

Differential Activation of a Latent Polyphenol Oxidase Mediated
by Sodium Dodecyl SulfateFERNANDO GANDÍA-HERRERO, MERCEDES JIMÉNEZ-ATIÉNZAR, JUANA CABANES,
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A kinetic study of the activity of soluble and membrane-bound latent polyphenol oxidase (PPO) extracted from beet root (*Beta vulgaris*) was carried out. For the first time, two types of behavior (hyperbolic and sigmoid) are reported in the same enzyme for PPO activation by the surfactant sodium dodecyl sulfate (SDS), depending on substrate nature. A kinetic model based on cooperative systems is developed to describe the activation effect of SDS, enabling the determination of the number of surfactant molecules binding to the enzyme in the activation process. The results indicate that the active site of the enzyme is not affected by SDS and that a stepwise conformational change favors the access of hydrophobic substrates compared to hydrophilic ones. Differential activation of PPO mediated by SDS may be of relevance in the control of PPO activity since the enzyme is able to express activity toward a specific substrate while remaining latent to others.

KEYWORDS: Polyphenol oxidase; tyrosinase; latency; activation; sodium dodecyl sulfate; *Beta vulgaris*; betalains

INTRODUCTION

Polyphenol oxidase, PPO (monophenol, *o*-diphenol:oxygen oxidoreductase; EC 1.14.18.1) is a copper-containing enzyme that 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) (1, 2). This enzyme has been found in microorganisms, animals, and plants, and thus PPO is responsible not only for browning in plants but also for melanization in animals. In the plant kingdom PPO has been detected in most fruits and vegetables and is predominantly located in the chloroplast thylakoid membranes (3–6). However, it is not an intrinsic membrane protein and it can be released from the thylakoids by sonication, mild detergent treatment, or proteases. The enzyme has also been detected in soluble fractions in homogenates from different vegetables (7).

PPO is a very important enzyme in the food industry since during the processing of fruits and vegetables any wounding may cause cell disruption and lead to quinone formation. The appearance of food and beverages may be affected but also the taste and nutritional value, often decreasing the quality of the final product (8, 9). Because of the considerable economic and nutritional loss induced by enzymatic browning in the commercial production of fruits and vegetables, numerous studies have been devoted to the biochemical and catalytic properties of PPO (7, 10).

The implication of PPO in the secondary metabolism of betalains has been proposed in plants belonging to the order Caryophyllales (11–13), like beet (*Beta vulgaris*). Interest in betalains has grown since their antiradical activity was characterized (14–16) and they are widely used as additives in the food industry because of their natural colorant properties and absence of toxicity, even at high concentrations (17, 18).

One unusual and intriguing characteristic of this enzyme is its ability to exist in an inactive or latent state (19, 20). PPO can be released from latency, or activated, by a variety of treatments or agents, including acid and base shock (21, 22), anionic detergents, such as sodium dodecyl sulfate (SDS) (5, 19, 23), proteases (3, 24), and fatty acids (25).

The use of SDS is of particular interest since few enzymes are known to be activated by this detergent, while many are inactivated. Thus PPO is active at high SDS concentrations (19), which would denature many other enzymes. The activation process by SDS detergent was found to involve a reorganization of protein tertiary structure (19, 26, 27). A limited conformational change due to binding of small amounts of SDS may induce or initiate the activation of the latent enzyme, and it was reported that the low optimum pH obtained for the latent enzyme was abolished in the presence of SDS. The possible relationship between these two activating factors (SDS and pH) has been studied in broad bean, grape, and lettuce PPOs (5, 28–30). Furthermore, the reversibility of the SDS-mediated activation of latent PPO from peach has been demonstrated by use of cyclodextrins as an agent able to trap SDS molecules (31). With respect to the physiologically relevant counterpart of the

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detergent, it has been suggested that lipids might fulfill this role (32), and SDS activation of tyrosinase has been demonstrated in vivo with *Terfezia clavaryi* sections (33). However, a kinetic study of the PPO activation by SDS depending on substrate nature has not been carried out.

Thus, the aim of this paper was to study the variations in the kinetic behavior of the latent beet root PPO through the activation process mediated by SDS, by use of different types of substrates. For the first time, we report a differential activation of PPO by SDS depending on the substrate nature. This kinetic behavior is modeled and interpreted in terms of a conformational change that modifies the access of the substrates to the active site of the enzyme but does not affect the structure of the active site itself.

MATERIALS AND METHODS

Plant Material. *Beta vulgaris* roots (granadina variety) were grown in an ecological plantation with no addition of pesticides. The roots were harvested, sliced, frozen in liquid nitrogen, and stored at -80°C until use.

Chemicals. PPO substrates and SDS were obtained from Sigma (Barcelona, Spain). All other chemicals were of analytical grade.

Subcellular Fractionation. Soluble and membrane fractions were obtained from red beet root slices, as previously described (34). Briefly, a beet root homogenate was centrifuged at 1000g for 10 min. The pellet, containing the wall fraction, was discarded, and the supernatant was centrifuged at 120000g for 40 min. The supernatant was considered as the soluble fraction, and the pellet, the membrane fraction. The soluble fraction was brought up to 35–85% $(\text{NH}_4)_2\text{SO}_4$. The salt content was removed by dialysis against 10 mM sodium phosphate buffer, pH 7.0. The extraction of PPO from the membrane fraction was carried out through treatment with Triton X-114 (34). To avoid possible activation of the PPO enzyme by endogenous proteases, PMSF (phenylmethanesulfonyl fluoride) and benzamidine hydrochloride were added before and after the dialysis to give a final concentration of 1 mM.

Spectrophotometric Assays. Monophenolase and diphenolase activities were determined spectrophotometrically at 25°C by measuring the appearance of reaction products in the medium. Catechol, 4-methylcatechol (4MC), and 4-*tert*-butylcatechol (4tBC) oxidations were followed by the appearance of the *o*-benzoquinone product ($\epsilon = 1450$, 1350, and $1150\text{ M}^{-1}\text{ cm}^{-1}$, respectively) at 390 nm (catechol) and 400 nm (4MC and 4tBC). Aminechrome formation for L-tyrosine and L-3,4-dihydroxyphenylalanine (L-DOPA) ($\epsilon = 3600\text{ M}^{-1}\text{ cm}^{-1}$) was monitored at 475 nm, and for tyramine and dopamine ($\epsilon = 3300\text{ M}^{-1}\text{ cm}^{-1}$), at 480 nm (35). SDS studies were performed in 50 mM sodium phosphate buffer, pH 6.5. Other conditions are detailed in the text and figure captions. The percentage of enzyme activation was calculated as % activation = [(activity – latent activity)/(maximum activity – latent activity)] \times 100. Spectrophotometric measurements were performed in a Kontron Uvikon 940 spectrophotometer (Kontron Instruments, Zurich, Switzerland).

Experiments were performed in triplicate and the mean and standard deviations were plotted.

Kinetic Data Analysis. The values of K_m and V_m on different substrates were calculated from measurements of the steady-state rate, V_{ss} , which was defined as the slope of the linear portion of the product accumulation curve. The lag period was estimated as the intercept on the abscissa axis obtained by extrapolation of the linear portion. Kinetic data analysis was carried out by linear and nonlinear regression fitting (36), by use of SigmaPlot Scientific Graphing for Windows version 8.0 (2001; SPSS Inc., Chicago, IL).

Protein Determination. Protein concentration was determined according to the Bradford Bio-Rad (Hercules, CA) protein assay with serum albumin as standard (37).

RESULTS AND DISCUSSION

The activation process of beet root PPO mediated by the anionic detergent SDS was studied with several monophenolic

and diphenolic substrates. The selected compounds were the L-tyrosine/L-DOPA and tyramine/dopamine pairs along with the hydrophobic diphenols catechol, 4MC, and 4tBC. The in-depth characterization of the enzymatic activation was carried out on the latent PPO obtained from soluble and membrane fractions of red beet root. The effect of the anionic detergent SDS has been studied in relation to the surfactant concentration, pH, and the nature of the substrate used to follow the enzymatic activity.

pH Study and Degree of Activation of PPO by SDS. pH is a determining factor in the expression of enzymatic activity; it alters the ionization states of amino acid side chains or the ionization of the substrate. PPO activity was measured, at different pH values, in the absence or presence of 0.69 mM SDS. At this detergent concentration the enzyme was fully active, as later demonstrated, and the concentration is below the cmc (critical micelle concentration) for the detergent at the experimental conditions (19, 38).

A pH variation in the absence and presence of SDS was carried out on the soluble PPO for all the selected substrates (Figure 1). In the absence of SDS, almost no activity was detected at acid pH values for most of the substrates (Figure 1) with the exception of the more hydrophobic ones, 4MC (Figure 1E) and 4tBC (Figure 1F). All the substrates showed an optimum pH over pH 5.5 under these conditions.

In the presence of SDS, the optimum pH value was the same for the monophenol as for the corresponding diphenol, and this value did not change in the absence of SDS. Thus, the optimum pH was 6.0 for the L-tyrosine/L-DOPA pair (Figure 1B,C) and 6.5 for tyramine (Figure 1A) and dopamine. The activation degree reached in the presence of SDS was 4-fold for L-tyrosine and tyramine, whereas for L-DOPA and dopamine it was 2-fold. For monophenols, the characteristic lag period (2) was also affected by the pH and a decrease could be observed when pH was increased, in both the absence and presence of SDS in the reaction medium (results not shown). With respect to hydrophobic substrates, the enzyme exhibited an optimum pH of 5.5 toward 4MC and 4tBC (Figure 1E,F). For catechol, the optimum pH observed was 7.0 (Figure 1D). A significant difference in the degree of activation by SDS was not found (around 2-fold) for these substrates. Thus the activation degree was higher for the enzyme acting on monophenols than on diphenols.

The pH profiles for all substrates studied in PPO from the membrane fraction were similar to those obtained with the soluble enzyme. However, the degree of activation changed dramatically. Beet root PPO bound to membrane was fully latent. In all cases the degree of activation was higher than 100-fold, in contrast to the less than 4-fold activation found for the soluble PPO. Figure 2 shows the pH profiles obtained for the most hydrophobic substrate, 4tBC (Figure 2A), and for the most hydrophilic L-tyrosine/L-DOPA pair (Figure 2B, inset). It is noticeable that, in the absence of SDS, the membrane PPO showed activity at acidic pH values (optimum pH = 4.5). However, this effect was not observed in the soluble PPO that was not totally latent (Figure 1C,F). Other authors (5, 19, 21, 23) have proposed that PPO activity can be induced by acid shocking, depending on the enzyme source. The presence of SDS eliminated the low optimum pH.

Differences in the pH profile found for beet root PPO depending on the presence of SDS for different substrates suggest that the activating effect of the detergent may be related to substrate nature. Therefore, the subsequent experiments were carried out to elucidate the mechanism for SDS activation.

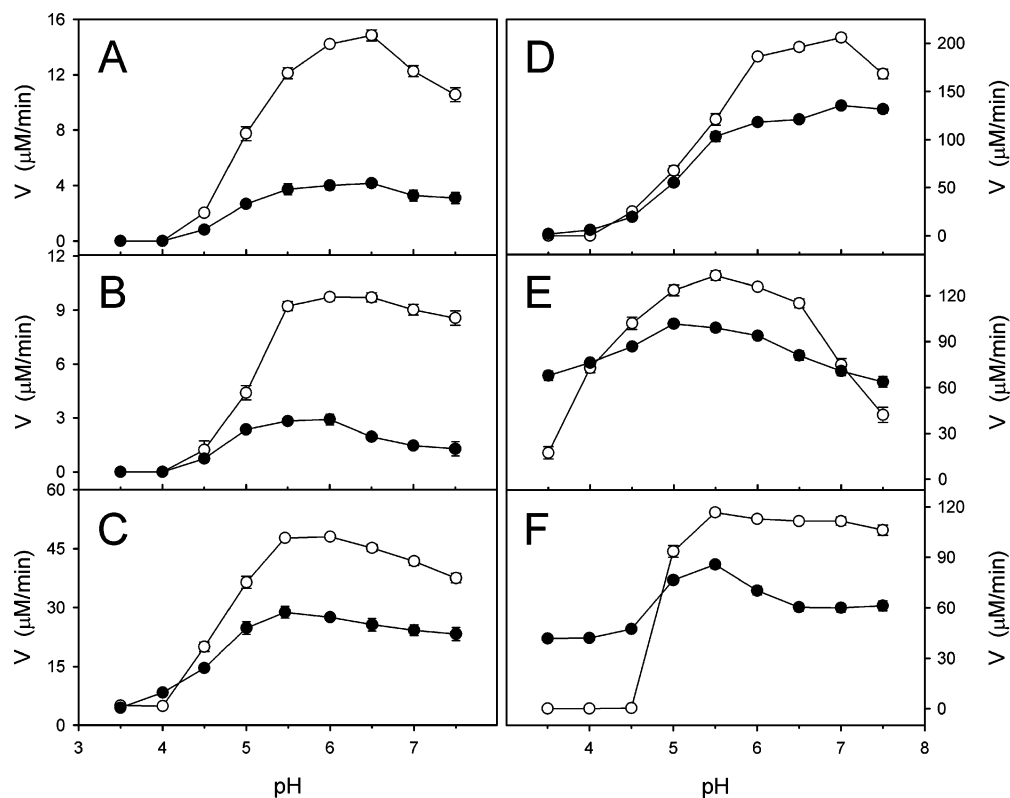


Figure 1. Effect of pH on the soluble PPO in 50 mM sodium acetate or sodium phosphate buffer (pH 3.5–7.5) in the presence (○) or absence (●) of 0.69 mM SDS. The reaction medium at 25 °C contained 3.0 mM tyramine (A), 0.75 mM tyrosine (B), 3.8 mM L-DOPA (C), 80.0 mM catechol (D), 10.0 mM 4MC (E), 15.0 mM 4tBC (F), and 5 $\mu\text{g}/\text{mL}$ soluble PPO for diphenols and 50 $\mu\text{g}/\text{mL}$ for monophenols.

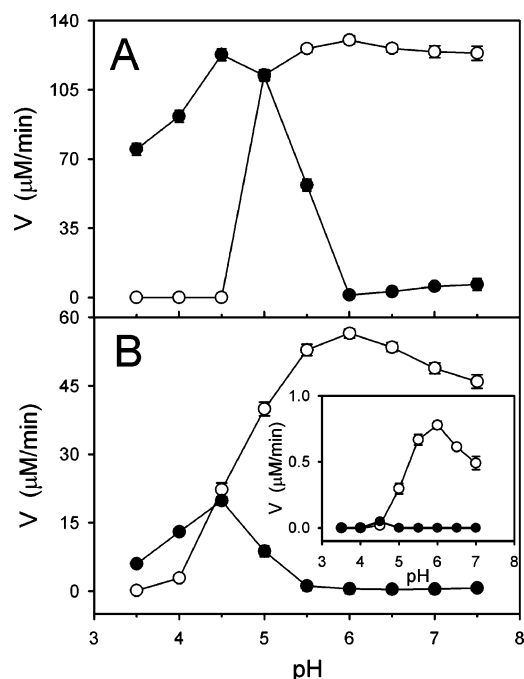


Figure 2. Effect of pH on the membrane-bound PPO in 50 mM sodium acetate or sodium phosphate buffer (pH 3.5–7.5) in the presence (○) or absence (●) of 0.69 mM SDS. The reaction medium at 25 °C contained 12.4 $\mu\text{g}/\text{mL}$ PPO extracted with TX-114 and 15.0 mM 4tBC (A), 3.8 mM L-DOPA (B), or 0.75 mM tyrosine (B, inset).

Effect of SDS Concentration Depending on the Substrate Nature. The effect of SDS concentration on the enzymatic activity at a saturating concentration for each substrate was studied. The soluble PPO activity showed hyperbolic behavior with the hydrophobic substrates catechol, 4MC, and 4tBC

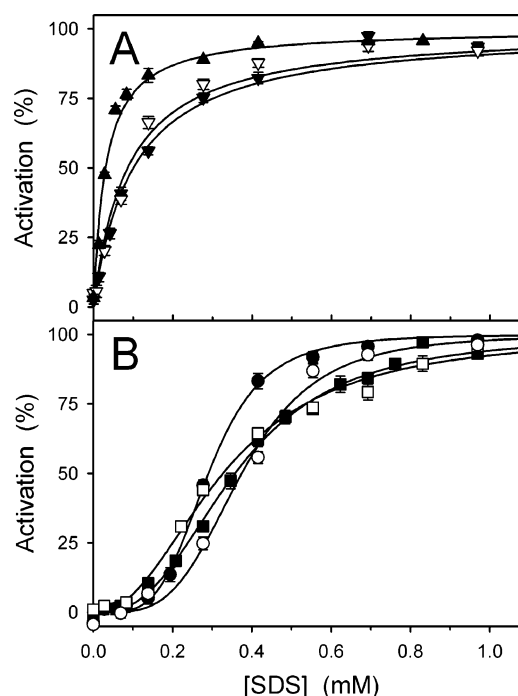


Figure 3. Percentage of soluble PPO activation at different SDS concentrations in the presence of catechol (▼), 4MC (▽), or 4tBC (▲) (A) or L-tyrosine (○), L-DOPA (●), tyramine (□), or dopamine (■) (B). The reaction medium at 25 °C contained 50 mM sodium phosphate, pH 6.5, 5 mg/mL soluble PPO for diphenols and 50 mg/mL for monophenols, and 80.0 mM catechol, 10.0 mM 4MC, or 15.0 mM 4tBC (A) or 0.75 mM tyrosine, 3.8 mM L-DOPA, 3.0 mM tyramine, or 5.0 mM dopamine (B).

(Figure 3A). However, PPO was activated in a sigmoidal manner with increasing SDS concentrations toward the L-

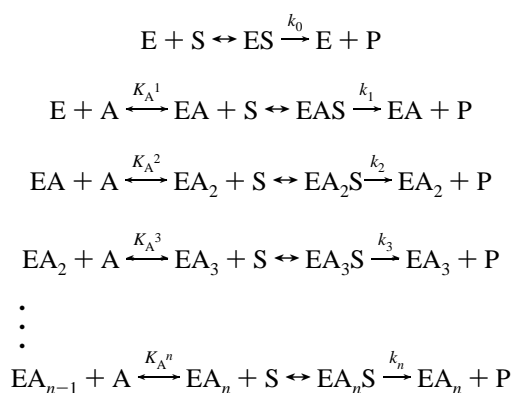
tyrosine/L-DOPA and tyramine/dopamine pairs (**Figure 3B**). The lag period observed in the monophenolase activity was also modified by the presence of SDS, reaching a minimum at SDS concentrations over 0.5 mM, at which 100% activation was observed (results not shown).

In the presence of hydrophobic substrates, the curvature of the hyperbole was related to the degree of hydrophobicity (4tBC > 4MC > catechol). Furthermore, the maximum activation for the hydrophobic substrates was reached at low SDS concentrations (near 0.1 mM) at which the degree of activation was negligible for the hydrophilic substrates tyramine, dopamine, L-tyrosine, and L-DOPA (**Figure 3B**). Therefore, as can be observed in **Figure 3**, for the soluble PPO, the behavior of the enzyme activation and the SDS concentration at which maximum activity was reached was different for each substrate.

The same effect could be observed for the PPO solubilized from the membrane fraction. **Figure 4** shows the activity of PPO toward the physiological substrate, L-DOPA, and toward the most hydrophobic, 4tBC, at different SDS concentrations. Because PPO from the membrane fraction was fully latent, at low SDS concentrations (0.1 mM) the enzyme activity was practically null against L-DOPA, whereas the enzyme showed maximum activity with 4tBC. Therefore, similar behavior was found for both the membrane and soluble fractions. These results strongly suggest that the conformational change needed for the enzyme to express its maximum activity toward a substrate is dependent on the substrate nature, with the binding of more SDS molecules (and higher SDS concentrations) for the more hydrophilic substrates being necessary.

Model for PPO Differential Activation Mediated by SDS.

A general kinetic model can be suggested to explain the differential activation of PPO depending on substrate nature described for beet root. In the absence of SDS the enzyme remains in a latent state with null (membrane fraction) or limited (soluble fraction) activity. The mechanism can be described by the following scheme, which is analogous to the model of Koshland et al. (39):



The positive kinetic cooperativity observed in the steady-state rate is due to the fact that the enzyme (E) undergoes conformational changes toward a more active form (EA_n) induced by the binding of discrete molecules of the activator (A). The SDS molecules are as monomers, since the detergent concentration is below the cmc under the assay conditions. For the same enzyme, the active form and thus the number of SDS molecules (n) needed to express the maximum activity varies depending on the nature of the substrate (S). Thus, a stepwise mechanism for activation allows the existence of PPO activity toward a substrate while the enzyme remains latent to others. The Hill equation fits the suggested mechanism, as demonstrated in the model of Koshland et al. (39), and for SDS activation,

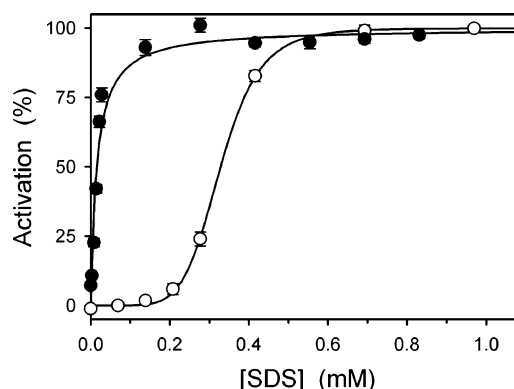


Figure 4. Percentage of membrane PPO activation at different SDS concentrations in the presence of 4tBC (●) and L-DOPA (○). The reaction medium at 25 °C contained 50 mM sodium phosphate, pH 6.5, 12.4 mg/mL PPO extracted with TX-114, and 15.0 mM 4tBC or 3.8 mM L-DOPA.

the Hill coefficient (n_H) relates to the number of SDS molecules binding to the original latent enzyme:

$$v = \frac{v_{\max}[A]^{n_H}}{K_H^{n_H} + [A]^{n_H}}$$

According to the proposed scheme, the presence of enzyme forms with partial activity before the maximum activation for each substrate is reached implies a lowering of the Hill coefficient and the possibility of obtaining nonnatural values. Thus, n_H calculated from activity versus [SDS] graphs indicates the minimum number of SDS molecules needed for the total activation of the enzyme.

For hydrophobic substrates (which show a hyperbolic behavior), n_H was 1, indicating that the enzyme is active toward these molecules since the first SDS monomer is bound to its surface at very low SDS concentrations. For hydrophilic substrates, the sigmoidal curve is described by n_H values ranging from 2.2 to 6.1. For soluble PPO, the values of n_H were 2.2 (tyramine), 2.7 (dopamine), 3.9 (L-tyrosine), and 4.2 (L-DOPA), indicating that the number of SDS molecules needed for activation is higher for more hydrophilic substrates. In the case of the membrane-bound PPO, more SDS molecules were necessary to show the maximum activity toward the most hydrophilic substrates. The Hill coefficients determined were 3.5 (L-tyrosine), 3.7 (dopamine), and 6.1 (L-DOPA).

Differences in the number of SDS monomers needed for activating the enzyme (n_H) make the differential activation of latent PPO possible. These results corroborate that the ability of SDS to activate the enzyme involves a limited conformational change due to the binding of small amounts of SDS (19). The access of hydrophobic substrates to the active site is favored since the first molecules of SDS are bound to the enzyme, while hydrophilic substrates require a deeper change for full access (activity).

Differential activation of PPO mediated by SDS, which would play an equivalent role to the physiological one fulfilled by lipids *in vivo* (32), enables activity toward a substrate while the same enzyme is latent to others. This may be of physiological relevance, mainly for the membrane-bound form of PPO. The data collected in the present study and the kinetic model proposed are in agreement with differential activation and positive cooperativity induced by SDS.

Effect of SDS on Enzyme Kinetics. The effect of SDS on the kinetic parameters was determined in order to characterize in detail the behavior of the enzyme toward each substrate. To

Table 1. Kinetic Parameters for Soluble and Membrane-Bound PPO Activity^a

substrate	SDS ^b	K _m (mM)	V _m (mM/min)	V _m /K _m (min ⁻¹)	optimum pH
Soluble Enzyme					
L-tyrosine	—	2.79	2.73 × 10 ⁻³	0.98 × 10 ⁻³	6.0
	+	1.98	8.73 × 10 ⁻³	4.41 × 10 ⁻³	6.0
L-DOPA	—	2.48	0.101	0.041	5.5
	+	2.32	0.200	0.086	6.0
tyramine	—	0.27	1.24 × 10 ⁻³	4.59 × 10 ⁻³	6.5
	+	0.35	4.32 × 10 ⁻³	0.012	6.5
dopamine	—	0.34	0.082	0.242	6.5
	+	0.35	0.193	0.559	6.5
catechol	—	8.94	0.275	0.031	7.0
	+	7.77	0.550	0.071	7.0
4MC	—	1.85	0.189	0.102	5.0
	+	1.60	0.353	0.221	5.5
4tBC	—	6.59	0.206	0.031	5.5
	+	4.04	0.385	0.095	5.5
Membrane-Bound Enzyme					
L-tyrosine	+	1.08	1.85 × 10 ⁻³	1.72 × 10 ⁻³	6.0
L-DOPA	+	2.21	0.077	0.035	6.0
dopamine	+	0.33	0.082	0.247	6.5
4tBC	+	3.95	0.161	0.041	6.0

^a All assays were performed in reaction media containing different substrate concentrations in 50 mM sodium phosphate buffer, pH 6.5 at 25 °C. The V_m values are normalized with the same protein concentration (12.4 μg/mL). ^b When SDS was present in the medium, the final concentration was 0.69 mM.

study whether the activation with SDS introduces or not a change in K_m and V_m values, both were determined in the absence and presence of 0.69 mM SDS at the pH value (pH 6.5) at which the degree of activation was higher for most of the substrates. The results obtained are summarized in Table 1. For the soluble fraction, the activation with SDS introduces a change in V_m values for all the substrates, with ~2-fold increase for diphenols and more than 3-fold increase for monophenols. The K_m value remained quite similar for most of them. Therefore, the increase in the catalytic power (V_m/K_m) through the presence of SDS was mainly due to the change in V_m (Table 1).

The K_m values for the L-tyrosine/L-DOPA pair of the SDS-activated soluble PPO are in the range of those values found for another betalain-producing PPO, like the one present in callus of *Portulaca grandiflora* (40). Tyrosine and DOPA are PPO substrates of physiological relevance in the first steps of biosynthesis of the plant pigments betalains in plants of the order Caryophyllales, which includes beet (11). As can be seen, soluble beet root PPO oxidizes a variety of monophenols and diphenols, and dopamine is the best substrate. Among the monophenols, tyramine was a better substrate than tyrosine.

For the membrane fraction, the kinetic parameters were determined in the presence of SDS at pH 6.5. PPO activity was barely detectable in the absence of SDS at this pH value, and therefore the kinetic parameters could not be evaluated. As can be observed in Table 1, the K_m values were quite similar to those evaluated for the soluble fraction.

We can conclude that the activation by SDS introduces a change in the catalytic power (V_m/K_m) for both the soluble and membrane PPO toward different substrates, which is mainly due to the increase of the V_m value. The insensitivity of K_m to the SDS presence is indicative of preservation of the active site, despite the conformational change provoked. The discrete molecules of SDS binding to PPO modify the tertiary structure of the enzyme, improving the accessibility of the substrates to the active site, without directly affecting its integrity. A differential substrate access mediated by the binding of SDS

monomers may constitute the basis of the differential activation. Thus, the existence of a regulatory peptide not related to the affinity of the enzyme fits with the data obtained in the present study. Its presence can be assumed in relation to the “shield region” described in hemocyanins to block the access of phenolic compounds, and also proposed in the structural model of a latent catechol oxidase (41, 42).

The results of this work regarding the differences in the pH profile of PPO according to the presence of SDS, the behavior of the enzyme activation for different substrates, and the SDS concentration at which maximum activity was reached support the existence of differential activation of PPO mediated by the surfactant SDS, depending on the substrate nature. The presence of sigmoidal or hyperbolic behavior in the activation process of the same latent enzyme is reported. For the first time, the conformational change provoked by the detergent is described by a stepwise kinetic model based on cooperativity, which allows the determination of the number of SDS molecules binding to PPO in the activating process. The kinetic scheme proposed might shed light on the mechanisms that regulate the enzyme activity in vivo, since it describes how PPO is able to express activity toward a specific substrate while remaining latent to others.

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