Proteolytic Activation of Latent Paraguaya Peach PPO. Characterization of Monophenolase Activity

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The kinetics of the activation process of latent peach PPO by trypsin was studied. By coupling this activation process to the oxidation of 4-*tert*-butylcatechol (TBC) to its corresponding quinone, it was possible to evaluate the specific rate constant of active PPO formation, k_3, which showed a value of 0.04 s^{-1}. This proteolytic activation of latent peach PPO permitted us to characterize the monophenolase activity of peach PPO for the first time using *p*-cresol as substrate, and it showed the characteristic lag period of the kinetic mechanism of monophenols hydroxylation, which depended on the enzyme and substrate concentration, the pH and the presence of catalytic amounts of *o*-diphenol (4-methylcatechol). The enzyme activation constant, k_{act}, was 2 μM.

Keywords: *Latent PPO; trypsin; peach; monophenolase activity*

INTRODUCTION

Polyphenol oxidase (PPO) is a copper-containing 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) (*1*). This enzyme has been found in an inactive or latent form in many fruits and vegetables (*2*−*5*). It can be activated by a variety of treatments such as low pH (*6*) or exposure to fatty acids (*7*), detergents (*5*, *8*, *9*), and proteases (*10*). Solubilization (*11*), the induction of limited conformational changes (*12*), as well as the dissociation of an enzyme−inhibitor complex (*13*), have all been proposed to account for this activation of latent PPO. However, the mechanism by which the enzyme is activated in vitro has not been clearly elucidated.

Treatment with proteases, such as trypsin and pancreatin, has been reported to activate and increase the solubilization of PPO (*7*, *9*, *14*), whereas treatment with chymotrypsin, papain, and carboxypeptidase had little effect (*14*). Trypsin activation seems to be the most effective protease in grape (*9*, *14*, *15*), broad bean (*10*), spinach (*7*), and mushroom (*5*) PPO. Besides solubilization, trypsin treatment has also been used to convert inactive pro-PPO from *Daucus carota* cell cultures to the active enzyme (*16*) and to interconvert PPO isozyme forms (*14*). In addition, Flurkey and Jen (*17*) used trypsin protease inhibitors (PMSF, Trasylol) to decrease the number of peach PPO isozyme forms during purification of the enzyme.

In 1987 King and Flurkey (*10*) reported that trypsin treatment of latent broad bean PPO generates an active enzyme form of slightly smaller molecular weight than the isolated enzyme. However, a detailed kinetic study of the activation process of plant PPO has not been developed.

In the present work, the effect of trypsin on the diphenolase and monophenolase activity of peach PPO was studied. The first activity, diphenolase, in the presence of trypsin, was used to kinetically characterize the process of enzyme activation. As regards the second activity, monophenolase, the presence of trypsin permitted us to characterize the complicated kinetic response of activated peach PPO toward monophenols.

MATERIALS AND METHODS

The peaches (*Prunus persica* cv. Paraguaya) used in this study were harvested at commercial maturity from a local growner in Molina de Segura, (Murcia, Spain).

Substrates were purchased from Sigma Chemical Co. (Madrid, Spain) and trypsin (110 U/mg) was purchased from Roche Molecular Biochemicals (Barcelona, Spain). Other reagents were of analytical grade.

Peach PPO was partially purified by phase-partitioning in Triton X-114, and yielded a fully latent enzyme showing no activity unless activated by detergents (SDS) (*18*) or proteases (trypsin).

Both diphenolase and monophenolase activities were measured using trypsin-activated peach PPO. For this, 35 μL of trypsin (1 mg/mL) and 35 μL of latent peach PPO (2 mg/mL) were incubated for 5 min. Diphenolase activity was measured spectrophotometrically at 400 nm using 4-*tert*-butyl-catechol (TBC) (ϵ = 1150 $M^{-1}cm^{-1}$) as substrate (*3*). The standard reaction medium contained 5 mM TBC and 50 ng of trypsin-activated peach PPO in 10 mM sodium phosphate buffer pH 6.5, in a total volume of 1 mL.

Monophenolase activity was also determined spectrophotometrically at 400 nm and 25 °C, using *p*-cresol as substrate (ϵ = 1433 $M^{-1}cm^{-1}$) (*19*) and trypsin-activated peach PPO. The monophenolase activity was not measured using *tert*-butylphenol (pair of TBC) as substrate because the TBC-quinone generated from this monophenol is so stable that it cannot be recycled, thus presenting the enzyme from leaving the lag period (*20*). The steady-state rate was calculated from the linear zone of the product accumulation curve after the lag period. In a total volume of 1 mL, the standard reaction medium included 70 μg of trypsin-activated peach PPO and 1 mM *p*-cresol in 10 mM sodium phosphate buffer pH 6.5. One

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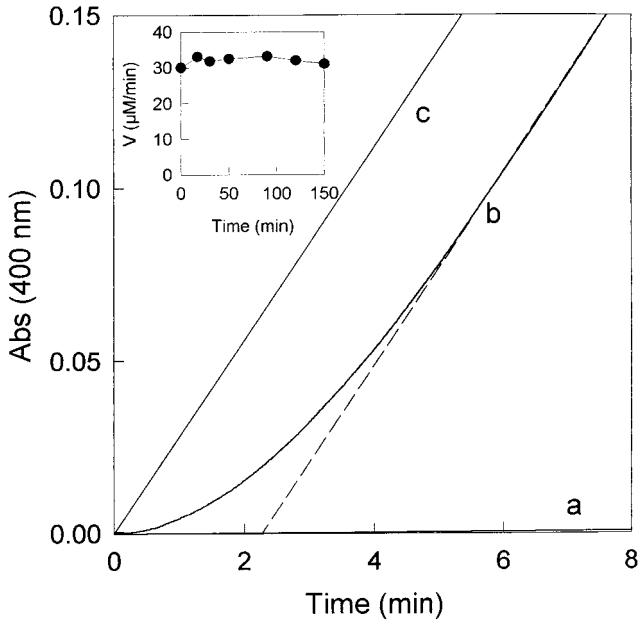


Figure 1. Time-course of TBC-quinone formation during trypsin activation of paraguaya peach PPO. The reaction medium at 25 °C included 5 mM TBC in 10 mM sodium phosphate buffer pH 6.5. The reaction was triggered with (a) 50 ng/mL of partially purified peach PPO without trypsin; (b) 1 μg/mL of trypsin and 50 ng/mL of partially purified peach PPO; (c) 50 ng/mL of latent PPO and 1 μg/mL of trypsin that was incubated for 5 min before adding 5 mM TBC to the reaction medium. Inset: effect of incubation time with trypsin on diphenolase activity of partially purified peach PPO. The reaction medium at 25 °C contained 5 mM TBC and 50 ng/mL peach PPO previously incubated with trypsin (35 μL trypsin (1 mg/mL) and 35 μL latent peach PPO (2 mg/mL)) for different times, in 10 mM sodium phosphate buffer pH 6.5. The reaction was started by the addition of TBC.

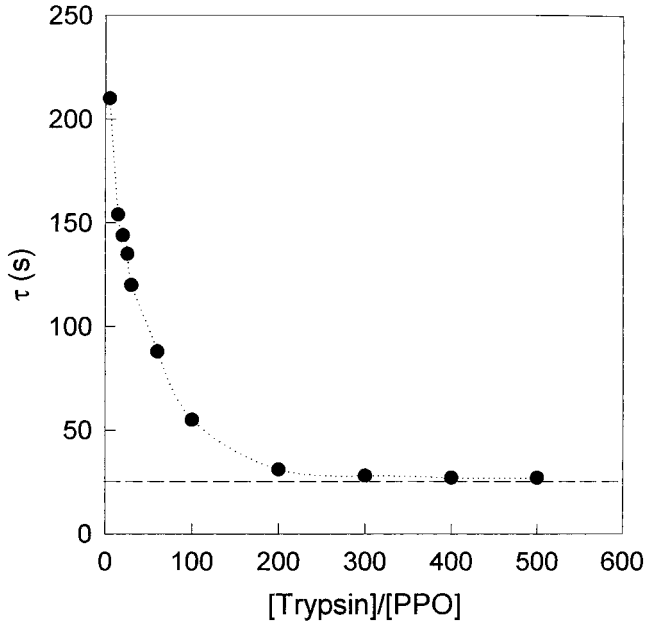


Figure 2. Effect of the [trypsin]/[PPO] ratio on lag period (τ). The reaction medium at 25 °C contained 5 mM TBC, 50 ng/mL of partially purified peach PPO, and increasing concentrations of trypsin (0.25−25 μg/mL) in 10 mM sodium phosphate buffer pH 6.5 in a final volume of 1 mL.

unit of enzyme in both cases was taken as the amount that produced 1 μmol of product per minute.

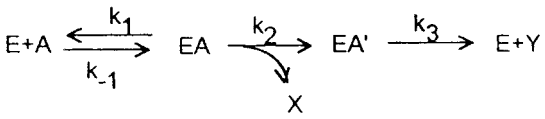
The kinetics of the activation process was measured by following diphenolase activity in a total volume of 1 mL containing 5 mM TBC, 50 ng of latent PPO, and variable concentrations of trypsin from 0.25 to 25 μg in 10 mM sodium phosphate pH 6.5.

RESULTS

Effect of Trypsin in Diphenolase Activity. Paraguaya peach PPO purified by the TX-114 method (*18*) showed no diphenolase activity unless it was activated with trypsin (Figure 1, curve a). The activation process was measured spectrophotometrically by following the appearance of the yellow TBC-quinone at 400 nm. This oxidation showed a lag period (τ) (Figure 1, curve b), which was determined by extrapolation of the linear portion of the product accumulation curve to [P] = 0. The linear portion showed that the system reached steady-state conditions. This lag period was abolished when latent peach PPO was incubated with trypsin prior to the oxidation of TBC (Figure 1, curve c). The enzyme was stable in these conditions for approximately 2 h (Figure 1, inset).

Figure 2 shows the decrease in the lag period observed with the [trypsin](μg/mL)/[latent PPO] (ng/mL) ratios up to 200, above which τ did not vary. This means that when [trypsin]≫[latent PPO] (a situation in which the reaction of proteolytic activation is under non-steady-state conditions), the τ values became independent of

Scheme 1. Kinetic Mechanism of PPO. M, monophenol; D, diphenol; Q, *o*-quinone.



trypsin concentration. Under these conditions, it is possible to obtain expressions relating τ with the kinetic constants, as first described by Hijazi and Laider (*21*) and later by Galindo et al., (*22*) for frog epidermis protyrosinase. They proposed a Michaelis−Menten mechanism with double intermediate (Scheme 1). In our case, *E* is trypsin, *A* is latent peach PPO, *EA* and *EA′* are intermediate complexes, and *X* and *Y* are the products of the proteolytic process. To determine the rate of active peach PPO formation and to ascertain the order in which the products are released, the oxidation reaction of TBC by PPO was coupled to the activating process.

In this coupled process the following set of conditions must be fulfilled: (a) [*E*] ≫ [*A*], (b) [TBC] ≫ [*A*], (c) the transient phase for the catalytic oxidation of TBC is negligible vs the transient phase of the activation process, and (d) *EA* and *EA′* do not react with TBC.

In this situation, the reaction represented in Scheme 1 will not reach the steady state because all the latent PPO will be converted to active enzyme. However, the oxidation of TBC will reach the steady state, and so the system formed by the two coupled reactions will attain the steady state. In this way, some information on the kinetic constants of the activation of latent peach PPO can be obtained by measuring the rate of TBC-quinone formation.

The rate of TBC-quinone formation under steady-state conditions should be proportional to active PPO concentration, and two possibilities may be considered, according to the equations described by Hijazi and Laider in 1972 (*21*) to describe the time-dependent formation of products *X* and *Y* in the proteolytic process:

(i) X is the active peptide after the proteolytic activation of latent PPO (Scheme 1):

$$TBC + O_2 \xrightarrow{X} TBC - quinone$$

In this case, the expression for the accumulation of P (TBC-quinone), for $t \gg 1$ can be written as

$$[P] = [A]^0Kt + [A]^0K\left(\frac{(-(k_{-1} + k_2 + k_2[A]^0) + \sqrt{(k_{-1} + k_2 + k_1[A]^0)^2 - 4(k_1k_2[A]^0)}/2)}{k_1k_2[E]^0} + \frac{(-(k_{-1} + k_2 + k_1[A]^0) - \sqrt{(k_{-1} + k_2 + k_1[A]^0)^2 - 4(k_1k_2[A]^0)}/2)}{k_1k_2[E]^0}\right) \quad (1)$$

where $[A]^0$ is the initial concentration of latent peach PPO and K is a proportionality constant.

This eq 1 represents a straight line, where the intercept with the time axis would provide

$$\tau = -\frac{(-(k_{-1} + k_2 + k_1[A]^0) + \sqrt{(k_{-1} + k_2 + k_1[A]^0)^2 - 4(k_1k_2[A]^0)}/2)}{k_1k_2[E]^0} + \frac{(-(k_{-1} + k_2 + k_1[A]^0) + \sqrt{(k_{-1} + k_2 + k_1[A]^0)^2 - 4(k_1k_2[A]^0)}/2)}{k_1k_2[E]^0} \quad (2)$$

(ii) Y is the active peptide after the proteolytic activation of latent PPO (Scheme 1):

$$TBC + O_2 \xrightarrow{Y} TBC - quinone$$

In this case the analytical expression for the accumulation of P (TBC-quinone) for $t \gg 1$, can be written as

$$[P] = [A]^0Kt + \frac{[A]^0K}{k_{-3}} \quad (3)$$

This equation also represents a straight line, where the intercept with the time axis is $1/k_3$ (*22*), the lag period, τ, in this case, being defined as

$$\tau = \frac{1}{-k_3} \quad (4)$$

Therefore, the two alternative analytical expressions for the lag period, τ, are given by eqs 2 and 4, depending on whether the active enzyme is X or Y, respectively.

The results presented in Figure 2 show that τ is independent of [trypsin] ($[E]^0$), when [trypsin] $\gg$ [latent PPO] ($[E]^0 \gg [A]^0$), which fits well with eq 4.

Effect of Trypsin on Monophenolase Activity. The lag period, characteristic of monophenolase activity of PPO, and the steady-state rate (defined as the slope of the linear part of the accumulation product curve) (Figure 3) were affected by a number of factors: enzyme concentration, pH, substrate concentration, and the presence of catalytic concentrations of *o*-diphenols.

An increase in enzyme concentration produced a linear increase in the monophenolase activity of latent peach PPO (Figure 4), as well as a shortening of the lag period (Figure 4). This behavior has been widely described in other PPOs (*23*−*25*).

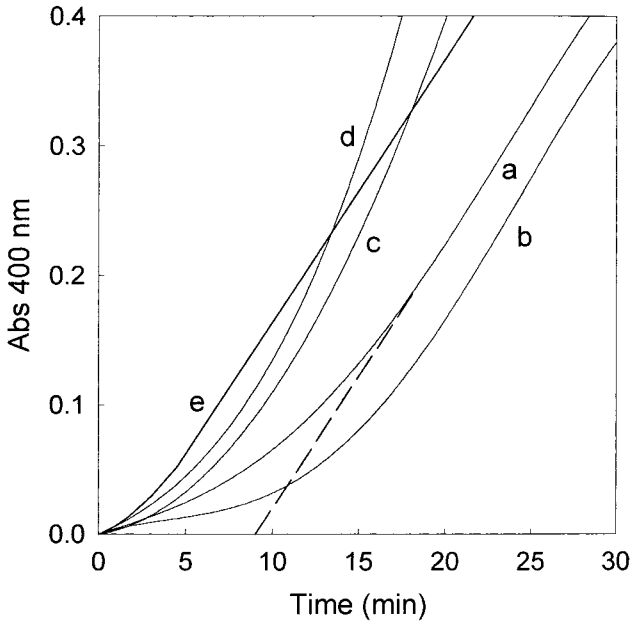


Figure 3. Monophenolase activity of partially purified peach PPO. (a) The reaction medium at 25 °C contained 10 mM sodium phosphate buffer pH 6.5, 1 mM *p*-cresol, and 70 μg/mL of trypsin-activated peach PPO. (b) 2 mM *p*-cresol. (c) 90 μg/mL trypsin-activated peach PPO. (d) pH 7.5. (e) 4-methyl catechol (4MC) 6.7 μM.

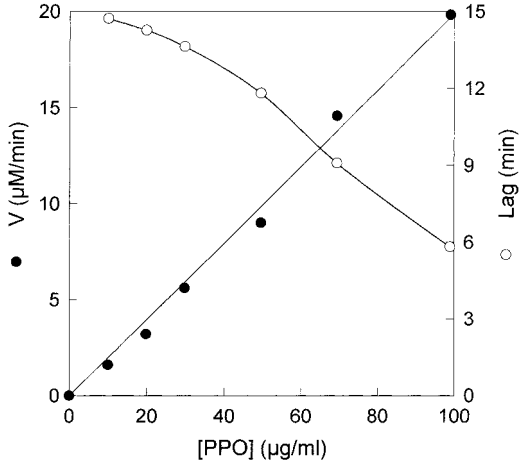


Figure 4. Effect of enzyme concentration on monophenolase activity of partially purified peach PPO (●) and on its lag period (○). The reaction medium at 25 °C included 1 mM *p*-cresol and increasing concentrations (0−100 μg/mL) of trypsin-activated peach PPO in 10 mM sodium phosphate buffer pH 6.5.

The monophenolase activity of trypsin-activated peach PPO toward *p*-cresol increased as the pH increased from pH 4.5 to 7.4, although no clearly defined maximum was reached (Figure 5). At pH values above 7.0, the formation of 4-methyl-*o*-benzoquinone was difficult to follow spectrophotometrically because of its great instability. The pH affected not only the enzyme activity but also the lag period, and a decrease in the latter was observed when pH was increased (Figure 5). These results are similar to those described for other plant PPOs (*19*, *26*, *27*), but are different from those obtained for Verdedoncella apple (*28*) and banana (*25*).

An increase in *p*-cresol concentration from 0.1 to 2.5

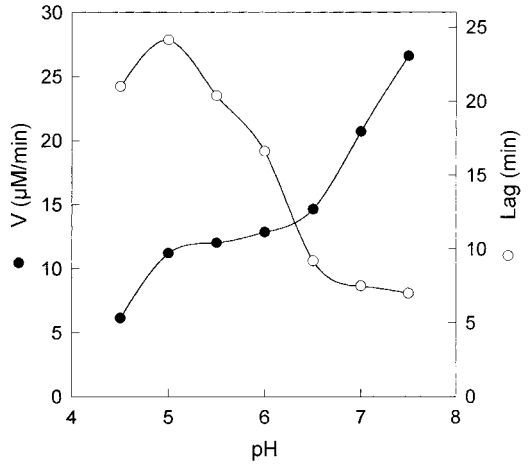


Figure 5. Effect of pH on monophenolase activity of partially purified peach PPO (●) and on its lag period (○). The reaction medium at 25 °C contained 1 mM *p*-cresol and 70 μg/mL of trypsin-activated peach PPO in 10 mM sodium acetate (pH 3.5−5.5) and 10 mM sodium phosphate (pH 6.0−7.5) buffers.

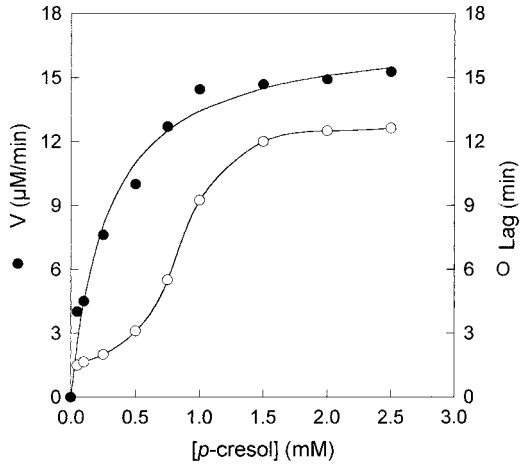


Figure 6. Effect of substrate concentration on monophenolase activity of partially purified peach PPO (●) and on its lag period (○). The reaction medium at 25 °C included 70 μg/mL of trypsin-activated peach PPO in 10 mM sodium phosphate buffer (pH 6.5) with different *p*-cresol concentrations.

mM (Figure 6) produced a sigmoidal increase in the lag period of partially purified peach PPO. However, the exact shape of the curve depends on the enzyme source used (*19*, *25*, *27*−*29*). The kinetic constants V_m and K_M obtained were 14.6 μM/min and 0.28 mM, respectively. This K_M was lower than that obtained using monastrell grape (*27*) or potato tuber as an enzyme source (*19*). Note that it is crucial to use a low level of monophenol when trying to accurately determine monophenolase activity, because the lag period greatly increases when monophenol concentration is high (Figure 6).

The effect of diphenols on the monophenolase activity of partially purified peach PPO was studied according to the protocol of Pomerantz and Warner (*30*). It was assumed that the *o*-diphenol binds to the activation center by a simple isotherm and that the lag period is proportional to the nonactive concentration at zero time, according to the empirical formula (*30*)

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

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. The open circles of Figure 7 show the decrease in the lag period observed as *o*-diphenol (4-methyl catechol) concentration was increased up to 20 μM, at which stage the lag was abolished. The steady-state rate was plotted against *o*-diphenol concentration (Figure 7, filled circles) and no changes were observed up to 20 μM, above which concentration the rate increased proportionally. The activation constant K_{act} was calculated from the reciprocal of the lag period in the presence of diphenol (Figure 7, inset) and the value obtained was 2 μM.

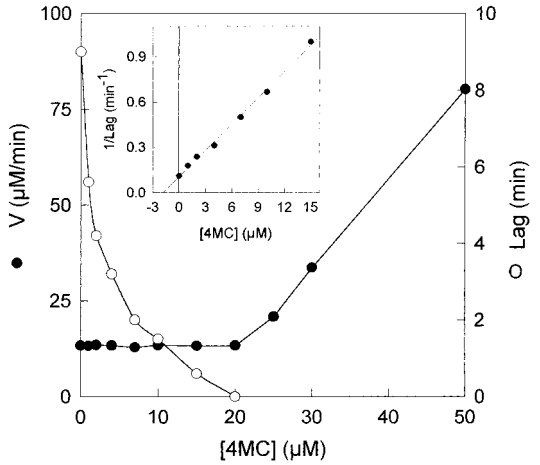
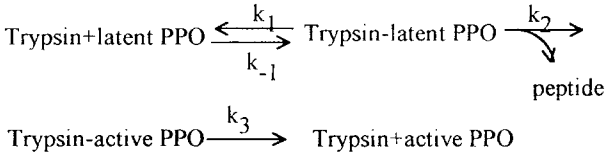


Figure 7. Effect of catalytic amounts of 4-methyl catechol (4MC) on monophenolase activity of partially purified peach PPO (●) and on its lag period (○). The reaction medium at 25 °C included 70 μg/mL of trypsin-activated peach PPO, 1 mM *p*-cresol, and different 4MC concentrations ranging from 0 to 50 mM in 10 mM sodium phosphate buffer (pH 6.5). Inset: evaluation of the activation constant, K_{act}, using the Pomerantz equation (*30*).

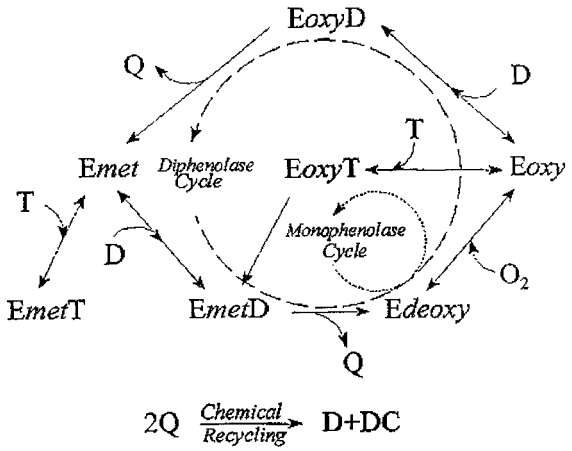
DISCUSSION

The native monophenolase activity of PPO from different sources, or activated by different processes (SDS, trypsin, etc.), shows a characteristic lag period which is related to its kinetic mechanism (for review see *1*). This lag period means that it is impossible to use monophenolase activity to characterize the activation process of latent peach PPO by trypsin. For this reason, diphenolase activity was used for this purpose.

In 1987 King and Flurkey (*10*) showed that trypsin treatment of broad bean PPO generated an active enzyme form with a slightly smaller molecular weight than the native enzyme. In our case, when latent paraguaya peach PPO was treated with trypsin, the lag period, τ, was independent of [trypsin] ($[E]^0$), when [trypsin]≫[latent PPO] ($[E]^0 \gg [A]^0$) (Figure 2) and fit well with eq 4, indicating that the active peptide was the product Y of Scheme 1, and X is the peptide of low molecular weight. Accordingly, the following mechanism is proposed for the proteolytic activation of latent peach PPO by trypsin:



Scheme 2



The value obtained for k_3 (0.04 s^{-1}) was much lower than that obtained by Galindo et al. (*22*) in the activation of frog epidermis pro-tyrosinase by trypsin (k_3 = 0.162 s^{-1}).

In this paper, by using the enzyme previously activated with trypsin, we were able to study the kinetic mechanism of monophenol hydroxylation by paraguaya peach PPO (monophenolase activity) which has not been previously described. The lag period obtained, characteristic of monophenolase activity of PPO, was affected by different factors (Figure 3), diminishing with the increase in enzyme concentration (Figure 4) and pH (Figure 5), and increasing with the increase in the substrate concentration (Figure 6). Moreover, this lag period was abolished in the presence of catalytic concentrations of *o*-diphenol (Figure 7).

Increasing PPO concentrations produced a proportional increase in the E_{oxy} form (Scheme 2), while the steady-state of *o*-diphenol activity was reached more quickly, thus diminishing the lag period (Figure 4). On the other hand, Increasing concentrations of monophenol in the reaction medium implies more enzyme in the dead-end complex $E_{met}M$ (Scheme 2) and more time to reach the steady-state. The lag period vs pH profile may be due to a combination of 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.

The monophenolase activity lag period can also be shortened or eliminated by the addition of reducing agents or *o*-diphenols, which act as co-substrates (*30*−*33*). The results presented in Figure 7 show the decrease in the lag period as *o*-diphenol (4-methyl catechol) concentration was increased up to 20 μM. At this stage the lag was abolished, indicating that the level of *o*-diphenol had reached the steady-state. The same steady-state rate, up to 4MC 20 mM indicates that the *o*-diphenol added was used as co-substrate and not as substrate (Figure 7, filled circles). Above 20 μM, the steady-state rate increased proportionally, indicating that the *o*-diphenol was being used as substrate for diphenolase activity (Figure 7). The value of activation constant K_{act} (2 μM), interpreted by Pomerantz and Warner (*30*) as the *o*-diphenol affinity constant for the activation center, was similar to that described for Airen grapes (*26*) and banana (*25*).

The results presented for the monophenolase activity of trypsin-activated peach PPO are in agreement with the mechanism previously described for PPO from other sources (*23*).

CONCLUSION

Latent peach PPO is activated by the proteolytic action of trypsin, yielding the second product of the proteolytic reaction as active peptide. This active peptide shows both diphenolase and monophenolase activities, over 4-*tert*-butyl catechol (TBC) (diphenol) and *p*-cresol (monophenol).The latter activity being possible to follow only after activating latent peach PPO with trypsin.

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