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EFFECT OF L-PROLINE ON MUSHROOM TYROSINASE

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Key Word Index—Tyrosinase; 4-methylcatechol; *p*-cresol; L-proline.

Abstract—The kinetic parameters for the catecholase and cresolase activities of mushroom tyrosinase have been determined both in the presence and in the absence of L-proline. L-Proline does not modify the kinetic parameters (V_{max} and K_m) of the catecholase activity, but modifies the V_{max} of the cresolase activity and affects the lag period and the steady-state rate of this activity. These results can be explained taking into account the complex mechanism proposed for tyrosinase.

INTRODUCTION

As has been indicated in the preceding paper [1], tyrosinase catalyses two different O_2-dependent reactions: hydroxylation of monophenols to *o*-diphenols (cresolase activity), and oxidation of *o*-diphenols to *o*-quinones (catecholase activity).

When tyrosinase acts on *p*-cresol or 4-methylcatechol, the 4-methyl-*o*-benzoquinone initially formed is reduced to 4-methylcatechol by the adducts formed between the quinone and either water or a nucleophile such as proline [1].

It is well known that tyrosinase has a lag period before expression of its cresolase activity. The lag period is decreased when sufficient *o*-diphenol is formed endogenously, as has been recently shown [2] for the formation of melanin from L-tyrosine; the proposed mechanism suggests that tyrosinase requires for the expression of cresolase activity on L-tyrosine a steady-state level of L-dopa, which can be modified by both enzymatic and chemical steps. The last one produces L-dopa and dopachrome from *o*-dopaquinone.

In the present paper, we study the possible application of the model [2] for plant tyrosinases to the substrates *p*-cresol/4-methylcatechol, since the chemical reduction of 4-methyl-*o*-benzoquinone is involved in the mechanism of tyrosinase [2]. We have examined the effect of the acceleration of these chemical steps by L-proline upon the expression of cresolase activity of tyrosinase by previously described spectrophotometric methods [1].

RESULTS

Measurement of enzymatic activity

Spectrophotometric determination of both activities of tyrosinase (cresolase and catecholase) on the substrates *p*-cresol and 4-methylcatechol was performed previously by measuring at 400 nm the appearance of 4-methyl-*o*-benzoquinone [3–5]. Similarly, the determination of catecholase activity on 4-methylcatechol in the presence of L-proline has been carried out by measuring at 520 nm [6, 7] the appearance of the addition product of L-proline and 4-methyl-*o*-benzoquinone.

These methods of measurement present different problems. For example, in the first method, the instability of 4-methyl-*o*-benzoquinone is not taken into account, while in the second the addition reaction of L-proline to 4-methyl-*o*-benzoquinone takes place very slowly [6]. Both problems can be eliminated if the enzymatic activities of tyrosinase are measured at the wavelengths where the isosbestic points for the formation of 4-methyl-*o*-benzoquinone from *p*-cresol/4-methylcatechol appear in the presence of L-proline. These isosbestic points have been calculated in the preceding paper [1].

In this study, we have measured cresolase activity at 377 nm ($\varepsilon = 1000\ M^{-1}\ cm^{-1}$), which is the wavelength of the isosbestic point obtained when 4-methylcatechol is oxidized with an excess of sodium periodate in the presence of L-proline. The choice of this wavelength was governed by the fact that when an excess of periodate is added to the reaction medium, the stoichiometry of 4-methyl-*o*-benzoquinone to 5-methyl-4-*N*-prolyl-*o*-benzoquinone is 1:1. The same situation holds for the enzymatic oxidation of *p*-cresol by tyrosinase when the system reaches the steady-state, since the level of *o*-diphenol accumulated in the steady-state is constant [2] and so the apparent stoichiometry of evolution of 4-methyl-*o*-benzoquinone is also 1:1.

Catecholase activity was measured at 370 nm ($\varepsilon = 830\ M^{-1}\ cm^{-1}$), which is the wavelength at which the isosbestic point is obtained [1] when 4-methylcatechol is oxidized with a sub-stoichiometric concentration of periodate in the presence of L-proline. In this situation the stoichiometry between 4-methyl-*o*-benzoquinone and 5-methyl-4-*N*-prolyl-*o*-benzoquinone is 2:1, the same situation pertains with catecholase activity when the steady-state is reached. In this case, the depletion of substrate during the reaction period is negligible and so the reaction rate is not appreciably modified. Therefore,

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Abbreviations: 4MC; 4-methylcatechol, dopachrome; 2-carboxy-2,3-dihydroindole-5,6-quinone, dopaquinone; 4-(2-carboxy-2-aminoethyl)-1,2-benzoquinone, *p*-cresol; 4-methylphenol.

4-methylcatechol recycled from 4-methyl-*o*-benzoquinone by the chemical steps does not modify the apparent rate of the enzymatic reaction either, the apparent stoichiometry of 4-methyl-*o*-benzoquinone to the final product being 2:1.

Effect of L-*proline on the cresolase activity*

When cresolase activity is followed spectrophotometrically at 377 nm (Fig. 1), a lag period is observed during enzymatic expression, as has been demonstrated for tyrosinase from varying sources [8, 9]. The presence of L-proline in the reaction medium increases significantly the steady-state rate (defined as the slope of the linear part of the product accumulation curve), and decreases the lag period present during the expression of cresolase activity, (defined as the intercept on the abscissa axis obtained on extrapolation of the linear part of the product accumulation curve).

Apparent kinetic parameters (V_{max} and K_M for cresolase activity were obtained from values of steady-state rates at different substrate concentrations by the equation of Lineweaver–Burk (results not shown). The apparent K_M (0.2 mM) was not affected by the presence of 2 mM L-proline in the reaction medium whereas the apparent V_{max} increased approximately two-fold. These results suggest that L-proline does not modify the affinity between the enzyme and the substrate *p*-cresol, but does modify the level of cresolase activity.

The effect produced on *p*-cresol oxidation by mushroom tyrosinase, by varying the concentration of L-proline (0–6 mM) is shown in Fig. 2. The activity expressed by tyrosinase increased in a hyperbolic form with L-proline concentration, reaching activations of 200%.

An inverse relationship between the lag period and the enzyme concentration can be observed when tyrosinase acts on *p*-cresol, in both the presence and absence of L-proline (Fig. 3). This result, together with the increase of steady-state rate observed in the presence of L-proline (Fig. 1), suggests that the lag period depends on cresolase activity expressed in the reaction medium rather than the enzyme concentration. To examine this possibility, these

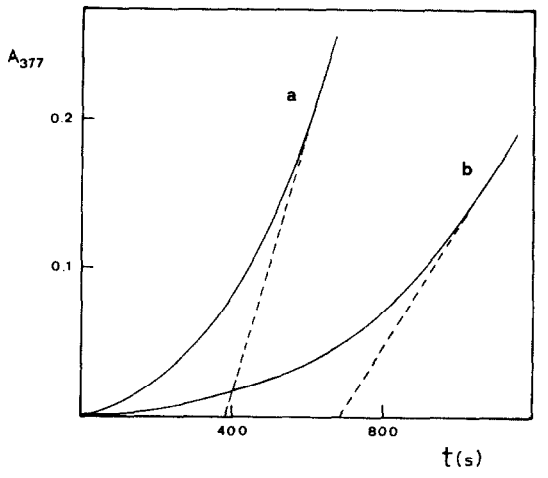


Fig. 1. Spectrophotometric measurements of cresolase activity at 377 nm. (a) In the presence of 2 mM L-proline, (b) in absence of L-proline. The reaction medium contained 1.6 mM *p*-cresol, mushroom tyrosinase (10 μg/ml) and 10 mM Na-Pi buffer, pH 6.0. The final volume was 2.5 ml.

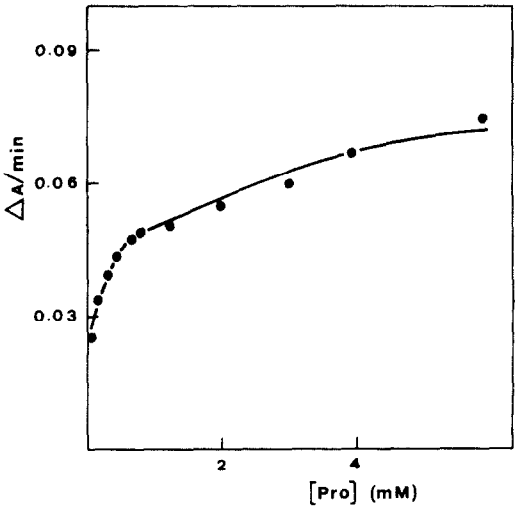


Fig. 2. Variation of cresolase activity with concentration of L-proline. Conditions were the same as those outlined in Fig. 1.

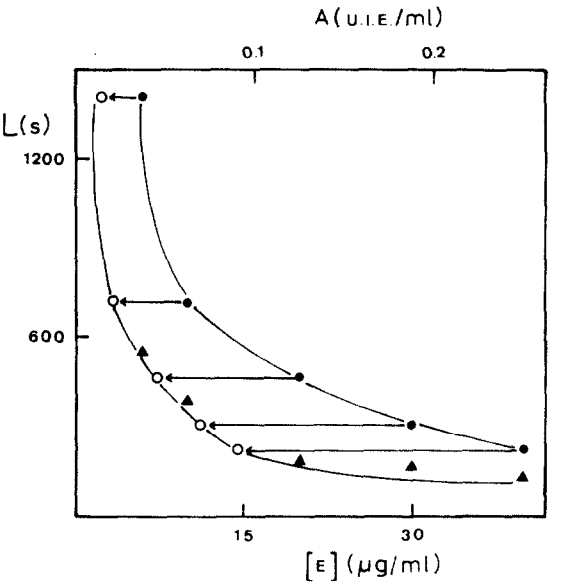


Fig. 3. Dependence of the lag period on cresolase concentration in the presence (▲) or in the absence (●) of 2 mM L-proline; and also with cresolase activity expressed in the reaction medium in the presence (▲) or in the absence (○) of L-proline. Experimental conditions were the same as those outlined in Fig. 1.

results were plotted as a function of cresolase activity. The same dependence between cresolase activity and the lag period was obtained in both the presence and absence of L-proline.

It is well known that the lag period of the cresolase activity of tyrosinase can be eliminated by *o*-diphenols and several reducing agents [10–15]. This effect has been interpreted as *o*-diphenols saturating an 'activation site' [12], according to the following empirical equation:

$$1/L = 1/l + 1/l \frac{[\text{Diphenol}]}{k_{act}} \qquad (1)$$

where L, and l represent, respectively, the lag period in the presence and absence of *o*-diphenol, and k_{act} the activation constant for the *o*-diphenol. From this equation, k_{act} is calculated and represents the affinity of *o*-diphenol to the 'activation site'.

The effect of 4-methylcatechol on the lag period of *p*-cresol oxidation is shown in Fig. 4. These results were plotted according to eqn (1) and a $k_{act}=0.2\ \mu M$ was obtained (Fig. 4B).

L-Proline also modifies the lag period shown by the cresolase activity (Fig. 1). It can be seen that a hyperbolic variation of the lag period, when L-proline was added to the reaction medium in a range of 0–6 mM was also obtained (Fig. 5A), although in this case a total elimination of the lag period did not occur. Fig. 5B shows the diminution of lag period by 4-methylcatechol in the presence of 4 mM L-proline. The lag period is eliminated, but a concentration of *o*-diphenol $\approx 20\ \mu M$ was necessary, that is a concentration ten times higher than the one necessary when L-proline was not present in the reaction medium. Furthermore, it can be pointed out that in the presence of L-proline the initial lag period was 280 seconds and in its absence the initial lag period was 680 seconds (Fig. 4).

In the presence of 4 mM L-proline the activation constant (k_{act}) evaluated for 4-methylcatechol by means of equation (1) was $k_{act}=1\ \mu M$ (results not shown), a value significantly higher than the one obtained in the absence of L-proline.

The results show that either in the presence or in the absence of L-proline (Figs 4 and 5), the steady-state rate for cresolase activity was not increased over the concentration range of 4-methylcatechol tested.

Effect of L-*proline on the catecholase activity*

Catecholase activity of mushroom tyrosinase was measured at 370 nm (as has been discussed previously).

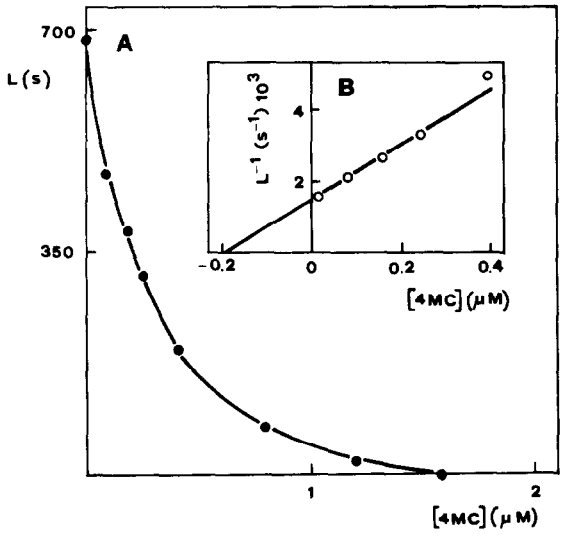


Fig. 4. (A) Influence of 4-methylcatechol on the lag period. The reaction medium contained 1.6 mM *p*-cresol, 4-methylcatechol (0–2 μM), mushroom tyrosinase (10 μg/ml) in Na-Pi buffer, pH 6.0. Final volume was 2.5 ml. (B) The same results plotted according to Pomerantz's equation [12].

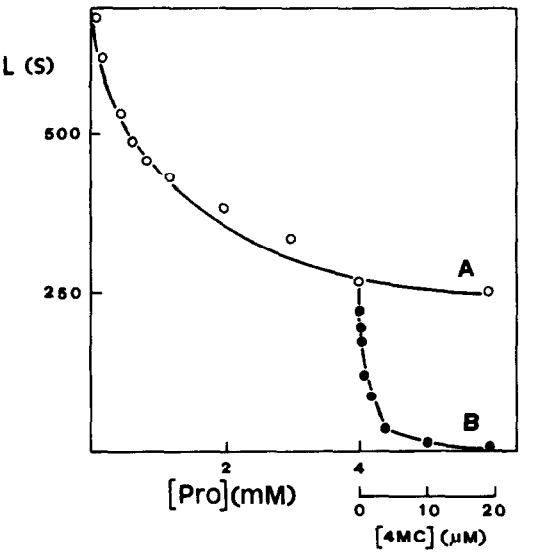


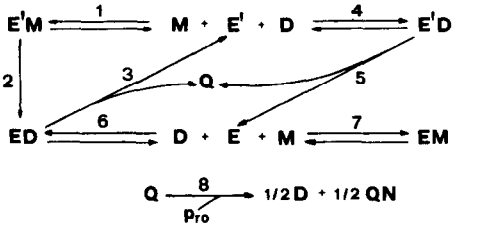
Fig. 5. (A) Influence of L-proline on the lag period of cresolase activity. (B) Elimination of the lag period by 4-methylcatechol in the presence of 4 mM L-proline. Experimental conditions were the same as those given in Fig. 1. A range of 0–20 μM of 4-methylcatechol was added to this medium in (B).

No lag period and no modification of V_{max} and K_M was observed in the presence and absence of L-proline at pH 6.0 and 25° (results not shown). The value obtained for K_M, 0.1 mM, was significantly higher than the one obtained for K_{act} (0.2 μM) (Fig. 4) when 4-methylcatechol acts as co-substrate of cresolase activity.

DISCUSSION

The results obtained in this paper show clearly the fundamental importance of the chemical steps involved in the transformation of the *o*-quinones involved in the mechanism of tyrosinase when attempting to explain the complex results observed during expressions of cresolase activity. All these effects can be explained by the model for the action of tyrosinase [2]. This model is based on:

(i) An internal mechanism of enzymatic catalysis [8], mainly based on structural studies of different forms of copper in the active site [16, 17] (Scheme 1), which included the possibility of union of monophenol at the



E Forms: Met-tyrosinase
E' Forms: Oxytyrosinase
M Monophenol
D Diphenol
Q *o*-Quinone
QN Product of addition of nucleophile to the quinone ring

Scheme 1.

met-tyrosinase, leading to a dead-end complex or inhibition complex.

(ii) The subsequent chemical reactions on the initial product of enzymatic activity that result in the transformation of half of the *o*-quinone into *o*-diphenol.

(iii) The presence of variable amounts (2–30%) of intrinsic oxytyrosinase in the resting enzyme [8].

According to this model, the lag period of cresolase activity can be interpreted as the result of a dynamic adjustment between enzymatic steps,, the competition established between monophenols and *o*-diphenols by oxy- and met-tyrosinase forms [18] and the chemical reactions of the net production of *o*-diphenol in the reaction medium. The system reaches the steady-state when the rate of transformation from *o*-diphenol to *o*-quinone by tyrosinase is the same as the rate of *o*-diphenol regeneration by chemical and enzymatic steps.

In this way, the effect produced by the addition of L-proline to the reaction medium must be attributed to the L-proline accelerating the regeneration of 4-methylcatechol (step 8 Scheme 1) due to an acceleration of steps 4 and 6 (Scheme 1). Therefore a higher level of *o*-diphenol will be reached in the steady-state and a minor amount of enzyme will remain in the inactive form (step 7), since the level of *o*-diphenol competes with this last step by means of step 6. So in the presence of L-proline in the reaction medium, it is necessary to add a higher concentration of 4-methylcatechol in order to eliminate the lag period (Fig. 5B). Since L-proline is not the cosubstrate of cresolase activity the lag period cannot be totally eliminated. On the other hand, in every case a different k_{act} was evaluated and so it cannot be considered as a real constant but as a parameter related to the level of *o*-diphenol in the steady-state.

EXPERIMENTAL

All materials used were the same as those of the preceding paper [1].

Determination of enzyme activity. Cresolase activity was measured at 377 nm, as has been previously discussed, at 25°. The reaction medium was: 10 mM Na-Pi buffer (pH 6.0), 1.6 mM *p*-cresol (i.e. in saturated conditions), L-proline as indicated in each case and an appropriate amount of enzyme so as not to cause oxygen depletion before reaching the steady-state.

Catecholase activity was measured at 370 nm, at 25°. The reaction medium was similar to that used for the measurement of cresolase activity, except that the *p*-cresol was replaced by 4-methylcatechol.

Measurements were carried out with an UV/Vis Perkin–Elmer Lambda-3 spectrophotometer on-line interfaced with a Perkin–Elmer computer model 3600 Data-Station. Temperature was controlled by using a Gilson bath and a digital Cole-Parmer thermistor with a SR ± 0.1°.

Protein concentration was determined by the method of ref. [19].

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