Monophenolase Activity of Latent Banana Pulp Polyphenol Oxidase

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The monophenolase activity of latent banana polyphenol oxidase (PPO) partially purified by using a combination of two aqueous two-phase systems based on Triton X-114 and PEG 8000 (Sojo et al. *J. Agric. Food Chem.* **1998**, *46*, 4924−4930) has been characterized in the presence of sodium dodecyl sulfate (SDS). The purification method used avoids the loss of monophenolase activity described for other banana pulp PPOs obtained by acetone powders. The activity presented a lag period before the steady-state rate was reached. The activity and lag period depended on the SDS, substrate, and enzyme concentration, the pH, and the presence of catalytic amounts of *o*-diphenol. The enzyme was characterized with an enzyme activation constant, K_{act}, of 2.4 μM. On the basis of these results we discuss why monophenolase activity is less studied (or detected) in plants based on the existence of two enzymatic forms, Eoxy and Emet, and the method used to purify the plant enzyme.

Keywords: *Polyphenol oxidase; monophenolase; monophenols; banana; tyramine; enzyme kinetics*

INTRODUCTION

Polyphenol oxidase (PPO; EC 1.14.18.1) is an oxidoreductase that is widely distributed in plants and, due to its poor specificity for several phenolic substrates, generally recognized as being responsible for the enzymatic browning reaction which occurs during the handling, storage, and processing of fruits and vegetables. The enzyme catalyzes two different reactions in the presence of molecular oxygen. The first, and the only specific reaction catalyzed by this enzyme, is the hydroxylation of monophenols to *o*-diphenols, a reaction that is usually termed monophenolase or cresolase activity [for a review, see Sánchez-Ferrer et al. (1995)]. The second, diphenolase activity, consists of the oxidation of the self-generated *o*-diphenols to the corresponding *o*-quinones, which are highly reactive molecules and which polymerize to brown, red, or black pigments depending on the natural components present in a given plant material. This last reaction can be achieved not only by PPO but also by other enzymes, such as peroxidase and laccase after cell decompartmentalization (Kalyanaraman et al., 1984).

A definitive way to confirm that an active PPO is present in a plant material is by determining the presence of monophenolase activity (Sánchez-Ferrer et al., 1988). However, there are very few studies of monophenolase activity in plant (Cabanes et al., 1987; Sánchez-Ferrer et al., 1988, 1989, 1993a,b; Valero et al., 1988; Ros et al., 1994; Espín et al., 1995a,b, 1996a,b, 1997), which is related to two factors: the lability of the enzyme during the purification process (Matheis et al., 1987) and the assay methods used to follow this activity (Sánchez-Ferrer et al., 1988, 1993a,b, 1995).

Banana pulp PPO is a clear example of the continuing disagreement as to whether the enzyme catalyzes both monophenolase and diphenolase activities. Palmer (1963) reported that the enzyme was very effective toward dopamine but inactive toward any monophenols, and similar results have been described by several authors (Galeazzi and Sgarbieri, 1981; Galeazzi et al., 1981). On the other hand, Deacon and March (1971), Montgomery and Sgarbieri (1975), and Oba et al., 1992 demonstrated the capability of different banana pulp PPOs to *o*-hydroxylate tyramine and *p*-cresol. In 1971, Thomas and Nair showed that an enzyme preparation from the skin tissues of banana fruits contained higher diphenolase and lower monophenolase activities than a preparation from pulp tissues. Later, Thomas and Janave (1986) postulated that monophenols were hydroxylated at a faster rate in the pulp tissues and that the diphenols thus formed were translocated to the skin, where their concentration was several times higher than in the pulp. However, no detailed kinetic study on this activity has been carried out.

The aim of this paper was to carry out a detailed kinetic study on the parameters that affect latent monophenolase activity in banana pulp.

MATERIALS AND METHODS

The bananas (*Musa acuminata* subgr. Cavendish var. Spanish Pequeña Enana) produced in the Canary Islands and used in this study were obtained from a commercial source in Murcia (Coplaca, S.A.). After sitting in ethylene gas in the dark for 24 h, the samples were stored at 17 °C for 72 h until they reached stage 4 with the peel "more yellow than green" (Sunday and Dismas, 1994) and with a soluble solids content of 10%.

Substrates were purchased from Sigma Chemical Co. (Madrid, Spain), and the other reagents were of analytical grade. Banana PPO was partially purified in a two-phase system with Triton X-114 (TX-114) and PEG 8000/phosphate.

Diphenolase activity was measured as described in a previous paper using dopamine as substrate (Sojo et al., 1998). Monophenolase activity toward tyramine was also determined spectrophotometrically at 480 nm at 25 °C ($\epsilon = 3300\ M^{-1}\ cm^{-1}$). The steady-state rate was calculated from the linear zone of the product accumulation curve after the lag period. In a total

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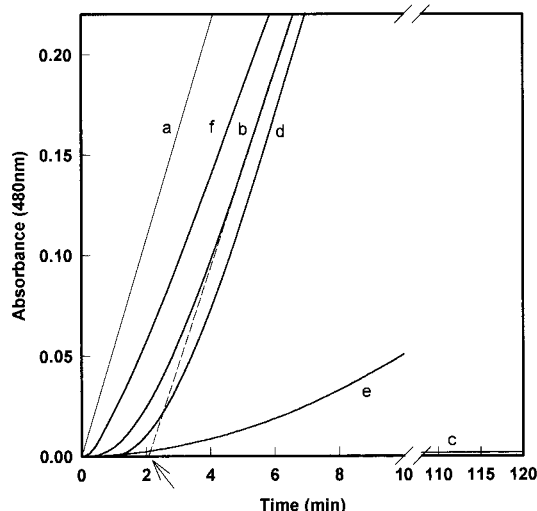


Figure 1. Enzymatic activities of banana PPO. (a) Diphenolase activity at 25 °C. The reaction medium included 7 μg/mL PPO and 2.5 mM dopamine in 10 mM phosphate buffer (pH 6.5). (b−f) Monophenolase activity at 25 °C in the standard reaction medium (b), in the standard reaction medium without SDS (c), in the standard reaction medium but with 2 mM of tyramine (d), in the standard reaction medium but with 14 μg/mL PPO (e), and in the standard reaction medium with the addition of *o*-diphenols to the reaction medium (10 μM dopamine) (f). The arrow indicates the lag period.

volume of 1 mL the standard reaction mixture included 35 μg of partially purified banana pulp PPO, 1 mM tyramine, and 2 mM SDS in 10 mM sodium phosphate buffer (pH 6.5). One unit of enzyme was taken as the amount that produced 1 μmol of product/min.

RESULTS

Latent banana pulp PPO, when partially purified by using consecutive phase partitioning in TX-114 and PEG 8000/phosphate (Sojo et al., 1998), shows both diphenolase (Figure 1a) and monophenolase activities (Figure 1b−f). The latter is characterized by a lag period, defined as the intercept on the abscissa obtained by extrapolation of the linear part of the product accumulation curve. This lag period has been reported for other PPOs from various sources when monophenolase activity is measured (Lerch, 1981; Robb, 1984; Sánchez-Ferrer et al., 1988; Nuñez-Delicado et al., 1996; Espín et al., 1997). The lag period and the steady-state rate (defined as the slope of the linear part of the accumulation product curve) were affected by SDS and substrate concentration, the pH, and the presence of catalytic concentrations of *o*-diphenols (Figure 1b−f)

Effect of SDS. Monophenolase activity of latent enzyme toward tyramine was measured in increasing SDS concentrations from 0.05 to 6 mM (Figure 2). It showed a clearly defined maximum at 2 mM, close to its critical micelle concentration (cmc; 1.36 mM) (Nuñez-Delicado et al., 1996), with a progressive decrease in activity thereafter; this profile is similar to those described for the latent enzyme when diphenolase activity was measured (Moore and Flurkey, 1991; van Gelder et al., 1997; Sojo et al., 1998).

The SDS concentration affected not only enzyme activity but also the lag period, which became shorter as SDS concentration increased, until it leveled out. This profile for the monophenolase activity of a latent PPO has not been described previously in the bibliography despite the number of latent PPOs studied (Sánchez-Ferrer et al., 1993b; Valero and García-Carmona, 1992; Chazarra et al., 1996; Nuñez-Delicado et al., 1996). To avoid any interference in the activation process of the latent banana pulp PPO when monophenolase activity was assayed, the other factors that affect this activity were assayed in the presence of 2 mM SDS.

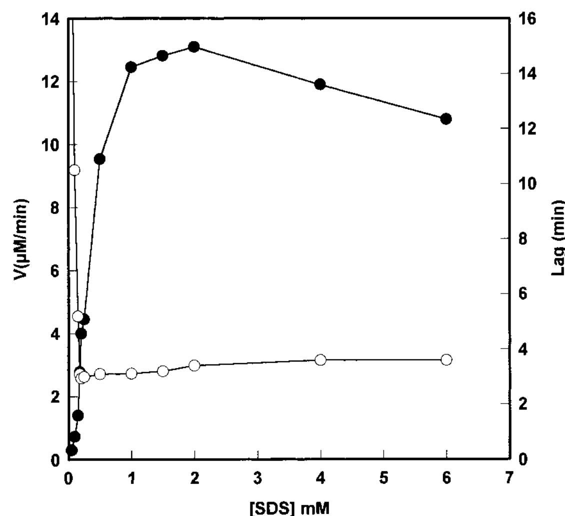


Figure 2. Effect of SDS concentration on monophenolase activity (●) and on its lag period (○). The reaction medium at 25 °C included 35 μg/mL partially purified PPO, 2 mM tyramine, and SDS ranging in concentration from 0 to 6 mM in 10 mM sodium phosphate buffer (pH 6.5).

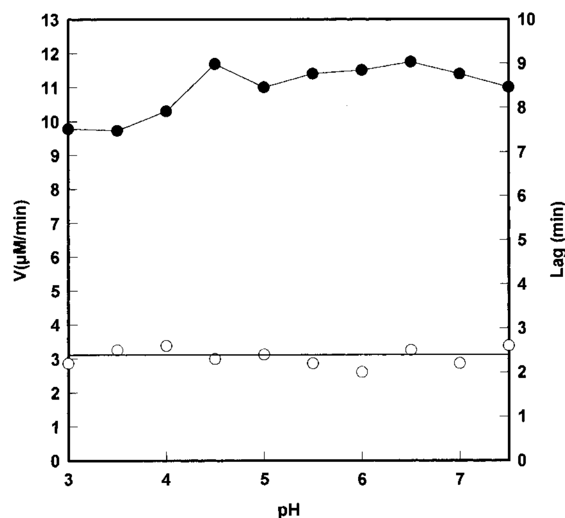


Figure 3. Effect of pH on monophenolase activity (●) and on its lag period (○). The reaction medium at 25 °C included 35 μg/mL partially purified PPO, 2 mM tyramine, and 2 mM SDS in 10 mM sodium phosphate buffer (pH 5.5−7.5) and sodium acetate pH (3.0−5.5).

Effect of pH. As in the case of diphenolase activity, the monophenolase activity of latent banana pulp PPO measured in the presence of SDS was unaffected by changes in the pH from 3.5 to 7.5 (Figure 3). The lag period was also unaffected. These results were in contrast with the well-defined pH profile described for a fully active banana pulp PPO with a maximum at pH 6.0 (Deacon and March, 1971) and with the pH profile of other plant PPOs (Cabanes et al., 1987; Sánchez-Ferrer et al., 1988; Valero et al., 1988; Ros et al., 1994; Espín et al., 1995a, 1996b, 1997).

The above-described flat pH profile was maintained even after 6 h of incubation at different pH values ranging between pH 3.0 and 7.5 (data not shown). This

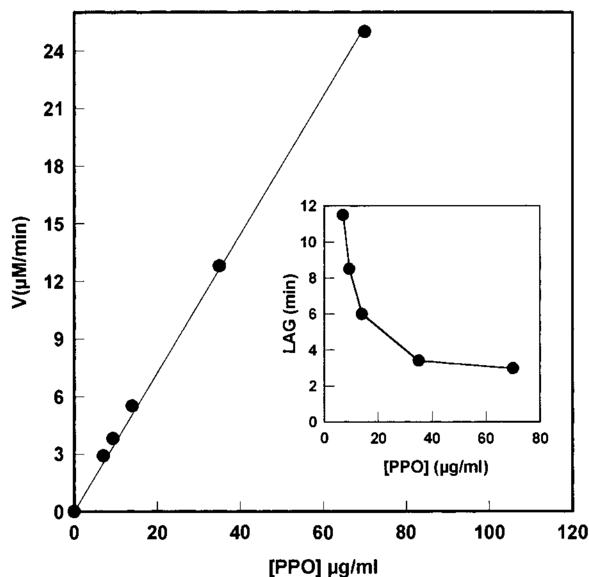


Figure 4. Effect of enzyme concentration on monophenolase activity of PPO and on the lag period. (Inset) The reaction medium at 25 °C included 2 mM tyramine and 2 mM SDS in 10 mM sodium phosphate buffer (pH 6.5) with different PPO concentrations.

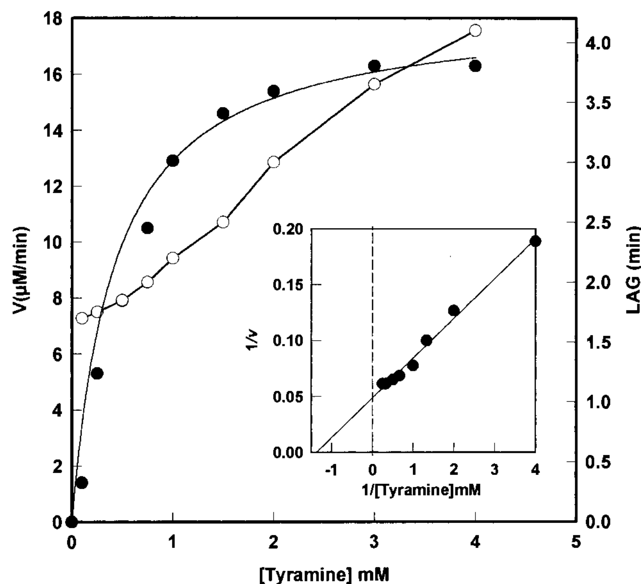


Figure 5. Effect of substrate concentration on monophenolase activity of PPO (●) and on the lag period (○). The reaction medium at 25 °C included 35 μg/mL partially purified PPO and 2 mM SDS in 10 mM sodium phosphate buffer (pH 6.5) with different tyramine concentrations. (Inset) Lineweaver–Burk plot.

enzyme stability at different pH values is of note and contrasts with the complete inactivation found by Deacon and March (1971) after 30 min at pH 3.5 when banana pulp PPO was purified using acetone powders.

Effect of Enzyme Concentration. The monophenolase activity of latent PPO was affected by enzyme concentration in the reaction medium, its steady-state rate increasing linearly (Figure 4) and the lag period decreasing hyperbolically (Figure 4, inset), a behavior that has been widely described in other PPOs (Cabanes et al., 1987; Ros et al., 1994; Espín et al., 1995a,b, 1996a,b, 1997).

Effect of Substrate Concentration. When the concentration of the naturally occurring monophenol (tyramine) was increased from 0.1 to 4 mM (Figure 5), the lag period of partially purified banana pulp PPO increased, in a similar way to that described for other plant PPOs (García-Carmona et al., 1988; Sánchez-Ferrer et al., 1988, 1993a; Espín et al., 1995a,b, 1996a,b, 1997), although the exact shape of the curve depends on the enzyme source used.

The kinetic constants, K_m and V_m, were evaluated from the data of Figure 5 (inset) by a Lineweaver–Burk plot and gave values of 0.75 mM and 20 μM/min, respectively. This K_m could not be compared with any other banana PPO data, even though Deacon and March (1971) used tyramine in their study, or with other PPOs because *p*-cresol was usually used as substrate (Sánchez-Ferrer et al., 1988, 1993a) or, more recently, with other synthetic monophenols in the presence of the nucleophile MBTH (Espín et al., 1995a,b, 1996a,b, 1997).

The catalytic efficiency (V_m/K_m) of diphenolase and monophenolase activities, which were evaluated after the respective values of K_m and V_m were obtained for dopamine (Sojo et al., 1998) and tyramine, was calculated. The diphenolase/monophenolase ratio was 4.7, which indicates that at pH 6.5, the oxidation of diphenols rather than that of monophenols is favored, as is usual for other plant PPOs (Sánchez-Ferrer et al., 1988, 1989, 1993a, 1995) and opposite to the postulate of Thomas and Janave (1986), in which some isoenzymes hydroxylated monophenols at a faster rate in the pulp than *o*-diphenols.

Effect of *o*-Diphenols. It is well-known that the monophenolase activity lag period can be shortened or eliminated by the addition of reducing agents or *o*-diphenols, which act as cosubstrates (Pomerantz and Warner, 1967; Duckworth and Coleman, 1970; Lerch and Ettlinger, 1972; García-Carmona et al., 1979). The effect of the latter compounds on the monophenolase activity of partially purified banana PPO was studied according to the protocol of Pomerantz and Warner (1967), assuming that the *o*-diphenol bound to the "activation center" by a simple isotherm and that the lag period was proportional to the nonactive enzyme concentration at zero time, using the empirical formula (Pomerantz and Warner, 1967)

$$\frac{1}{l} = \frac{1}{L} + \frac{1}{L}\frac{[o\text{-diphenol}]}{K_{act}}$$

where l, L, and K_{act} represent the lag period in the presence of *o*-diphenol, the lag period in the absence of *o*-diphenol, and the diphenol activation constant, respectively. Pomerantz and Warner (1967) interpreted the K_{act} as the *o*-diphenol affinity constant for the activation center. Figure 6 shows the decrease in the lag period as *o*-diphenol concentration was increased to 50 μM, at which stage the lag was abolished. To check that the *o*-diphenol added was used as cosubstrate and not as substrate, the steady-state rate was plotted against *o*-diphenol concentration (Figure 7), and no changes were observed up to 50 μM. Above this concentration the rate increased proportionally, indicating that the *o*-diphenol was being used as substrate for the diphenolase activity.

The activation constant K_{act} was calculated from the reciprocal of the lag period in the presence of diphenol (Figure 6, inset). The value obtained was 2.4 μM, which is lower than that described for potato PPO (Sánchez-Ferrer et al., 1993a).

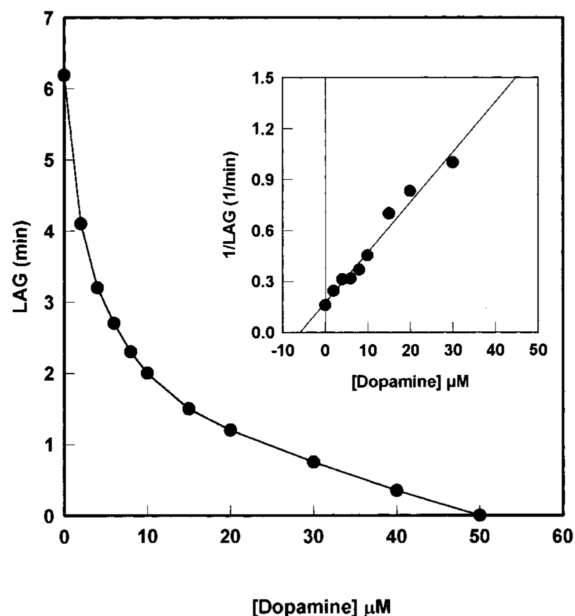


Figure 6. Influence of dopamine in shortening of the lag period. In the standard reaction medium with 2 mM tyramine, 14 μg/mL PPO, and 2 mM SDS, the concentration of dopamine ranged from 0 to 50 μM. (Inset) Evaluation of the activation constant, K_{act}, by the Pomerantz equation (Pomerantz and Warner, 1967).

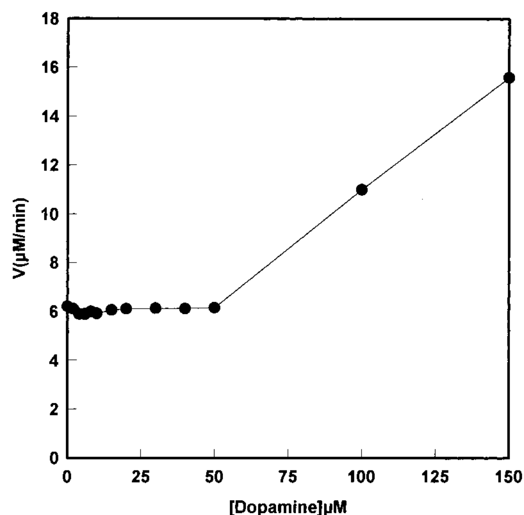


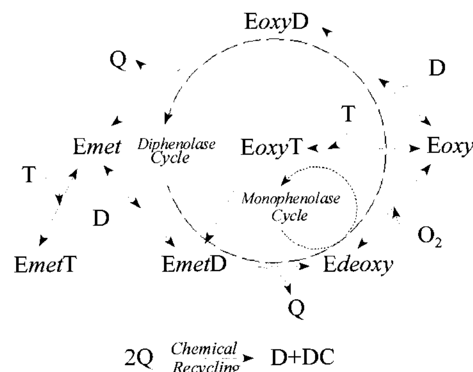
Figure 7. Effect of catalytic amounts of dopamine on the steady-state rate of monophenolase activity. The conditions are the same as in Figure 6.

DISCUSSION

To understand the results of this paper and how monophenolase activity works, it is necessary to describe the kinetic mechanism of the enzyme proposed by Cabanes et al. (1987). This model takes into account the occurrence of three forms of the enzyme (E*met*, E*oxy*, and E*deoxy*) and the chemical redox recycling of the *o*-quinones formed by the enzyme (Scheme 1). When a diphenol is present in the medium, this substrate binds to both to E*oxy* and E*met* to give E*oxy*D and E*met*D intermediates, which, in turn, give rise to two quinones. In our case, these two dopamine quinones redox recycle to regenerate one *o*-diphenol (dopamine) and one dopaminechrome (DC). This cycle shows no lag period when dopaminechrome accumulation is followed (Figure 1a).

During the monophenolase cycle, the binding of monophenol to the E*oxy* form renders E*met*D, which gives a quinone. During binding of the monophenol to the E*met*, a portion of the enzyme is scavenged from the catalytic turnover as a dead-end complex (E*met*T). Exit from this nonactive E*met*-monophenol form is possible only by upsetting of the equilibrium with the *o*-diphenol obtained by recycling the quinones in the chemical reaction. The time needed to produce the catalytic level of diphenol for the steady-state rate to be maintained is the lag period shown by this activity (Figure 1b−f), which may last from minutes to hours (Figure 1b), depending on a variety of factors:

Scheme 1. Catalytic Cycle for the Oxidation of Monophenol and Diphenol Substrates to *o*-Quinones by PPO in the Presence of O_2[a]



[a] For details, see text. T, tyramine; D, dopamine; Q, *o*-quinone; DC, dopaminochrome.

(a) Amount of Eoxy Form in the Enzymatic Extract. This amount ranges from 2 to 30% depending on the source (Lerch, 1981). At the low level of this range are the latent PPOs, which need to be activated before monophenolase activity can be measured (Figure 1c−f). Of the activated agents, SDS is the most effective (Figure 2). Because the E*oxy* present in the medium increased when PPO concentration increased and because the steady state of *o*-diphenol activity was reached more quickly, the lag period diminished (Figure 4, inset).

(b) Monophenol Concentration. Increasing amounts of monophenol in the reaction medium imply more enzyme in the dead-end complex, E*met*T (Figure 5), and more time to reach the steady state. Note that it is critical to use a low level of monophenol when trying to detect monophenolase activity, because the lag period greatly increases when monophenol concentration is high (Figure 5).

(c) pH. The appearance and disappearance of the lag period in the monophenolase activity at different pH values may be due to a combination of both changes in the affinity of the E*met* form for the monophenol and the speed of the recycling chemical reactions, which are faster at neutral pH values. This combination of factors normally gives a bell-shaped profile (Sánchez-Ferrer et al., 1993a; Espín et al., 1995a,b, 1996a,b, 1997), although the results obtained in this paper using banana PPO clearly show no dependence on pH, as in the case of diphenolase activity (Sojo et al., 1998).

(d) Presence of o-Diphenols. The addition of catalytic amounts of *o*-diphenol to the reaction medium diminished the lag period until it was abolished (Figure 6), indicating that the level of *o*-diphenol in the steady state has been reached. If this level is exceeded, the excess *o*-diphenol is used by the diphenolase catalytic cycle, and activity increases (Figure 7).

(e) Purification Method Used. Because both monophenolase and diphenolase cycles overlap, a true PPO must

show both activities; the loss of monophenolase activity during purification reported in the bibliography must be due to a dramatic loss of enzymatic activity produced by acetone powder, reducing agents, or insoluble resins, which affect the monophenolase rather than the diphenolase activity (Sánchez-Ferrer et al., 1988). Thus, the use of mild purification methods based on TX-114 as first proposed by our group (Sánchez-Ferrer et al., 1989, 1993a−c, 1994a,b; Nuñez-Delicado et al., 1996; Chazarra et al., 1996) and then used by other groups (Espín et al., 1995a,b, 1996a,b, 1997; Fraignier et al., 1995) seems to be a suitable method to obtain a PPO showing both activities.

(f) Chemical Characteristics of the Monophenol Used To Test Monophenolase Activity. As described above, a crucial step in the catalytic cycle is the chemical recycling of quinones. Thus, if a monophenol, such as *tert*-butylphenol, which gives the water stable *tert*-butyl-*o*-quinone, is used (Sánchez-Ferrer et al., 1993a,c), no monophenolase can be detected unless H_2O_2 is used to obtain the E*oxy* form during the reaction (Jiménez and García-Carmona, 1996). Recently, coupled reactions of quinones and MBTH, first described by Winder and Harris (1991), have been tried in the determination of monophenolase activity (Espín et al., 1995a, 1996a).

CONCLUSION

In conclusion, although the basis for determining monophenolase activity in plant PPO was described at the end of the 1980s by our group in our studies of the kinetic mechanism (Cabanes et al., 1987), and despite its experimental determination in plants (Sánchez-Ferrer et al., 1988) and the development of mild purification methods based on TX-114 (Sánchez-Ferrer, 1989, 1994a,b), very few plant PPO monophenolase activities have been kinetically characterized (Cabanes et al., 1987; Sánchez-Ferrer et al., 1988, 1993a,b; Valero et al., 1988; Ros et al., 1994; Espín et al., 1995a,b, 1996a,b, 1997). This paper describes how the use of an additional phase partitioning step in PEG 8000/phosphate might help in characterizing monophenolase activity in other rapidly oxidizing plant material, which generally requires the use of acetone powder.

ABBREVIATIONS USED

TX-114, Triton X-114; PPO, polyphenol oxidase; SDS, sodium dodecyl sulfate; PEG 8000, polyethylene glycol Pm. 8000 Da.

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