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A Kinetic Study of *p*-Cresol Oxidation by Quince Fruit Polyphenol Oxidase

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The monophenolase activity of quince pulp polyphenol oxidase was characterized by extracting samples using a combination of a two-phase partition step in Triton X-114, followed by a PEG 8000/phosphate partition step, and a final ammonium sulfate fractionation between 30 and 75%. The purification method avoids the loss of cresolase activity described in another quince pulp polyphenol oxidase. The activity was characterized by a lag period, whose duration depended on the substrate concentration, the pH, and the presence of catalytic amounts of *o*-diphenol. By increasing the concentration of *o*-diphenols, it was possible to evaluate the enzyme activation constant, K_{act}, which showed a value of 4.5 μM for 4-methylcatechol. A general kinetic mechanism for this enzyme is used to explain the loss of activity that normally occurs during quince pulp polyphenol oxidase purification.

KEYWORDS: Quince; polyphenol oxidase; monophenolase; lag period

INTRODUCTION

In common with many fruits and vegetables, quince fruit contains polyphenol oxidase (PPO; EC 1.14.18.1, monophenol dihydroxy-L-phenylalanine:oxygen oxidoreductase), which catalyzes two different reactions, each using molecular oxygen: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of the *o*-diphenols to *o*-quinones (diphenolase activity) (for a review, see ref *1*). The quinones thus formed are highly reactive substances, which normally react further with other quinones, amino acids, or proteins to produce the colored compounds that are responsible for losses in nutrient qualities (*2*). This is also a severe problem in the isolation of plant enzymes. The successful prevention of these reactions in the pulp, where most of the PPO occurs (*3*), involves a detailed study of the enzyme's biochemical properties before a technological process can be developed (*4*).

PPO was seen to be the enzyme responsible for the browning of quince fruit, in which it acted by oxidizing the naturally occurring phenolic substances, as described by Yagar and Sagiroglu (*5*), although no activity was detected toward monophenols. The lability of monophenolase activity during the purification of several plant and fruit PPOs is well-known (*3*, *6*), resulting from the changes in the structure of the protein during purification (*7*) due to covalent binding with polyphenol pigments. The use of drastic methods (acetone powders) or ammonium sulfate fractionation which harms the enzyme has been associated with this loss in monophenolase activity, whereas gentle methods based on nonionic detergents preserve it (*1*).

The present paper shows that the joint use of Triton X-114 and PEG 8000/phosphate preserves the monophenolase activity of quince fruit pulp PPO, permitting it to be kinetically characterized for the first time. .

MATERIALS AND METHODS

Quince fruits (*Cydonia oblonga* var. Gigante de Vranja) grown in Don Benito (SOLVEFRUIT S. L., Badajoz, Spain) were obtained from a local supermarket. Biochemicals were purchased from Fluka (Madrid, Spain) and used without further purification.

Enzyme Purification. Latent quince fruit PPO was extracted and partially purified in a sequential two-phase system based on Triton X-114 and PEG-8000/phosphate. A total of 100 g of fresh quinces was homogenized with 100 mL of 4% (w/v) Triton X-114 in 100 mM sodium phosphate buffer (pH 7.3). The homogenate was subjected to phase partitioning at 37 °C for 15 min. The turbid solution obtained was centrifuged at l0000*g* for 15 min at 25 °C. The dark detergent-poor supernatant (crude enzyme extract) was subjected to additional phase partitioning to remove the remaining phenols. For this, new Triton X-114 was added to a final concentration of 6% (w/v), and the mixture was held at 37 °C for 15 min. After centrifugation of the sample at 25 °C at 10000*g*, the supernatant of 6% (w/v) Triton X-114, which contained the soluble quince PPO, was subjected to a partition system based on PEG-8000 (5% w/w)/potassium phosphate buffer (pH 7.0). After 15 min at room temperature, the sample was centrifuged at 10000*g* for 10 min at 25 °C. The bottom clear phosphate-rich phase containing the quince PPO was brought to 30−75% saturation with $(NH_4)_2SO_4$. The final solution was centrifuged at 60000*g* for 30 min at 4 °C. The precipitate obtained with 75% ammonium sulfate was collected and dissolved in a minimum volume of water.

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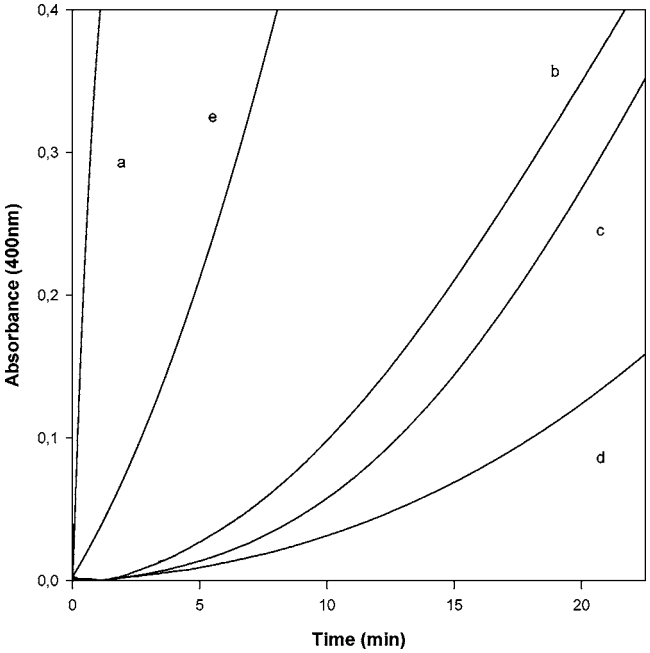


Figure 1. Enzymatic activities of soluble quince fruit pulp PPO. (a) Catecholase activity at 25 °C. The reaction medium included 0.5 μg/mL PPO and 2.5 mM 4-methylcatechol in 50 mM phosphate buffer pH 7.0. (b–e) Cresolase activity at 25 °C. The reaction medium contained (b) 16 μg/mL PPO and 2.5 mM *p*-cresol in 50 mM sodium phosphate buffer pH 7.0. (c–d) The same reaction medium as (b) except for (c) different substrate concentration (5 mM *p*-cresol), (d) different pH (pH 5), and (e) the addition of *o*-diphenols to the reaction medium (4-methylcatechol 15 μM).

Enzyme Activity. Monophenolase and diphenolase activities were determined spectrophotometrically at 400 nm with *p*-cresol and 4-methylcatechol, respectively (*2*). The steady-state rate in monophenolase activity was calculated from the linear zone of the product accumulation curve after the lag period. The standard reaction mixture included, in a total volume of 1 mL, 16 μg/mL soluble quince PPO and 2.5 mM *p*-cresol in 50 mM sodium phosphate buffer pH 7. One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of 4-methyl-*o*-quinone per minute ($\epsilon_{400} = 1433\ M^{-1}\ cm^{-1}$).

RESULTS

The enzyme obtained from quince fruit pulp by using a combination of two different aqueous two-phase systems in Triton X-114 and PEG-8000/phosphate was a real polyphenol oxidase because it shows both diphenolase (**Figure la**) and monophenolase activities (**Figure 1b−e**). 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 was measured (*8−11*). The lag period and the steady-state rate (defined as the slope of the linear part of the accumulation product curve) were affected by substrate concentration, the pH, and the presence of catalytic concentrations of *o*-diphenols (**Figure 1b−e**).

Effect of pH. Monophenolase activity toward *p*-cresol increased as the pH increased from pH 3.5, although no clearly defined maximum was reached (**Figure 2**). At pH values above 7.5, it was difficult to follow the formation of 4-methyl-*o*-benzoquinone spectrophotometrically because of its great instability.

The pH affected not only enzyme activity but also the lag period, and a decrease in the latter was observed when the pH was increased (**Figure 2**). These results are similar to those described for other plants PPO (*9*, *11*, *12*) but differ from the flat pH profile obtained for apple (*13*) and banana (*14*).

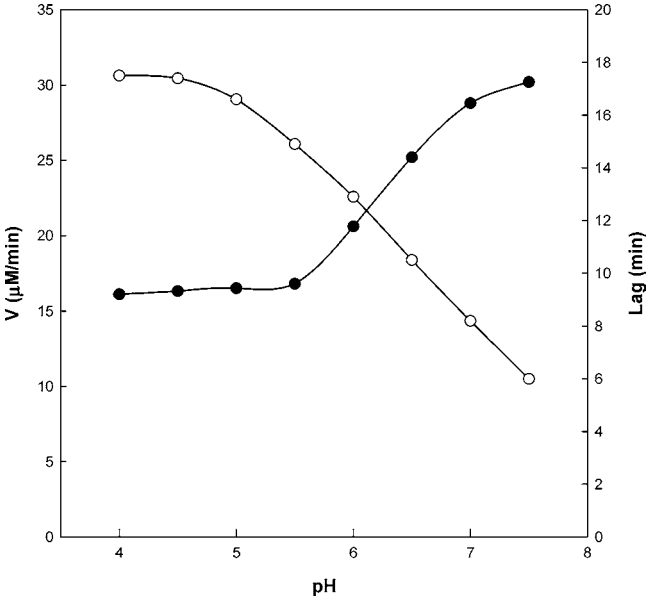


Figure 2. Effect of pH on cresolase activity (●) and on its lag period (○). The reaction medium at 25 °C included 16 μg/mL partially purified quince PPO and 2.5 mM *p*-cresol in 50 mM sodium acetate (pH 3.5–5.5), and sodium phosphate (pH 5.5–7.5).

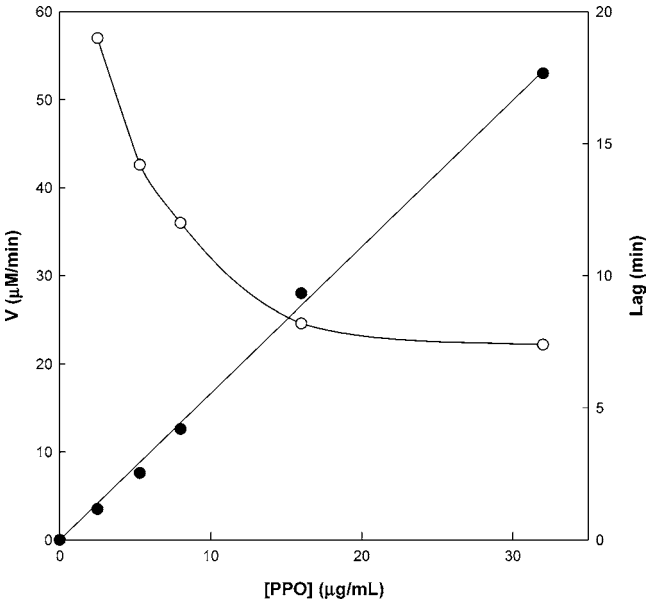


Figure 3. Effect of PPO concentrations on its cresolase activity (●) and on the lag period (○). The reaction medium at 25 °C included 2.5 mM *p*-cresol in 50 mM sodium phosphate buffer pH 7 and different PPO concentrations (0–32 μg/mL).

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 3**) and the lag period decreasing hyperbolically (**Figure 3**), a behavior that has been widely described in other PPOs (*12*, *15−17*).

Effect of Substrate Concentration. An increase in *p*-cresol concentration from 0 to 7.5 mM (**Figure 4**) produced an increase in the lag period of quince PPO, similar to that described for other plant PPOs (*9*, *11*). However, the explanation of the

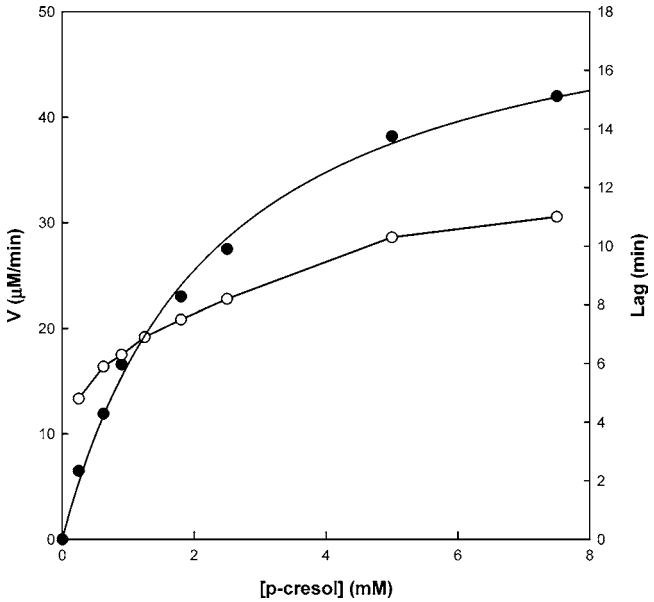


Figure 4. Effect of substrate concentration on cresolase activity of quince PPO (●) and on the lag period (○). The reaction medium at 25 °C included 16 μg/mL of partially purified PPO in 50 mM sodium phosphate buffer pH 7.0 with different *p*-cresol concentrations.

increase in the lag period profile is complex because the shape of the curve depends on the enzyme source used.

The kinetic constants, V_m and K_M, were evaluated from the data of **Figure 4** by a nonlinear regression to the Michaelis−Menten equation using the data obtained at pH 7.0. The values obtained were 54.7 μM/min and 2.2 mM, respectively. This K_M value for *p*-cresol cannot be compared with the only work on quince fruit PPO (*5*) because no activity was detected toward *p*-cresol due to the drastic purification method used, but it is higher than that obtained with banana (*14*), potato (*18*), and peach (*19*). Moreover, this K_M value is higher than the one obtained in this enzyme for 4-methylcatechol (1.2 mM) (data not shown).

Effect of *o*-Diphenols. The effect of *o*-diphenols on the cresolase activity of partially purified quince fruit pulp PPO was studied according to the protocol of Pomerantz and Warner (*20*), assuming that the *o*-diphenol was 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 (*20*):

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

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.

Figure 5 shows the decrease in the lag period as *o*-diphenol concentration was increased up to 30 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 6**), and no changes were observed up to 30 μM. Above this concentration the rate increased proportionally, indicating that the *o*-diphenol was being used as a substrate for the catecholase activity.

The activation constant K_{act} was calculated from the reciprocal of the lag period in the presence of diphenol (**Figure 5**, inset). The value obtained was 4.5 μM, higher than the K_{act} found for

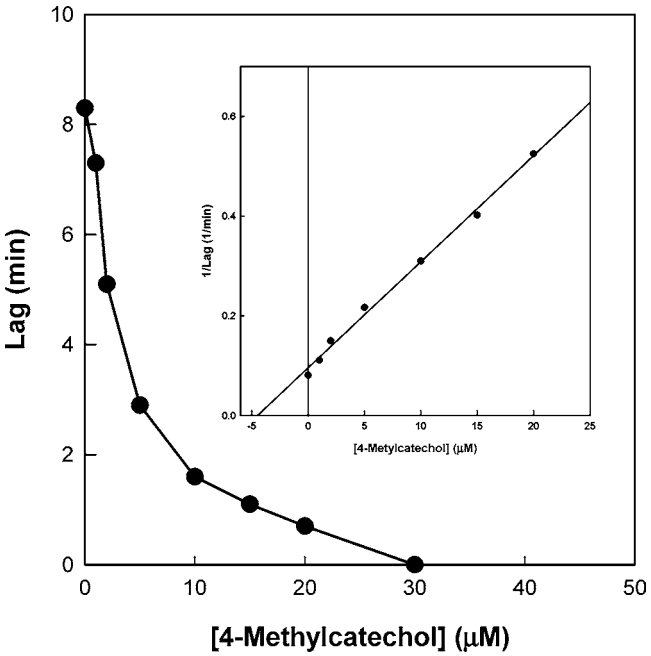


Figure 5. Influence of 4-methylcatechol on the shortening of lag period. In the standard reaction medium, the concentration of 4-methylcatechol ranged from 0 to 50 μM. (Inset) Evaluation of the activation constant, K_{act}, by the Pomerantz equation (*22*).

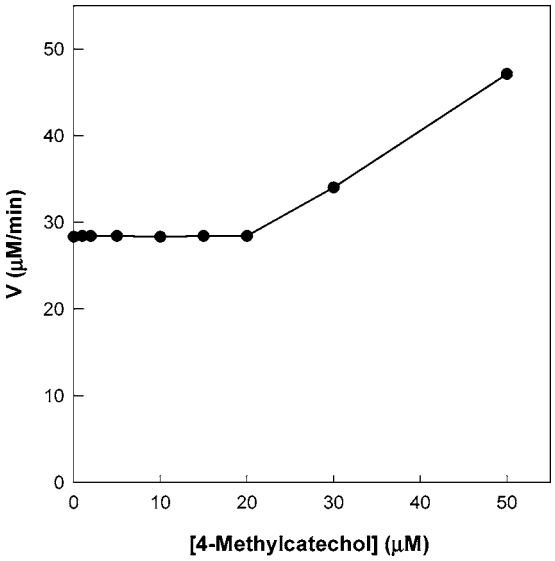


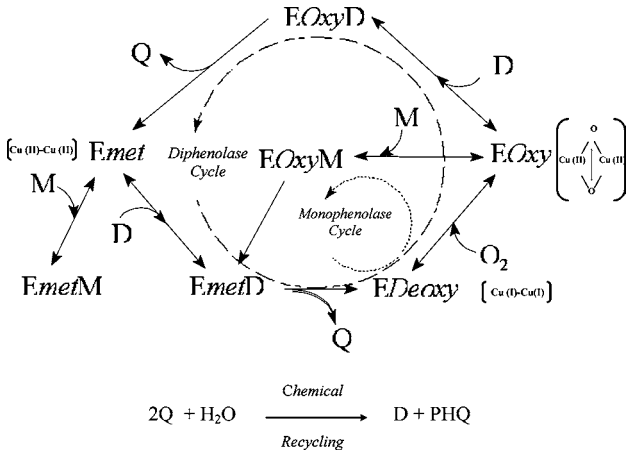
Figure 6. Effect of catalytic amounts of 4-methylcatechol on the steady state of cresolase activity. The conditions are the same as in **Figure 5**.

other plant PPOs (*9*, *21*, *22*) but the same value as that described in potato tuber (*18*).

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. (*12*). 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, as a result of 4-methyl-*o*-benzoquinones unstability, it undergoes water addition, generating the corresponding trihydroxy. The latter

Scheme 1. Enzymatic-Chemical-Chemical Mechanism Proposed for Cresolase Activity[a]



[a] M, *p*-cresol; D, 4-methylcatechol; Q, 4-methyl-*o*-benzoquinone; PHQ, 2-hydroxy-*p*-benzoquinone. E_{oxy} (oxotyrosinase consist of two tetragonal Cu (II) atoms); E_{met} (met-tyrosinase contains two tetragonal copper (II) ions antiferromagnetically coupled); E_{deoxy} (deoxotyrosinase has a bicuprous structure [Cu (I)-Cu (I)]).

compound gives rise to a redox reaction with another 4-methyl-*o*-benzoquinone, rendering the corresponding 2-hydroxy-*p*-benzoquinone and one *o*-diphenol (4-methylcatechol) (*23*). This cycle shows no lag period when 4-methyl-*o*-benzoquinone accumulation is followed (**Figure la**).

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 ($E_{met}M$). Exit from this nonactive E_{met}-monophenol form is possible only by upsetting 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**−**e**), which may last from minutes (**Figure 1b**) to hours, depending on a variety of factors:

(a) 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 different profiles, from bell-shaped to no clearly defined maximum (**Figure 2**).

(b) Amount of E_{oxy} Form in the Enzymatic Extract. This amount ranges from 2 to 30% depending on the source (*8*). Because the E_{oxy} present in the medium increased when the PPO concentration increased and because the steady state of *o*-diphenol activity was reached more quickly, the lag period diminished (**Figure 3**).

(c) Monophenol Concentration. Increasing amounts of monophenol in the reaction medium involves more enzyme in the dead-end complex, $E_{met}T$, and more time to reach the steady state (**Figure 4**). 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 4**).

(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 5**), indicating that the level of *o*-diphenol in the steady-state had been reached. If this level is exceeded, the excess *o*-diphenol is used by the diphenolase catalytic cycle, and activity increases (**Figure 6**).

(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 (*9*). Thus, mild purification methods based on Triton X-114 (*24*, *25*), or a combination of Triton X-114 and PEG-8000/phosphate as described in this paper seem to be suitable methods for obtaining 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 *p*-cresol, which quinone redox recycle, is used, monophenolase activity can be detected (**Figure 1b**−**e**). In contrast, if the monophenol gives a water-stable *o*-quinone, such as *tert*-butylphenol, no monophenolase activity can be detected unless H_2O_2 is used to obtain the E_{oxy} form during the reaction (*23*).

In conclusion, although the basis for determining monophenolase activity in plant PPO was described at the end of the 1980s in studies of the kinetic mechanism (*12*), and despite its experimental determination in plants (*9*) and the development of mild purification methods based on TX-114 (*23*, *24*), only a few plant PPO monophenolase activities have been kinetically characterized. This paper describes how the use of an additional phase partitioning step in PEG 8000/phosphate might help in preserving quince monophenolase activity, because the only study on quince PPO (*5*) found no activity toward *p*-cresol.

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ABREVIATIONS USED

PPO, polyphenol oxidase; PEG-8000, poly(ethylene glycol); TX-114, Triton X-114.

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