Phytochemistry, Vol. 20. No. 6, pp. 1215–1217, 1981.
Printed in Great Britain.

0031-9422/81/061215-03 $02.00/0

HYSTERESIS OF MUSHROOM TYROSINASE: LAG PERIOD OF CRESOLASE ACTIVITY

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(*Revised received* 21 *October* 1980)

Key Word Index—*Agaricus bispora*; fungi; mushroom; tyrosinase; cresolase; lag period of tyrosinase.

Abstract—Mushroom tyrosinase presents a lag period in the expression of its cresolase activity depending on enzyme and substrate concentration in the reaction medium. At a fixed enzyme concentration in the reaction medium, the lag period depends on the concentration of previously stored enzyme. Experimental data are in agreement with the existence of a hysteretic process in which the lag period corresponds to a slow dimerization step of the enzyme induced by the substrate which could combine with the non-polymeric form of the enzyme. The K_m for L-tyrosine of the polymeric enzyme was 0.15 mM.

INTRODUCTION

Tyrosinase or polyphenoloxidase (EC 1.14.18.1) is a copper protein widely distributed in the phylogenetic scale, being responsible for melanization in animals and browning in plants. The enzyme catalyzes two different reactions: cresolase activity, or hydroxylation of monophenols (L-tyrosine) to *o*-diphenols (L-dopa) using oxygen and catecholase activity, or oxidation of *o*-diphenols (L-dopa) to *o*-quinone (L-dopa-quinone) using oxygen.

The existence of a lag period for cresolase activity of tyrosinase was pointed out some time ago [1–4]. This lag period can be shortened or eliminated by the presence of diphenols [4–8]. On the other hand, it is known that several tyrosinases, like those from mammals [8], plants [9] and mushroom [10], behave like auto-associating systems, in processes which depend on enzyme concentration.

In this paper we have studied the lag period of mushroom cresolase as a function of three different variable experimental conditions: enzyme concentration in the storage medium and in the reaction medium, and substrate concentration in the reaction medium. Results have been interpreted considering both the concept of enzymatic hysteresis developed by Frieden [11] and the theoretical treatment of Kurganov *et al.* [12] for auto-associating enzyme in the presence of substrate.

RESULTS AND DISCUSSION

Tyrosinase activity, when tyrosine is used as substrate, shows a lengthened transient phase before reaching the steady-state. When product is plotted versus time of reaction, the lag time (τ) can be estimated by extrapolation of the linear portion of the curve to the abscissa (Fig. 1).

The τ value for different tyrosinases has been given, in some cases, as constants: 0.5 min, in *Vibrio tyrosinaticus* [13] and nearly 2 hr in hamster melanoma [14]. However, it has been shown for mushroom tyrosinase that τ values change with the enzyme concentration in the reaction medium [10]. This dependency can be analysed after the enzymatic hysteresis concept developed by Frieden [11].

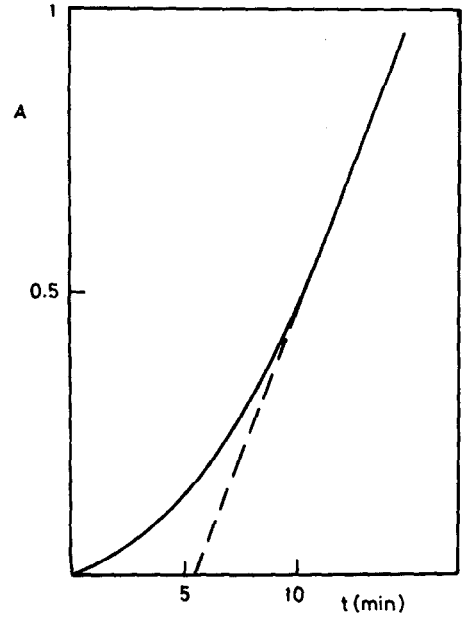

Fig. 1. Time course of the reaction. Product accumulation was measured as ΔA_{305} in the standard reaction medium containing 2 mM L-tyrosine and 12.5 μg of enzyme of a concentration of 0.5 mg/ml; a lag period τ = 5.5 min was determined.

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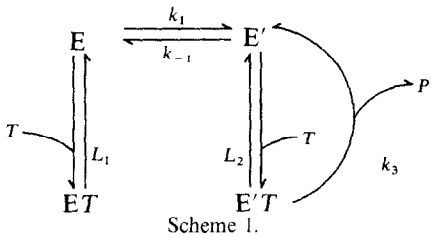
Because of the dependence of τ on enzyme concentration, it is necessary to reject the possibility of a first-order transition model like the following:



Scheme 1.

in which E' would be the catalytic active form of the enzyme. In this model,

$$\tau = \frac{1}{K} = \frac{t_{1/2}}{0.692}, \qquad (1)$$

L_1 and L_2 are the equilibrium constants and T is the concentration of tyrosine. The rate constant k_3 represents breakdown of the E'T complex to give product. Because K is a function of only T, L_1, L_2, k_{-1} and k_{+1}, τ would not be affected by changes in enzyme concentration.

If we accept the possibility of a second-order transition we can suppose the existence of a slow polymerization process mediated by the substrate as proposed by Frieden [11]. In these circumstances Kurganov *et al.* [12] have developed a theoretical study deducing that, when there is a sufficiently high enzyme concentration in the reaction medium, it must be realized that:

$$\log \tau = -\log [\mathrm{E}] + c, \qquad (2)$$

or

$$1/\tau = \frac{1}{10^c} [\mathrm{E}], \qquad (3)$$

where c is a function of L_1', L_2', T, k_1' and k_{-1}' (see Scheme 2).

Therefore plotting $1/\tau$ vs [E] will yield a straight line. This was actually obtained as shown in Fig. 2. A different approximation has been tried based on the known capacity of association–dissociation of mushroom tyrosinase [10] which depends on enzyme concentration, the formation of polymeric forms being favoured when the concentration is high.

Several preparations of enzyme of different concentrations were kept for a time, *ca* 48 hr, to reach equilibrium, and aliquots containing the same milli-units of enzyme were taken from the samples and their respective lag periods were analysed. The results obtained are shown in Fig. 2 and they are in agreement with a hysteretic behaviour of the cresolase activity of tyrosinase. Thus, lag periods increased as enzyme preparations were more diluted in which case less polymeric forms of the enzyme would exist. As can be observed in Fig. 2, linearity was obtained for each one of the three different initial preparations of enzyme used (0.1 mg/ml, 0.5 mg/ml, 1 mg/ml).

On the other hand, the effect of substrate concentration on the lag period can be seen in Fig. 3. The lag period was longer with increasing concentration of tyrosine, showing an inflection point. From Fig. 3, a K_m of 0.15 mM has been determined for tyrosine and the active polymeric form of the enzyme. This value was similar to that previously obtained by Fourche *et al.* [15] for this enzyme. Since lag

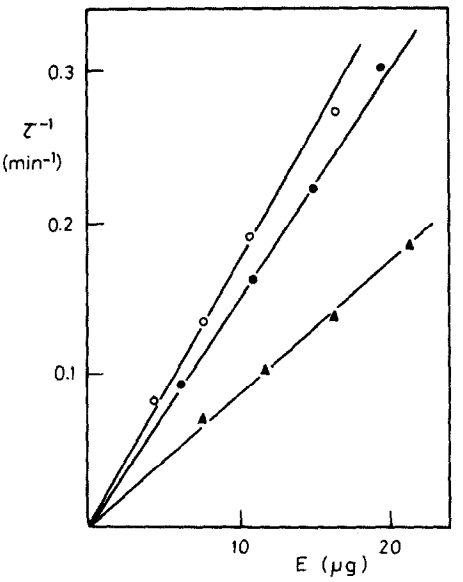
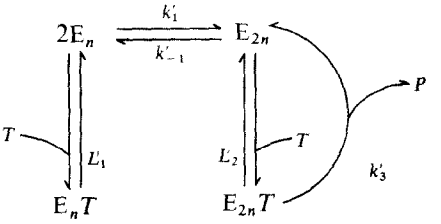


Fig. 2. Representation of the variation of the lag period with the enzyme concentration present in the reaction medium according to equation 3 (see text), measured according to the conditions established in Fig. 1. ○———○. Enzyme concentration 1 mg/ml; ●———●, enzyme concentration 0.5 mg/ml; ▲———▲, enzyme concentration 0.1 mg/ml.

period increases when substrate concentration increases, without reaching a plateau, it is possible to hypothesize that the non-polymeric form of the enzyme has a lower substrate affinity than the polymeric one.

The existence of a lag period for the cresolase activity of tyrosinase has previously been interpreted in different ways. Thus, with tyrosinase of hamster melanoma [14] it was suggested that non-enzymatic reactions produced small quantities of dopa from tyrosine, and that the so formed dopa behaved as an activator of cresolase. However, with tyrosinase of *Vibrio tyrosinaticus* [13] it was speculated that dopa or any reduced substrate was closely bound to the bacterial enzyme.

Nevertheless, the previous hypotheses do not explain satisfactorily kinetic data like those presented in this paper and which are similar to others obtained by us with tyrosinase from frog epidermis [16]. Up to now, no adequate explanation has been given for the existence of a lag time when tyrosinase acts on monophenols whose quinones are very stable, as in the case of *p*-coumaric acid [4]. However, all our data are in agreement with Scheme 2,



Scheme 2.

where L_1' and L_2' are equilibrium constants. The rate constant k_3' represents breakdown of the $E_{2n}T$ complex to give product.

Hysteretic behaviour, in this mechanism, signifies the existence of a system of polymerization $2E_n$

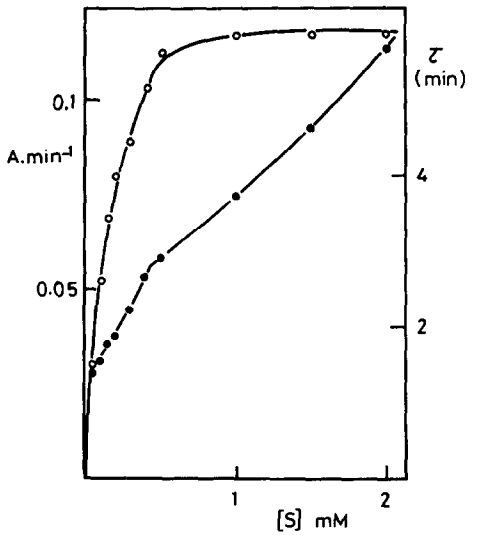


Fig. 3. ○———○, Representation of Michaelis–Menten for the different final velocities measurable after the respective lag periods; ●———●, representation of the variation of the lag period with L-tyrosine concentration; the conditions of measurement are those established in Fig. 1.

(inactive) $\rightleftharpoons E_{2n}$ (active) induced by substrate, resulting in a lag in the response of the enzyme to changes in the ligand level (Scheme 2). The mechanism would also explain previously described phenomena of auto-association with the concentration described in mushroom tyrosinase [10]. The dependence of lag period on substrate concentration could be explained by the fact that the non-polymeric form of the enzyme has a certain degree of affinity towards the substrate, and thus, tyrosine would avoid the polymerization of the enzyme to the active form, E_{2n}.

EXPERIMENTAL

Mushroom tyrosinase was obtained from Sigma Chemical Co. at 4000 U/mg, L-tyrosine from Merck and other products were reagent grade without further purification.

Cresolase activity was determined at 25° in a medium containing 0.01 M NaPi buffer pH 7 and 2 mM L-tyrosine. At zero time enzyme soln was added and formation of dopachrome was spectrophotometrically recorded at 305 nm [17]. One unit of the enzyme produces 1 μmol of dopachrome per min. The molar extintion of dopachrome was taken as 9600 M cm.

Protein determination was carried out by the method of ref. [18] using bovine serum albumin as standard.

REFERENCES

1. Deacon, W. and Mansh, H. V., Jr. (1971) *Phytochemistry* **10**, 2915.
2. Hughes, L. and Price, G. M. (1976) *J. Insect Physiol.* **22**, 1005.
3. Andersen, S. O. (1978) *Insect Biochem.* **8**, 143.
4. Carmona, F. G., Pedreño, E., Galindo, J. D. and Cánovas, F. G. (1979) *Analyt. Biochem.* **95**, 433.
5. Pomerantz, S. H. (1966) *J. Biol. Chem.* **241**, 161.
6. Pomerantz, S. H. and Warner, M. C. (1967) *J. Biol. Chem.* **242**, 5308.
7. Cánovas, F. G., Carmona, F. G., Galindo, J. D., Pedreño, E. and Lozano, J. A. (1979) *Rev. Esp. Fisiol.* **35**, 209.
8. Hearing, V. J., Jr., Ekel, T. M., Montagne, P. M., Hearing, E. D. and Nicholson, J. M. (1978) *Arch. Biochem. Biophys.* **185**, 407.
9. Harel, E., Mayer, A. M. and Lehman, E. (1973) *Phytochemistry* **12**, 2649.
10. Duckworth, H. W. and Coleman, J. E. (1970) *J. Biol. Chem.* **245**, 1613.
11. Frieden, C. (1970) *J. Biol. Chem.* **245**, 5788.
12. Kurganov, B. I., Dorozhko, A. I., Kagan, Z. S. and Yakalev, V. A. (1976) *J. Theor. Biol.* **60**, 287.
13. Pomerantz, S. H. and Murthy, V. V. (1974) *Arch. Biochem. Biophys.* **160**, 73.
14. Pomerantz, S. A. and Li, J. P. C. (1973) *Yale J. Biol. Med.* **46**, 541.
15. Fourche, J., Jensen, H., Neuzil, E. and Bellergade, B. (1977) *C.R. Acad. Sci.* **284**, 2163.
16. Carmona, F. G., Cánovas, F. G. and Lozano, J. A. (1980) *Int. J. Biochem.* **11**, 325.
17. Carmona, F. G., Cánovas, F. G. and Lozano, J. A. (1981) *Analyt. Biochem.* (submitted).
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.