Characterization of Catecholase and Cresolase Activities of Eggplant Polyphenol Oxidase

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In the present paper the catecholase and cresolase activities of eggplant polyphenol oxidase (PPO) are described. To preserve the latter activity, a partially purified enzyme was used. Peroxidase was removed from the preparation to avoid its interference with PPO during phenol oxidation. The partially purified eggplant PPO was fully active. The catecholase/cresolase ratio of 41.1 indicated that, in a pH close to the physiological, diphenol oxidation predominates over monophenol oxidation. The characteristic lag phase of the cresolase activity is modulated by the pH, the monophenol and diphenol concentrations, and the enzyme's concentration. The effect of several inhibitors was also tested, and the K_i values of the two most effective (tropolone and 4-hexylresorcinol) were determined.

Keywords: *Browning; catecholase; cresolase; eggplant; polyphenol oxidase; Solanum melongena*

INTRODUCTION

Tissue browning in fruits and vegetables damaged by mechanical injury during harvesting, postharvest storage, or processing is one of the main causes of quality loss (Mathew and Parpia, 1971; Flick et al., 1977). This reaction, which is mainly catalyzed by polyphenol oxidase (EC 1.14.18.1; PPO), gives rise to the formation of *o*-quinones, which subsequently polymerize, leading to the appearance of brown pigments (Nicolas et al., 1994). The problem is especially important in plants with a high phenol content such as potato and eggplant (Matheis, 1987a,b; Sakamura and Obata, 1963).

Because of these deleterious effects of enzymatic browning on fruits and vegetables, a considerable number of inhibitors of PPO activity have been identified. Reducing agents have been widely used but may have adverse health effects and can also react with other components in the food system (McEvily et al., 1992). Another important group of browning inhibitors is formed by compounds that are structurally analogous to phenolic substrates, the inhibitory capability of which will depend on the enzyme source and the substrate used (Mayer and Harel, 1979).

PPO is a bifunctional copper protein complex that catalyzes two different reactions: cresolase activity (the hydroxylation of monophenols to *o*-diphenols) and catecholase activity (the oxidation of *o*-diphenols to *o*-quinone). The first activity shows a lag period (Pomerantz, 1966; García-Carmona et al., 1979) that has been explained by taking into account the chemical steps of the tyrosinase reaction which are necessary for the production of catalytic amounts of *o*-diphenol (Cabanes et al., 1987a), whereas the catecholase activity shows no slow-transition phenomena.

Potato (*Solanum tuberosum* L.) is the only member of the Solanaceae family in which PPO, from both tuber and leaf, has been widely characterized (Matheis, 1987a,b; Sánchez-Ferrer et al., 1993a,b). Although eggplant (*Solanum melongena* L.) fruits are a rich source of PPO, it has been only partially characterized (Knapp, 1965; Sakamura et al., 1966; Fujita and Tono, 1988), and there is no reference to its cresolase activity. The absence of such data could be a consequence of the lability of cresolase activity (Mayer and Harel, 1979; Matheis, 1987a). In addition, no effective inhibitor for this enzyme has been found (Almeida and Nogueira, 1995).

The aim of the study described in the present paper was to characterize the catecholase and cresolase activities of PPO in eggplant fruits. With the purification method reported in this paper, both preservation of cresolase activity and removal of interfering peroxidase were achieved. The activities of different compounds used as PPO inhibitors are also reported.

MATERIALS AND METHODS

Plant Material. Purple eggplant fruits (*S. melongena* L., cv. Belleza negra) at harvest maturity were obtained from the local market.

Reagents. Ascorbic acid, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Bradford reagent, kojic acid, 4-methylcatechol (4-MC), L-mimosine, *p*-cresol, and tropolone were purchased from Sigma Chemical Co. (Madrid, Spain). *tert*-Butylcatechol (TBC) and ammonium sulfate were from Fluka (Madrid, Spain). Bovine serum albumin (BSA) was from Bio-Rad (Madrid, Spain). 4-Hexylresorcinol and hydrogen peroxide was purchased from Aldrich (Madrid, Spain). The rest of the reagents were of analytical grade.

Enzyme Purification. Eggplants (120 g) were peeled, cut into small pieces, and homogenized with 300 mL of cold 2 mM ascorbic acid in 50 mM phosphate buffer, pH 7.0. The resulting homogenate was filtered through four layers of gauze and centrifuged at 20000*g* for 10 min at 4 °C. The supernate was brought to 40% saturation with solid ammonium sulfate under continuous stirring at 4 °C. After 1 h, the solution was centrifuged at 60000*g* and the pellet resuspended in a small volume of 50 mM phosphate buffer, pH 7.0. The enzyme remained stable at −20 °C for >3 months.

Enzyme Activity. Catecholase activity was determined spectrophotometrically at 400 nm (Sánchez-Ferrer et al., 1989) with TBC ($\epsilon = 1682\ M^{-1}\ cm^{-1}$) or 4-MC. Both cresolase activity

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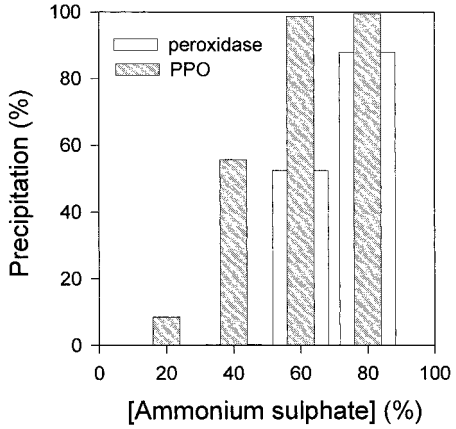

Figure 1. Percentage of PPO and peroxidase precipitated with different concentrations of ammonium sulfate. Plant material was homogenated as described under Materials and Methods and precipitated with different concentrations of ammonium sulfate. After centrifugation, the peroxidase activity and the catecholase activity of PPO in the pellet were measured.

toward *p*-cresol and catecholase activity toward 4-MC were also measured spectrophotometrically by the appearance of 4-methyl-*o*-benzoquinone at 400 nm ($\epsilon = 1350\ M^{-1}\ cm^{-1}$), as has been described by Mayer et al. (1966).

Cresolase activity is characterized by a lag period that depends on different factors, such as substrate and enzyme concentration, the presence of *o*-diphenols, and the pH. Cresolase activity in the steady-state rate was calculated from the linear part of the product accumulation curve after the lag period.

Experiments were performed in triplicate and the mean and SD plotted. One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of *tert*-butylquinone/min.

Peroxidase activity was followed at 414 nm in a reaction medium containing 1 mM ABTS and 0.4 mM H_2O_2 in 0.1 mM phosphate buffer, pH 5.5 ($\epsilon_{414} = 31100\ M^{-1}\ cm^{-1}$; Arnao et al., 1990).

Protein Determination. The protein content was measured according to the dye binding method of Bradford (1976) using BSA as a standard.

RESULTS

Purification. Peroxidases have been assumed to be involved in enzymatic browning by numerous authors (Nicolas et al., 1994; Richard-Forget and Gauillard, 1997). Thus, to prevent eggplant peroxidase from interfering with the measurement of PPO, the activity of the first enzyme was also followed during the purification process. Figure 1 shows the percentage of peroxidase and PPO precipitated at different ammonium sulfate concentrations. When the homogenate was brought to 40% ammonium sulfate, 56% of the total PPO activity was precipitated and only 0.2% of the total amount of peroxidase. The specific activity of this extract was 2.5 units/mg of protein. Thus, despite the small purification factor obtained, the PPO/peroxidase ratio increased 280 times.

Effect of pH. The pH profiles of catecholase activities was very similar for both 4-MC and TBC. The enzyme was active between pH 4 and 9 and showed a broad maximum at pH ~5.5 (Figure 2A). The effect of pH was also assayed for cresolase activity, with a sharp increase observed at pH 7.5 (Figure 2B). The pH also influenced the length of the lag period of the cresolase activity, which was shorter as pH increased (Figure 2B).

Effect of Substrate Concentration. The kinetic parameters (V_{max} and K_m) of *p*-cresol oxidation were

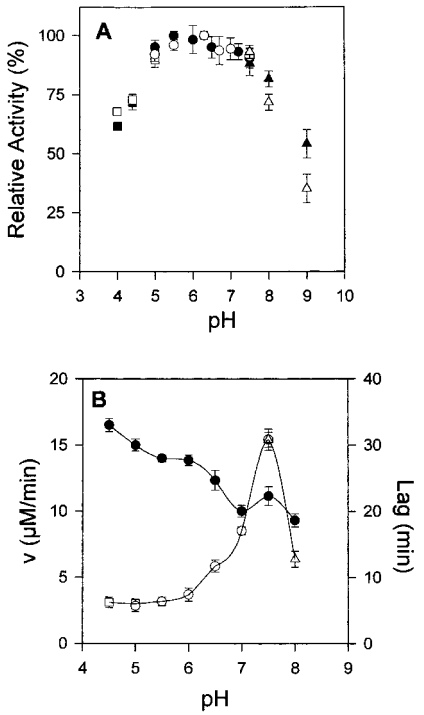

Figure 2. (A) Effect of pH on the catecholase activity of PPO. The reaction medium at 25 °C consisted of 2.5 mM TBC (solid symbols) or 4-MC (open symbols), 0.2 unit of PPO, and 10 mM acetate (pH 4.0−5.0, squares), phosphate (pH 5.0−7.5, circles), or borate (pH 7.5−9.0, triangles). (B) Effect of pH on cresolase activity (open symbols) and on its lag period (solid symbols). The reaction medium at 25 °C included 0.5 unit of PPO and 4.75 mM *p*-cresol in the same buffers as used in (A).

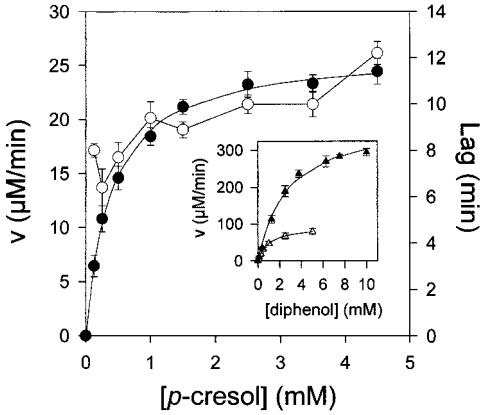

Figure 3. Effect of substrate concentration on cresolase activity of PPO (●) and on the lag period (○). The reaction medium consisted of 1.0 unit of PPO in 10 mM phosphate buffer, pH 7.5, with different *p*-cresol concentrations. (Inset) Effect of substrate concentration on catecholase activity of PPO. The reaction medium consisted of 0.08 unit of PPO and different concentrations of TBC in 10 mM sodium phosphate buffer, pH 5.5 (△), or 4-MC in 10 mM phosphate buffer, pH 7.5 (▲). Solid lines represent the fitting by nonlinear regression to eq 1.

studied at pH 7.5 using 10 mM sodium phosphate buffer. The initial rate showed a hyperbolic dependence with respect to *p*-cresol concentration (Figure 3). K_m and V_{max} were calculated by nonlinear regression fitting (Marquard, 1963) of the experimental points to the following equation:

$$v = \frac{V_{max}[TBC]}{K_m + [TBC]} \quad (1)$$

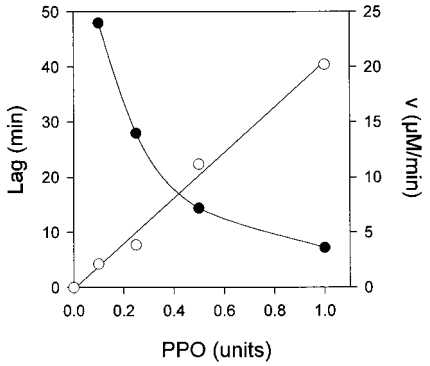

Figure 4. Effect of enzyme concentration on the initial rate (○) and on the lag period (●) of the cresolase activity. The reaction medium contained 4 mM *p*-cresol and different amounts of PPO in 10 mM phosphate buffer, pH 7.5.

This fitting is represented by the solid line between the experimental points. The values obtained [0.38 mM and 47.9 μM min^{-1} (mg of protein)$^{-1}$] were similar to those reported for the cresolase activity of grape and potato (Sánchez-Ferrer et al., 1988, 1993a).

Increasing the *p*-cresol concentration from 0.125 to 4.5 mM produced an increase in the lag period (Figure 3) similar to that reported for other plant PPOs (Lavollay et al., 1975; García-Carmona et al., 1988; Sánchez-Ferrer et al., 1988, 1993a).

The kinetic constants, K_m and V_{max}, for the catecholase activity were obtained as described for the cresolase activity and gave values of 1 mM and 1.9 mM min^{-1} (mg of protein)$^{-1}$, respectively, for TBC and 2.7 mM and 14 mM min^{-1} (mg of protein)$^{-1}$, respectively, for 4-MC (Figure 3, inset). The K_m and V_{max} with 4-MC were determined at pH 7.5 to compare these results with those obtained for the oxidation of *p*-cresol.

Effect of Enzyme Concentration. Figure 4 shows the linear dependence between the enzyme concentration and the steady-state rate obtained for the cresolase activity. The same results were obtained for the catecholase activity. The lag period presented an inverse dependence with enzyme concentration (Figure 4, solid circles), which is similar to that obtained for PPO from other sources (Valero et al., 1988; Sánchez-Ferrer et al., 1988).

Effect of *o*-Diphenols. We used the equation of Pomerantz and Warner (1967) to study the effect of 4-MC on the cresolase activity of eggplant PPO:

$$\frac{1}{l} = \frac{1}{L} + \frac{1}{L}\frac{[o\text{-diphenol}]}{K_{act}} \qquad (2)$$

In eq 2 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.

The effect of catalytic amounts of 4-MC in the lag period of the cresolase activity is shown in Figure 5. The activation constant K_{act} was calculated from the reciprocal of the lag period in the presence of diphenol (Figure 5B). The value obtained (7.9 μM) was similar to that obtained by Sánchez-Ferrer et al. (1993a) for potato PPO and higher than the K_{act} found for other PPOs (Duckworth and Coleman, 1970; Lerch and Ettlinger, 1972; Sánchez-Ferrer et al., 1988). The K_{act} was ~340 times smaller than the K_m for 4-MC.

Effect of Different Inhibitors. We tested the effect of different compounds normally used as PPO inhibitors on the catecholase activity of eggplant PPO using TBC as substrate. Table 1 shows the percentage of inhibition obtained with these compounds.

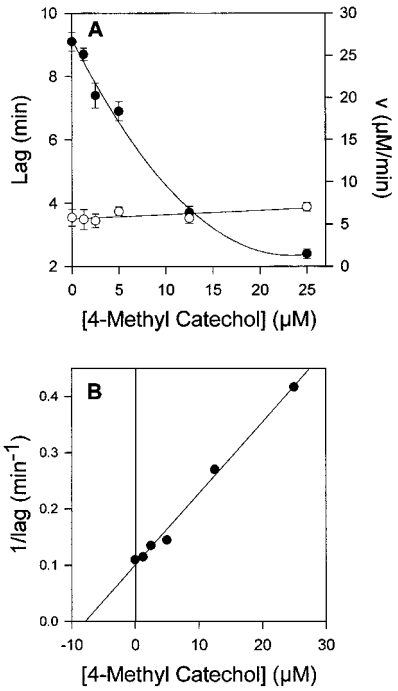

Figure 5. (A). Influence of 4-MC on the shortening of the lag period (●) and on the initial rate (○) of the cresolase activity. Different concentrations of 4-MC were added to the reaction medium consisting of 0.5 mM *p*-cresol and 0.5 unit of PPO in 10 mM phosphate buffer, pH 7.5. (B) Evaluation of the K_{act} constant by eq 2.

Table 1. Percentage of Inhibition of Catecholase Activity by Substrate Analogues

	1 mM	0.1 mM	50 μM
L-mimosine	8	7	0.4
kojic acid	35	12	7
4-hexylresorcinol	65	20	14
tropolone	91	74	61

L-Mimosine, a slow-binding inhibitor of mushroom tyrosinase (Cabanes et al., 1987b), displayed a limited inhibitory effect, which was within the range of values obtained with potato PPO (Sánchez-Ferrer et al., 1993b). To confirm that the pH had no effect on the inhibition caused by kojic acid, the effect of this compound was tested at different pH values (data not shown), the results not differing from those obtained at pH 5.5 (Table 1). The percentage of inhibition obtained with kojic acid was higher than that recently reported for banana PPO (Sojo et al., 1998).

When a kinetic analysis of the inhibition was carried out with tropolone and 4-hexylresorcinol (Figure 6), both compounds showed competitive inhibition, which was analyzed by plotting 1/v versus 1/[TBC] at several inhibitor concentrations. The K_i obtained for tropolone was 16 μM, similar to the value obtained for 4-hexylresorcinol (50 μM).

DISCUSSION

Plant PPOs are nuclear-encoded chloroplast proteins, which are found in plastids, although reports of their occurrence in other cell compartments are quite abundant (Mayer and Harel, 1979). PPO is often described as being tightly bound to thylakoids but has also been

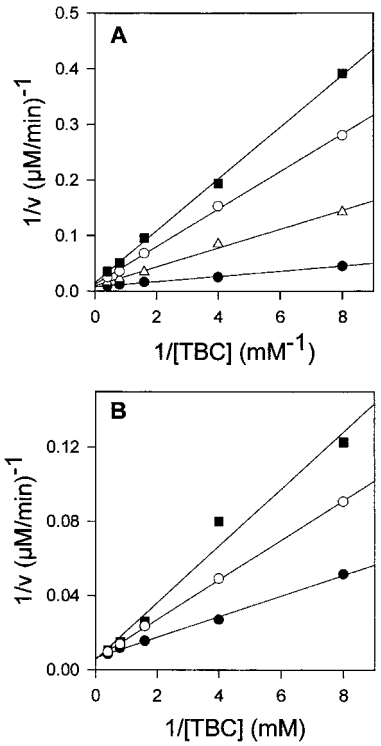

Figure 6. Lineweaver−Burk plots for the inhibition of the catecholase activity of PPO with (A) tropolone [(△) 20 μM; (○) 50 μM; (■) 100 μM] or (B) 4-hexylresorcinol [(○) 50 μM; (■) 100 μM]. In each graph a control without inhibitor is represented (●). The reaction medium contained 0.1 unit of PPO and 10 mM phosphate buffer, pH 5.5.

observed in plastid envelopes and the thylakoid lumen (Koussevitzky et al., 1998). In higher plants, the enzyme is mostly membrane bound and found in nonsenescing tissue, although more enzyme becomes soluble as the fruit ages (Murata et al., 1997). The partially purified eggplant PPO was fully active, and so it was not necessary to activate any latent form, as has been described for other plant PPOs using different treatments that include trypsin (Tolbert, 1973), pH (Valero and García-Carmona, 1992), and detergent (King and Flurkey, 1987). Such fully active PPO showed a preference for water soluble substrates such as 4-MC (Figure 3, inset). This pattern is characteristic of mature tissues (Sánchez-Ferrer et al., 1993b), whereas, in general, in leaves and developing tissues PPO is membrane bound and extracted in a latent form (Tolbert, 1973). The two main advantages of the extraction method used in this paper were the removal of interfering peroxidases (Figure 1) and the preservation of the cresolase activity of PPO.

The effect of variations in pH on catecholase and cresolase activities differed (Figure 2). In the case of the catecholase activity, the profile was the same whether 4-MC or TBC was used, and no clear optimum pH was defined. This broad maximum is in agreement with the results reported by Knapp (1965) and Fujita and Tono (1988) when chlorogenic acid was used as substrate. The pH optimum obtained for the cresolase activity was slightly higher than the observed for potato (Sánchez-Ferrer et al., 1993a). No instability of the 4-methyl-*o*-benzoquinone was observed at pH 7.5 or 8.0, possibly because we used a buffer with a lower ionic strength. This different pattern for catecholase and cresolase activities has also been observed in other plant PPOs (Sánchez-Ferrer et al., 1988).

The catecholase/cresolase ratio was obtained after the catalytic efficiency (V_{max}/K_m) had been calculated for both activities using 4-MC and *p*-cresol at pH 7.5. The obtained value of 41.1 indicated that, at pH close to the physiological, diphenol oxidation predominates over monophenol oxidation, as has been reported by other authors (Matheis, 1987a).

It has been reported that the characteristic lag period of the cresolase activity can be reduced by adding catalytic amounts of reducing agents or *o*-diphenols to act as cosubstrates (Pomerantz and Warner, 1967; Duckworth and Coleman, 1970; Lerch and Ettlinger, 1972; García-Carmona et al., 1979). Figure 5A shows the effect of different concentrations of 4-MC in the lag period (solid circles) and the activity of the enzyme when the same concentrations of 4-MC were used in the reaction medium in the presence of a constant concentration of *p*-cresol (open circles). This last plot indicates that 4-MC is used as cosubstrate of the cresolase activity and not as substrate for the catecholase activity of PPO.

The results obtained with partially purified eggplant PPO agree well with the mechanism proposed for other PPOs (García-Carmona et al., 1982; Cabanes et al., 1987a). The model proposed by this group takes into account the occurrence of three forms of the enzyme (E_{met}, E_{oxy}, and E_{deoxy}) and the chemical redox recycling of *o*-quinones formed by the enzyme. In brief, during catecholase activity, *o*-diphenol (D) binds to both E_{oxy} and E_{met} to give $E_{oxy}D$ and $E_{met}D$ intermediates, respetively, which give rise to two *o*-quinones. These two *o*-quinones recycle to regenerate one *o*-diphenol and one *p*-hydroxyquinone.

During the cresolase cycle, the binding of monophenol (M) to the E_{oxy} form renders $E_{met}D$, which gives a quinone; however, during the binding of monophenol to E_{met} (with no catalytic activity on monophenols), a portion of tyrosinase is scavenged from the catalytic turnover as a dead-end complex ($E_{met}M$). The enzyme slowly re-enters the catalytic cycle by means of the *o*-diphenol obtained by recycling in the chemical reactions. The lag period corresponds to the time needed to reach the catalytic level of diphenol for the steady state to be maintained. Thus, the duration of the lag period depends on a number of factors, which can be summarized as follows:

(a) pH. The appearance and disappearance of the lag period in the monophenolase activity at different pH values (Figure 2B) may be due to a combination of both changes in the afinity of the E_{met} form for the monophenol and the speed of the recycling chemical reactions, which are faster at neutral pH values.

(b) Substrate concentration. An increase in the concentration of *p*-cresol brings about an increase in the lag phase (Figure 3) because more enzyme enters the dead-end complex $E_{met}M$.

(c) Enzyme concentration. An increase in PPO concentration leads to an increase in activity and a decrease in the length of the lag phase (Figure 4) because the E_{oxy} present in the medium increases when PPO concentration increases, and the steady-state amount of *o*-diphenol necessary to eliminate the lag phase is reached more quickly.

(d) Diphenol concentration. The addition of catalytic amounts of 4-MC (an *o*-diphenol) to the system diminishes the lag period of the cresolase activity (Figure 5A)

until it is abolished. If the level of 4-MC is exceeded, the excess is transformed by the catecholase activity of PPO.

Tropolone was the most effective inhibitor of eggplant PPO (Table 1). This compound is structurally analogous to orthodiphenolic substrates of PPO and an effective copper chelator. Depending on the source of the enzyme and on the substrate used to carry out the experiment, mixed-type inhibition (Moore and Flurkey, 1990) and simple competitive inhibition (Valero et al., 1991; Sojo et al., 1998) have been reported. The competitive inhibition of TBC oxidation by tropolone that we observed confirms that this activity was not the result of a contamination of the crude extract by peroxidases (Figure 1).

Knapp (1965) reported the competitive inhibition of eggplant PPO by resorcinol using chlorogenic acid as substrate. The K_i obtained in these conditions was 23 mM. Introduction of a hydrophobic substituent in the 4-position increased the inhibitory potential of these compounds (Jiménez and García-Carmona, 1997). Using TBC as substrate we found a K_i of 50 μM for 4-hexylresorcinol. This compound, which is claimed to be nontoxic, nonmutagenic, and noncarcinogenic, is beginning to be used in the food industry for browning control (Frankos, 1991).

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