPO-B) and various substrate concentrations in 50 mM sodium acetate buffer, pH 5.5 without (○) or with 0.69 mM SDS (●).

in the lag period led to a null value and even negative values were attained (Figure 5). In other words, a rise in the initial *o*-diphenol concentration ([D]$_o$) shortened the time required for the steady state level of *o*-diphenol ([D]$_{ss}$) to be reached. When [D]$_o$ = [D]$_{ss}$, the value of [D]$_{ss}$ was reached quickly and no lag period was detected in the initial monophenolase activity. However, when [D]$_o$ > [D]$_{ss}$, the system must first consume the excess of *o*-diphenol (D) and then gradually consumes the monophenol (M) and D before the steady state is finally reached. In these conditions, there was a burst in the activity, which was characterized by negative values of the lag period. Therefore, the physical meaning of the "+" or "−" sign of the lag period corresponded to the type of assay conditions with a substeady state or oversteady state level of [D]$_o$, respectively. It should be noted that the $V_{ss}$ was the same for every *o*-diphenol concentration chosen.

**Diphenolase Activity.** The diphenolase activity was followed by measuring the action of polyphenol oxidase on dopamine. The kinetic characterization of the diphenolase activity of PPO on dopamine led to the determination of $K_m$ and $V_m$ at pH 6.8 (Table 1). For the soluble enzyme as well as for the membrane-bound enzyme, the $K_m^D$ values were slightly higher than the $K_m^M$ values, whereas $V_m^D$ were markedly higher than $V_m^M$ when the same PPO concentration was used (Table 1). Therefore, the catalytic efficiency of PPO on diphe-

nol ($V_m^D/K_m^D$) increased up to 18 times with respect to that with monophenol ($V_m^M/K_m^M$), this ratio being quite similar to the ones calculated for tyramine/dopamine with tyrosinase from mushroom and grape (Ros et al., 1994). As in the case of monophenolase activity, the catalytic efficiency on diphenol was nearly 2 times higher for PPO-B than for PPO-S.

In order to study whether or not the activation with SDS introduces a change in $K_m$ and $V_m$ values, the kinetic parameters using dopamine were determined in the absence and presence of SDS at pH 5.5. Activation by SDS was studied at this pH because the highest activating effect of SDS was found here, whereas at pH 6.8 without SDS, the activity is negligible (Escribano et al., 1997).

The results obtained when dopamine concentration was varied in the presence and absence of SDS are shown in Figure 6. By fitting these data to the Michaelis−Menten equation, the kinetics parameters were evaluated. The effect of SDS on these parameters is shown in Table 1. The presence of SDS decreased the $K_m$ for dopamine and increased the $V_m$ with PPO-S as well as PPO-B, resulting in a 6.5-fold increase in the catalytic efficiency ($V_m/K_m$) of the activated PPO-S, whereas only a 4-fold increase was found in the case of the activated PPO-B. Comparing the catalytic efficiency on dopamine for the two latent enzyme forms, PPO-B showed a higher ($V_m/K_m$) value than the soluble enzyme form (Table 1). However, the kinetics parameters for the monophenolase activity of the latent enzyme could not be evaluated because of its undetectable activity without SDS in the reaction medium.

In this paper, we have studied the monophenolase activity of PPO from table beet on L-tyramine. All these results could be satisfactorily explained with the mechanism previously described for PPO from other sources and it was mentioned in the Introduction (Sanchez-Ferrer et al., 1995) (Scheme 1). This model takes into account the occurrence of the three forms of the enzyme (indicated as $E_{met}$, $E_{oxy}$, $E_{deoxy}$) and the chemical recycling of the *o*-quinones formed by the enzyme (step 9 in Scheme 1). This model is fundamentally based on the following:

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**Scheme 1. Reaction Mechanism for the Monophenolase and Diphenolase Activities of PPO[a]**
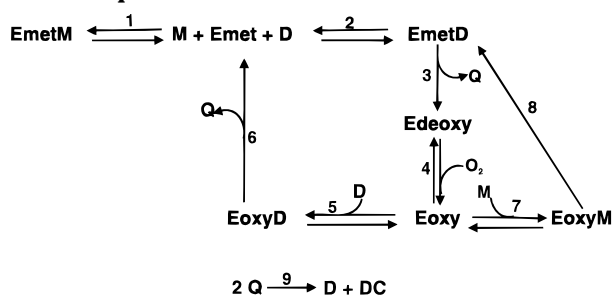


$^a$ M, monophenol; D, *o*-diphenol; Q, *o*-quinone; DC, aminochrome; $E_{met}$, met-PPO or oxidized form of PPO with $Cu^{2+}-Cu^{2+}$ in the active site; $E_{oxy}$, oxy-PPO or the oxidized and oxygenated form with $Cu^{2+}-Cu^{2+}$ in the active site; $E_{deoxy}$, desoxy-PPO or reduced form with $Cu^{+}-Cu^{+}$ in the active site (Sanchez-Ferrer et al. 1995).

During diphenolase activity, *o*-diphenol (D) binds both to $E_{oxy}$ (step 5) and $E_{met}$ (step 2), rendering $E_{oxy}D$ and $E_{met}D$ intermediates, which give rise to two *o*-quinones (Q) (steps 3 and 6). These two *o*-quinones recycle to regenerate one *o*-diphenol (D) and one aminechrome (DC) (step 9). The lack of a lag period can be explained by the binding and transformation of *o*-diphenols into *o*-quinones by the $E_{met}$ and $E_{oxy}$ forms.

During monophenolase activity, the binding of monophenol (M) to the $E_{oxy}$ form renders $E_{met}D$ (step 8), which gives rise *o*-quinone (step 3), the binding of monophenol to $E_{met}$ (with no catalytic activity on monophenols) (step 1) scavenges a portion of tyrosinase from the catalytic turnover as a dead-end complex $E_{met}M$ in the steady state of monophenolase activity. The enzyme slowly re-enters the catalytic cycle (step 2) by means of the diphenol obtained by recycling in the chemical reactions (step 9), being transformed into the $E_{oxy}$ form (steps 2–4); this puts an end to the lag period and produces the level of diphenol necessary to maintain the steady state. Therefore, the lag period can only be interpreted as a dynamic equilibrium between the enzymatic and chemical steps to obtain the steady state diphenol concentration. To reach such a concentration, the presence of a small amount of $E_{oxy}$ form in the native PPO and the recycling steps are necessary. The former requirement stems from the fact that $E_{oxy}$ is the only enzyme form able to transform monophenol into $E_{met}D$, which subsequently renders *o*-quinone. The enzymatically generated quinones produce new molecules of *o*-diphenols, which drive the $E_{met}M$ inactive forms into the catalytic cycle, thus generating new *o*-diphenol molecules until the steady state is reached.

An increase in the amount of enzyme means that more $E_{oxy}$ is accessible to the catalytic cycle, which means that there is more enzymatic activity and so the level of *o*-diphenol in the steady state is reached more quickly, and therefore, the lag period diminishes (Figure 3). On the other hand, an increase of monophenol concentration in the reaction medium leads to more enzyme draining into the dead-end complex $E_{met}M$, which implies that a higher level of *o*-diphenol is necessary for the steady state to be reached, and so, the lag period increases (Cabanes et al., 1987; Rodriguez-Lopez et al., 1992) (Figure 4).

The addition of small amounts of *o*-diphenol concentrations at the start of the reaction leads to a shortening of the lag period, due to the lower time required for the attainment of the corresponding level of *o*-diphenol for the steady state to be reached. Thus, when the level of

*o*-diphenol added is higher than the level required to reach the steady state, the system yields a burst in activity but it always reaches the same steady state rate (Figure 5).

In conclusion, the kinetic characterization of the soluble and the membrane-bound table beet polyphenol oxidase has been carried out. The two enzyme forms showed both monophenolase and diphenolase activities. The PPO-B presented a higher catalytic efficiency on tyramine and dopamine than the PPO-S. Both enzyme forms have similar $K_m^M$ values for the monophenol. However, the $K_m^D$ for the diphenol was slightly higher for the PPO-B in all assay conditions. Both enzyme forms were extracted in its latent state. The activation by SDS was studied for dopamine. The presence of SDS decreased the $K_m^D$ and increased the $V_m^D$ in both soluble and membrane-bound forms.

## ABBREVIATIONS USED

PPO, polyphenol oxidase; SDS, sodium dodecyl sulfate; TX-114, Triton X-114; PPO-B, membrane-bound polyphenol oxidase; PPO-S, soluble polyphenol oxidase; $K_m^M$, $K_m$ value for monophenol; $K_m^D$, $K_m$ value for diphenol; $V_m^M$, maximum rate of the monophenolase activity; $V_m^D$, maximum rate of the diphenolase activity; $(V_m^M/K_m^M)$, catalytic efficiency of PPO toward monophenol; $(V_m^D/K_m^D)$ catalytic efficiency toward diphenol.

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