Monophenolase Activity of Polyphenol Oxidase from Verdedoncella Apple

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Apple polyphenol oxidase (PPO) has been isolated and partially purified by using two sequential phase partitionings with Triton X-114 reported here for the first time. The enzyme showed monophenolase activity when assayed on (p-hydroxyphenyl)propionic acid (PHPPA) with 3-methyl-2-benzothiazolinone hydrazone (MBTH) in a new and reliable continuous spectrophotometric method, with high sensitivity, accuracy, and precision. The initial monophenolase activity showed a lag period (τ) prior to the attainment of the steady state rate (V_{ss}). Both kinetic parameters, τ and V_{ss}, depended on the pH, the enzyme and substrate concentrations, and the presence of catalytic amounts of *o*-diphenol. These dependencies can be explained in terms of a reaction mechanism involving one single active site, two enzyme forms, E_{met} and E_{oxy}, and a set of nonenzymatic reactions from *o*-quinones to chromophoric MBTH–quinone adduct, with regeneration of one molecule of *o*-diphenol. The effect of pH was related with two significant pK_a values of the free enzyme forms but not of enzyme–substrate complexes. This kinetic characterization was essential for understanding and choosing optimal assay conditions for determining enzyme activity and concentration when using PHPPA as a monophenolic substrate of apple PPO.

Keywords: *Apple; tyrosinase; polyphenol oxidase; enzyme kinetics; monophenols; hydroxyphenylpropionic acid; MBTH*

INTRODUCTION

Enzymatic browning in fruits and vegetables is often an undesirable reaction that is responsible for unpleasant sensory qualities and losses in nutrient quality. The prevention of this reaction has always been a challenge to food scientists (Matheis, 1987). The main enzyme involved in this reaction is tyrosinase or polyphenol oxidase (EC 1.14.18.1; PPO), which has been the subject of several reviews (Vamos-Vigyazo, 1981; Robb, 1984; Mayer, 1987; Nicolas et al., 1994; Sánchez-Ferrer et al., 1995). PPO is a copper enzyme which in the presence of oxygen catalyzes two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) which, in turn, are polymerized to brown, red, or black pigments (Mason, 1955; Mathew and Parpia, 1971; Prota, 1988).

Diphenolase activity of apple PPO (Janovitz-Klapp et al., 1990a; Nicolas et al., 1994) as well as the effect of many inhibitors on this activity (Janovitz-Klapp et al., 1990b) has been widely studied. However, there are only a few works on apple monophenolase activity (Walker, 1964; Raa and Overeem, 1968). This lack of information stems from the lability of the enzyme during the purification process (Matheis, 1987). This phenomenon is well-known in other plant PPOs (Mayer and Harel, 1979) and results from changes in the structure of the protein during purification (Walter and Purcell, 1980). Goodenough et al. (1983) detected monophenolase activity of apple PPO with the phenolic compounds phloridzin and *p*-coumaric acid, by using NMR spectroscopy and HPLC. These techniques are sensitive but expensive and are not widely used. Furthermore, they are used in a discontinuous way, which limits their applicability to slow processes, and are less reliable because only a small number of data points is possible per assay.

The aim of this study was the detection and the kinetic characterization of the monophenolase activity of apple var. Verdedoncella PPO. The enzyme will be partially purified by using a two-phase partitioning method in Triton X-114 (Bordier, 1981) with some modifications. The monophenolase activity will be assayed by means of a reliable continuous spectrophotometric method with the presence of the nucleophile MBTH. The method is based on that previously proposed for mushroom PPO (Rodríguez-López et al., 1994). This method was based on previous works involving nonenzymatic reactions of the *o*-quinones produced by PPO in the absence (García-Carmona et al., 1982) and in the presence (Cabanes et al., 1987a; García-Carmona et al., 1987, 1988; Valero et al., 1988; Rodríguez-López et al., 1994) of nucleophiles. The kinetics of the monophenolase activity of apple PPO will be investigated with special emphasis on the effects of pH and enzyme and substrate concentration (García-Cánovas et al., 1982; García-Carmona et al., 1982; Cabanes et al., 1987b; Tudela et al., 1987; Serna et al., 1990; García-Moreno et al., 1991, 1994; Rodríguez-López et al., 1991, 1992). From such study the reaction mechanism of the monophenolase-catalyzed reaction can be inferred.

MATERIALS AND METHODS

Reagents. Apples from the variety Verdedoncella, picked in Murcia, Spain, at commercial maturity and immediately stored at 5 °C, were used as enzyme source.

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Substrates and MBTH were purchased from Sigma Chemical Co. (Madrid, Spain), and all other reagents were of analytical grade.

Triton X-114 (TX-114) was obtained from Fluka (Madrid, Spain) and condensed three times prior to use as described by Bordier (1981) but using sodium phosphate buffer (PB) (pH 7.3) containing 20 mM EDTA. The detergent phase of the third condensation had a concentration of 22% TX-114 (w/v).

Preparation of PPO. Apple PPO was extracted and partially purified using the method of Bordier (1981) with some modifications (Sánchez-Ferrer et al., 1989, 1993).

The apples were washed and hand-peeled. A 150 g sample was homogenized with 75 ml of cold buffered 0.1 M sodium phosphate (pH 7.3), 20 mM EDTA, and 6% (w/v) TX-114 for 2 min. The homogenate was kept at 4 °C for 60 min before being centrifuged at 100000*g* for 45 min at 4 °C. The supernatant was collected and used as a crude enzyme extract. It was subjected to temperature-induced phase partitioning by increasing the TX-114 concentration by an additional 8% (w/v) at 4 °C and then warming to 35 °C for 15 min. The solution became turbid due to the formation, aggregation, and precipitation of large micelles of detergent that contained hydrophobic proteins and phenolic compounds. This solution was centrifuged at 8000*g* for 15 min at 25 °C. The detergent-rich phase was discarded, and the supernatant was subjected to an additional phase-partitioning step with 8% (w/v) TX-114. The protocol was repeated once more to remove the remaining phenols. The clear supernatant containing soluble apple PPO was acidified to pH 5.0. The acidified supernatant was kept for 1 h at 4 °C before being centrifuged at 100000*g* for 30 min at 4 °C. The pellet was discarded and the supernatant brought to 30% saturation with solid $(NH_4)_2SO_4$ under continuous stirring at 4 °C. After 15 min, the solution was centrifuged at 80000*g* for 30 min at 4 °C and the pellet discarded. Additional $(NH_4)_2SO_4$ was added to the clear supernatant to give 80% saturation and stirred for 30 min at 4 °C. The solution was centrifuged at 100000*g* for 30 min and the precipitate dissolved in a minimal volume of deionized water. The salt content was removed by a desalting column of Sephadex G-25. The enzyme was stored at −30 °C with a 10% loss of the original activity after 8 months and no discoloration. This simple and fast protocol gave a 10-fold purification of the enzyme extract which preserved its monophenolase activity.

Other Methods. Protein was determined according to the method of Bradford (1976) using bovine serum albumin as standard.

Enzymatic Assays. The use of MBTH as a nucleophilic agent on some *o*-quinones generated by PPO had previously been described for the measurement of diphenolase (Winder and Harris, 1991) and monophenolase (Rodríguez-López et al., 1994) activities of PPO.

The diphenolase activity was assayed spectrophotometrically at 500 nm using as substrates (3,4-dihydroxyphenyl)-propionic acid (DHPPA) with MBTH and is reported here for the first time (ϵ = 40 000 M^{-1} cm^{-1}). The standard reaction mixture contained 10 mM substrate, 1 mM MBTH, 2% DMF, 50 mM sodium acetate buffer (AB) (pH 4.6), and 1 μg/mL soluble PPO.

The monophenolase activity was also determined spectrophotometrically at 500 nm with (*p*-hydroxyphenyl)propionic acid (PHPPA) with MBTH and is reported for the first time in the literature. The standard reaction mixture included 1 mM MBTH, 2% DMF, and 50 mM AB pH (4.6) as well as the different PHPPA and PPO concentrations detailed below.

One unit of enzyme was taken as the amount that produced 1 μmol of the adduct/min. Experiments were performed at 25 °C in triplicate, and the corresponding mean values were plotted.

The spectrophotometric assays were carried out with a Perkin-Elmer Lambda-2 spectrophotometer on-line interfaced with a compatible PC for further data analysis. All of the assays were carried out at 25 °C with a Haake D1G circulating water bath equipped with a heater/cooler and controlled by a Cole-Parmer digital thermometer with a precision of ±0.1 °C.

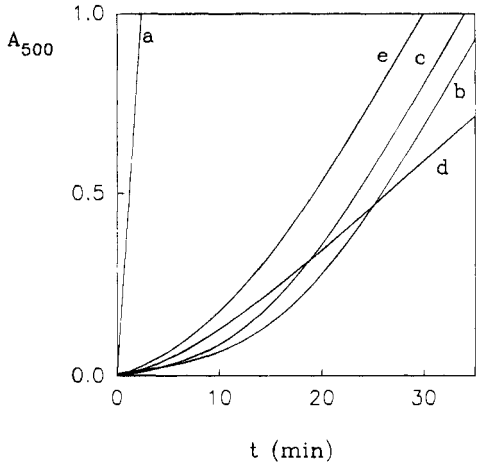


Figure 1. Enzymatic activities of soluble apple PPO: (a) diphenolase activity [the reaction medium included 1 μg/mL PPO, 10 mM DHPPA, 1 mM MBTH, and 2% DMF in 50 mM sodium acetate buffer (AB) (pH 4.6)]; (b–e) monophenolase activity [the reaction medium contained (b) 22 μg/mL PPO and 12 mM PHPPA in 50 mM AB (pH 4.6); (c, d) same as (b) except for (c) different substrate concentration (10 mM PHPPA), (d) different pH (pH 5.4), and (e) the addition of *o*-diphenol to the reaction medium (1 μM)].

Data Analysis. Kinetic data analysis was carried out by using linear and nonlinear regression fittings (Marquardt, 1963), using the Sigma Plot 5.0 program (Jandel Scientific, 1992).

Determination of the enzyme concentration was carried out by lowering the PPO concentration to a level where V_{ss} was 10-fold higher than the rate of nonenzymatic oxidation of the PHPPA substrate (blank rate). The cuvette contained a saturating concentration of monophenolic substrate and enough MBTH concentration to trap all of the generated *o*-quinone. Ten blank cuvettes were assayed for the determination of the limit of detection (LOD) and the limit of quantitation (LOQ) of the method. The precision of the method was also evaluated from 10 activity assays at each one of the three enzyme concentrations used.

RESULTS

Apple PPO was partially purified by using two sequential phase partitionings with Triton X-114 as described above. The enzyme had both diphenolase (Figure 1, curve a) and monophenolase activities (Figure 1, curves b–e). The latter was characterized by a lag period (τ), defined as the intercept on the abscissa axis 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 substrate concentrations, as well as the presence of catalytic concentrations of *o*-diphenols.

Effect of pH. Monophenolase activity toward PHPPA increased as the pH was increased from pH 3.6 and showed a maximum at pH 4.6 (Figure 2). The pH affected not only the enzyme activity but also the lag period. Both plots, V_{ss} *vs* pH and τ *vs* pH, showed the same profiles (Figure 2). These results were not similar to those described for other plant PPOs (Cabanes et al., 1987b; Sánchez-Ferrer et al., 1988; Valero et al., 1988) but were in agreement with the results of Ros et al. (1994) for other PPO sources.

Effect of Enzyme Concentration. An increase of enzyme concentration produced a linear increase in V_{ss} (Figure 3) as well as a shortening in the lag period (Figure 3). This behavior has been widely described

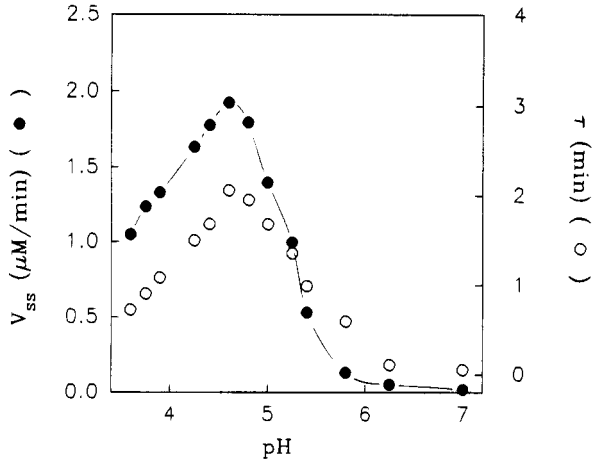


Figure 2. Effect of pH on monophenolase activity (●) and on its lag period (○). The reaction medium included 88 μg/mL partially purified PPO, 1 mM MBTH, 2% DMF, and 0.5 mM PHPPA in 50 mM AB (pH 3.6–5.6) and sodium phosphate buffer (PB) (pH 5.8–7.0).

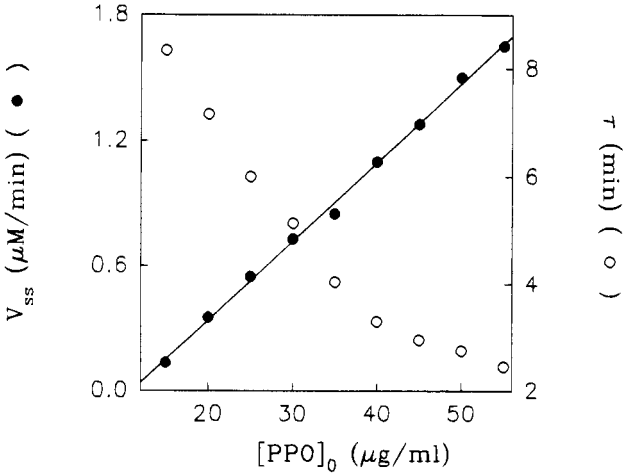


Figure 3. Effect of enzyme concentration on monophenolase activity of PPO (●) and on its lag period (○). The reaction medium included 0.5 mM PHPPA, 2% DMF, and 1 mM MBTH in 50 mM AB (pH 4.6) with different PPO concentrations (10–5 μg/mL).

from several PPO sources (Cabanes et al., 1987b; Ros et al., 1994).

Effect of Monophenol Concentration. An increase in PHPPA concentration from 0.3 to 14 mM (Figure 4) produced an increase in both the steady state rate and lag period, similar to that described for other plant PPOs (Lavollay et al., 1975; García-Carmona et al., 1988; Sánchez-Ferrer et al., 1988; Ros et al., 1994).

The kinetic constants V_{max} and K_m were evaluated from the data in Figure 4 by using nonlinear regression fitting (Marquardt, 1963). The V_{max} and K_m obtained at the optimum pH were 6.5 μM/min and 2.8 mM, respectively. The K_m values increased when the pH was either lower or higher than 4.6 (optimum pH) (Table 1). However, no change was observed in the V_{max} values (Table 1).

Effect of *o*-Diphenol Addition. The lag period observed in the monophenolase activity of apple PPO toward PHPPA was modified by the addition of different concentrations of DHPPA (Figures 5 and 6). An increase in the DHPPA concentration shortened the lag period, although above a certain concentration a burst in activity resulted. It should be noted that the V_{ss} is the same for every *o*-diphenol concentration chosen. The

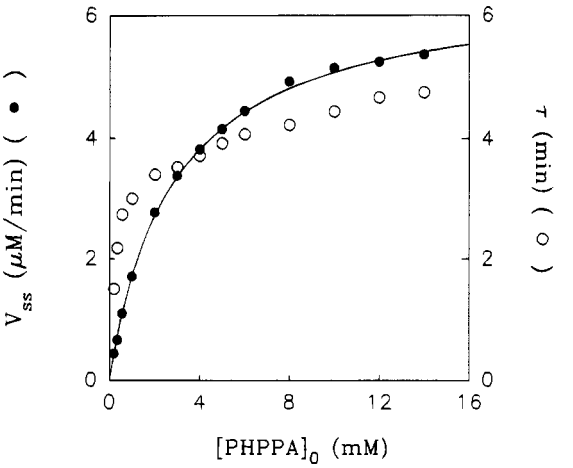


Figure 4. Effect of PHPPA concentration on monophenolase activity of PPO (●) and on its lag period (○). The reaction medium included 88 μg/mL of partially purified PPO, 1 mM MBTH, and 2% DMF in 50 mM AB (pH 4.6) with different PHPPA concentrations (0.3–4 mM).

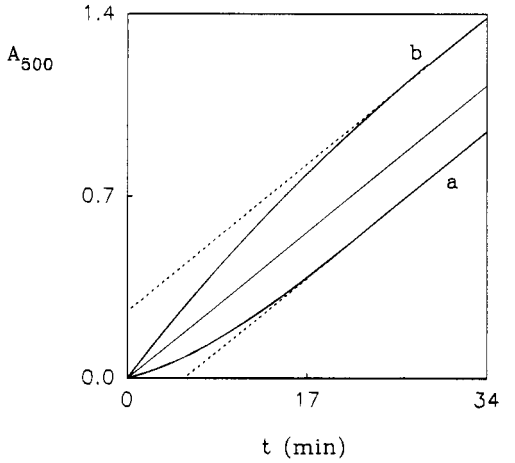


Figure 5. Influence of DHPPA on the lag period of monophenolase activity. In the standard reaction with 66 μg/mL PPO, 1 mM PHPPA, 1 mM MBTH, 2% DMF, and 50 mM AB (pH 4.6), the concentration of DHPPA ranged from 0 (a) to 20 μM (b).

Table 1. K_m and V_{max} Values for Monophenolase Apple PPO Activity as a Function of pH Using PHPPA with MBTH

pH	K_m (mM)	V_{max} (μM/min)
3.9	5.50 ± 0.02	6.51 ± 0.06
4.6	2.80 ± 0.03	6.50 ± 0.07
5.0	4.01 ± 0.04	6.45 ± 0.06
5.4	5.80 ± 0.04	6.47 ± 0.08

above shortening in the lag period led to a null value and even negative values being attained (Figures 5 and 6). In other words, a rise in the initial *o*-diphenol concentration ($[D]_0$) shortened the time required for the steady state level of *o*-diphenol ($[D]_{ss}$) to be reached. When $[D]_0 \cong [D]_{ss}$, the value of $[D]_{ss}$ was reached quickly and no lag period was detected in the initial monophenolase activity (Figures 5 and 6). However, when $[D]_0 > [D]_{ss}$, the system must first consume the excess of *o*-diphenol (D) and then gradually consume 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 τ (Figures 5 and 6). Therefore, the physical meaning of the increase $|\tau|$ was equivalent to a longer pre-steady-state transient phase. The physical meaning of the sign $+\tau$

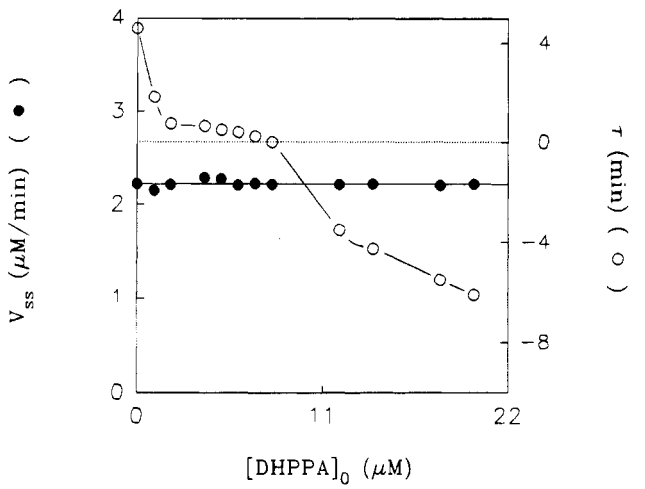


Figure 6. Effect of catalytic amounts of DHPPA on the steady state rate of monophenolase activity (●) and on the lag period (○). The conditions are the same as in Figure 5.

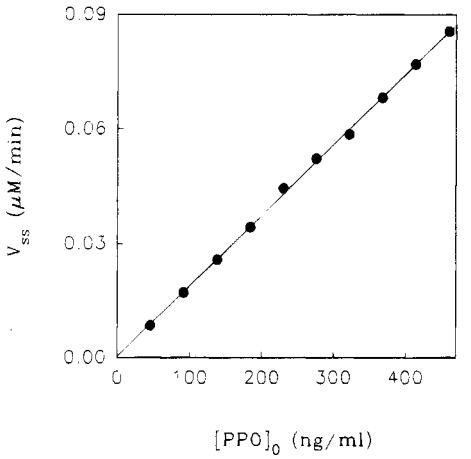


Figure 7. Effect of enzyme concentration on monophenolase activity of PPO using PHPPA with MBTH. LOD and LOQ values were determined (see text for details). The reaction medium contained 10 mM PHPPA, 1 mM MBTH, 2% DMF, 50 mM AB (pH 4.6), and different concentrations of PPO (46–460 ng/mL).

or $-\tau$ corresponded to the type of assay conditions with a sub-steady-state or over-steady state level of $[D]_0$, respectively.

Determination of the Enzyme Concentration. Consideration of the above factors allows for the determination of the optimal monophenolase assay conditions on PHPPA. Thus, a LOD of 6.8 ng/mL and a LOQ of 12.8 ng/mL were obtained (Figure 7). Furthermore, from 10 assays at 46, 230, and 460 ng/mL enzyme concentrations, CV of 5.8%, 2.9%, and 1.4%, respectively, were obtained for the corresponding V_{ss} data values (Figure 7). Therefore, this new continuous spectrophotometric method for measuring the monophenolase activity of PPO on PHPPA with MBTH was reliable and highly sensitive.

DISCUSSION

Partial Purification of PPO. Apple PPO was extracted and partially purified by using two sequential phase partitionings with Triton X-114 as detailed above. The main advantage of this method lies in the preservation of both PPO monophenolase and diphenolase activities. Other more drastic methods lead to the loss of the monophenolase activity of PPO from other biological sources, such as fruits and vegetables (Mayer and Harel, 1979; Walter and Purcell, 1980; Matheis, 1987). The purification procedures used by other authors (Janovitz-Klapp et al., 1989; Trejo-González and Soto-Valdez, 1991; Zhou, et al., 1993) might have provoked some changes in the structure of the enzyme with a consequent loss of the monophenolase activity. This activity has a catalytic power, V^M_{max}/K^M_m, around one hundred times lower than the catalytic power of the diphenolase activity, V^D_{max}/K^D_m (Rodríguez-López et al., 1992; Ros et al., 1994). Therefore, slight changes in the concentration and/or functionality may not affect the diphenolase activity, whereas the monophenolase activity may be seriously altered. In fact, if this occurs, the monophenolase activity cannot be detected with the most widely used techniques such as spectroscopy or polarography.

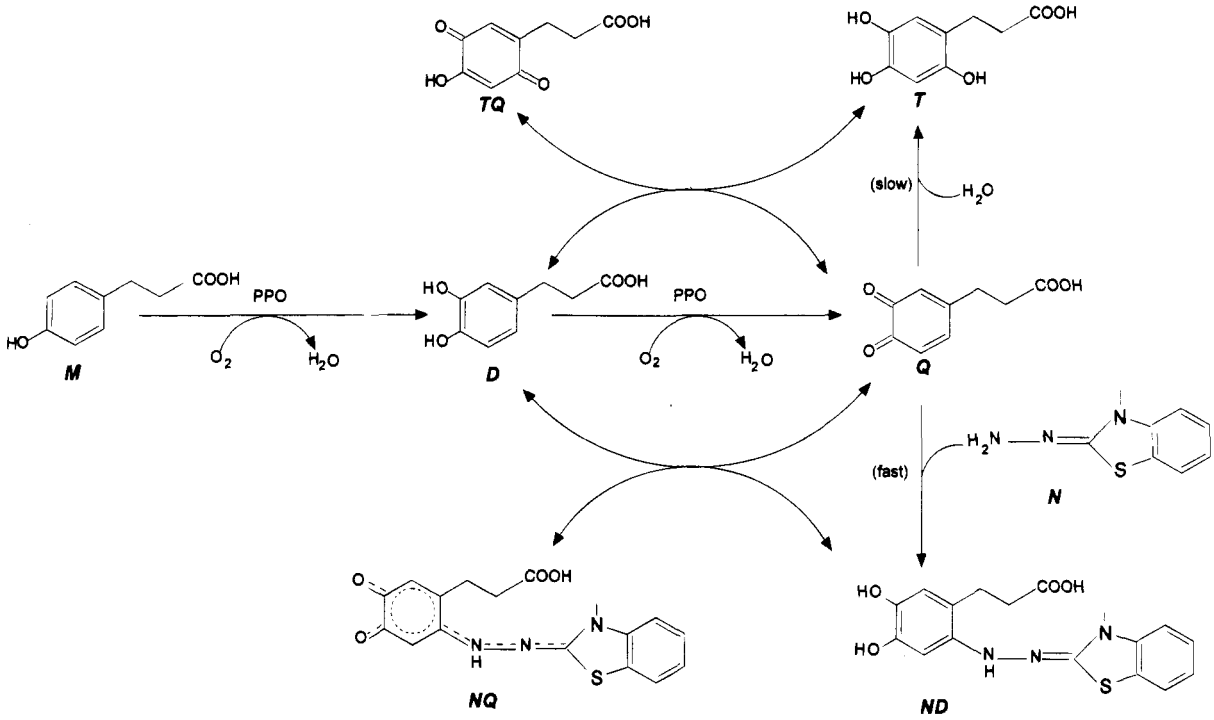
Reaction Mechanism. The results presented for the monophenolase activity of partially purified apple PPO (Schemes 1 and 2) are in agreement with the mechanism previously described for PPO from other sources (García-Cánovas et al., 1982; García-Carmona et al., 1982; Cabanes et al., 1987b; Rodríguez-López et al., 1992).

Figure 5 shows that the lag period is shortened by the addition of small amounts of *o*-diphenol concentrations. In other words, the system needs a minimal amount of *o*-diphenol to reach the steady state which, in this case, is obtained from the enzyme transformation of monophenol to *o*-diphenol and from the oxidation of the adduct by the *o*-quinone generated by the enzyme. In addition, the *o*-diphenol is oxidized by the E_{oxy} form of the enzyme, while the latter evolves from the E_{oxy} to the E_{met} form. For the *o*-diphenol generation and consumption rates to be equal, a certain time is necessary. This time is the lag period. 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. An increase in the level of monophenol means that a higher level of *o*-diphenol is required for the steady state to be reached. This can be understood because both ligands, monophenol and *o*-diphenol, compete for the E_{met} and E_{oxy} forms.

Increasing PPO concentrations produce a proportional increase of the E_{oxy} form in the native state, which means that there is more enzymatic activity and the level of *o*-diphenol in the steady state is reached more quickly and, so, τ diminishes (Figure 3). On the other hand, increasing monophenol concentrations in the medium imply that a higher level of *o*-diphenol is necessary for the steady state to be reached because both, monophenol and *o*-diphenol, are in direct relation (Cabanes et al., 1987b; Rodríguez-López et al., 1992) and, so, the lag period increases (Figure 4).

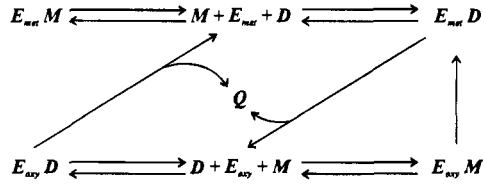
Effect of pH. The steady state rate (V_{ss}) shows a bell-shaped profile with regard to pH (Figure 2). This could indicate the existence of two significant pK_a values (3.8 and 5.3) in the enzyme activity. Changes in the pH affect the K_m, but not the V_{max}, values from Table 1. This indicates that the pH affects only the interaction of the substrates to the free enzyme and not the transformations of the enzyme–substrate complexes. These dependencies on pH were similar to that previously observed for diphenolase activity (Tudela et al., 1987; García-Moreno et al., 1994). The effect of the pH on the nonenzymatic reactions of the *o*-quinones generated by PPO has also been characterized elsewhere

Scheme 1. Sequence of Reactions for the Monophenolase and Diphenolase Activities of PPO in the Presence of a Chromogenic Nucleophile[a]



[a] M, monophenol; D, *o*-diphenol; T, triphenol; Q, o-quinone; TQ, triphenolquinone; N, chromogenic nucleophile; ND, nucleophile–diphenol colorless adduct; NQ, nucleophile–quinone chromophoric adduct (García-Cánovas et al., 1982; García-Carmona et al., 1982; Cabanes et al., 1987; Rodríguez-López et al., 1992, 1994; Ros et al., 1994).

Scheme 2. Kinetic Reaction Mechanism Proposed To Explain the Monophenolase and Diphenolase Activities of PPO in the Presence of a Chromogenic Nucleophile[a]



$$2Q + N \longrightarrow D + NQ$$

[a] M, monophenol; D, *o*-diphenol; Q, *o*-quinone; N, chromogenic nucleophile; NQ, nucleophile–quinone chromophoric adduct; E_{met}, met-PPO; E_{oxy}, oxy-PPO (García-Cánovas et al., 1982; García-Carmona et al., 1982; Cabanes et al., 1987; Rodríguez-López et al., 1994).

(García-Cánovas et al., 1982; Serna et al., 1990; García-Moreno et al., 1991; Rodríguez-López et al., 1991).

Thus, at the optimum pH (4.6; Table 1), the Michaelis–Menten constant of PPO for PHPPA is lower, and therefore the E_{met} form, which is inactive on monophenols, has higher affinity toward them. Thus, τ increases because there is less enzyme available to react with the *o*-diphenol initially. On the other hand, at pH values lower or higher than the optimum, the lag period is shorter because the affinity of the enzyme toward the monophenol diminishes (Figure 2).

There are some PPO sources whose extracts show apparent low or null monophenolase activity. This occurs when the native enzyme has a low proportion of the E_{oxy} form (Scheme 1). This causes an increase in the lag period and a lower steady state rate.

ABBREVIATIONS USED

AB, sodium acetate buffer; CV, coefficient of variation; DHPPA, (3,4-dihydroxyphenyl)propionic acid; DMF, *N*,*N*′-dimethylformamide; EDTA, ethylenediaminetetraacetic acid; LOD, limit of detection; LOQ, limit of quantitation; MBTH, 3-methyl-2-benzothiazolinone hydrazone; PB, sodium phosphate buffer; PHPPA, (*p*-hydroxyphenyl)propionic acid; PPO, tyrosinase or polyphenol oxidase (EC 1.14.18.1); TX-114, Triton X-114.

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