Study of stereospecificity in mushroom tyrosinase

Juan Carlos ESPÍN*, Pedro Antonio GARCÍA-RUIZ†, José TUDELA* and Francisco GARCÍA-CÁNOVAS*[1]

*GENZ: Grupo investigación Enzimología, Departamento de Bioquímica y Biología Molecular–A, Facultad de Biología, Universidad de Murcia, A. Correos 4021, E-30080 Espinardo, Murcia, Spain, and †Departamento de Química Orgánica, Facultad de Química, Universidad de Murcia, Spain

This paper reports experiments on the stereospecificity observed in the monophenolase and diphenolase activities of mushroom tyrosinase. Several enantiomorphs of monophenols and *o*-diphenols were assayed: L-tyrosine, D,L-tyrosine, D-tyrosine; L-α-methyltyrosine, D,L-α-methyltyrosine; L-dopa, D,L-dopa, D-dopa; L-α-methyldopa, D,L-α-methyldopa; L-isoprenaline, D,L-isoprenaline and D-isoprenaline. The V_{max} values obtained for each series were the same. The electronic densities on the carbon atoms in the *meta* (C-3) and the *para* (C-4) positions of the benzene ring were determined by NMR assays. This value is related to the nucleophilic power of the oxygen atom belonging to the hydroxy group, which could explain the V_{max} values experimentally obtained for the monophenolase and diphenolase activities of mushroom tyrosinase. The spatial orientation of the ring substituents led to lower K_m values for L-isomers than for D-isomers. However, the V_{max} values were the same for each series of isomers because spatial orientation did not affect the NMR value of C-4. Therefore mushroom tyrosinase showed stereospecificity in its affinity towards its substrates (K_m) but not in the transformation reaction rate (V_{max}) of these substrates.

INTRODUCTION

Tyrosinase (monophenol, *o*-diphenol:oxygen oxidoreductase, EC 1.14.18.1) is a copper enzyme that is widely distributed throughout micro-organisms, plants and animals. It is of central importance in such processes as vertebrate pigmentation and the browning of fruits and vegetables [1]. Different tyrosinases obtained from several biological sources have similar structural and functional characteristics [2].

The active site of tyrosinase consists of two copper atoms and three states: 'met', 'deoxy' and 'oxy' [3–8]. Structural models for the active site of these three forms have been proposed [9–13]. This enzyme catalyses the *ortho*-hydroxylation of monophenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) [2,14]. The monophenolase activity is coupled to its diphenolase activity and to the non-enzymic reactions from the corresponding *o*-quinones [15–30].

Several monophenols and *o*-diphenols are chiral tyrosinase substrates (L-, D,L- and D-enantiomers). Some works have reported the stereoselective action of several tyrosinases on these enantiomers [31,32], although this phenomenon has not been systematically characterized. Wilcox et al. [11] reported stereospecificity for tyrosinase from *Neurospora crassa*. L-Tyrosine, D-tyrosine, L-dopa and D-dopa were assayed and their kinetic constants were obtained. The results did not show a determined sequence in the K_m and V_{max} values when the different isomers were compared. Khan and Pomerantz [33] reported the same V_{max} value for L-tyrosine and D-tyrosine, as well as for L-dopa and D-dopa, by using avocado polyphenol oxidase. The same pattern was obtained for *Vibrio tyrosinaticus* tyrosinase [34]. Different results were obtained for mammalian [35] and *Pseudomonas* tyrosinase [36]. However, all these contradictory results might simply have arisen from the assay methods used, which might not have rendered reliable and precise results.

Catalytic stereospecificity has been reported in other enzymes such as horseradish peroxidase and lactoperoxidase. This phenomenon was approached with ESR spin stabilization techniques combined with optical methods by Ferrari et al. [37], with the substrates L-dopa and D-dopa. The kinetic parameters, K_m and V_{max}, of these enantiomers were found to differ. The peroxidase reaction mechanism might justify these results.

In a recent paper [13] the lack of quantitative results on the monophenolase activity of tyrosinase was reported. The aim of our paper is the systematic study of the stereospecificity of mushroom tyrosinase in its action on several chiral monophenolic and *o*-diphenolic substrates. Monophenolase and diphenolase activities of mushroom tyrosinase were determined spectrophotometrically by using the chromogenic nucleophile 3-methyl-2-benzothiazolinone hydrazone (MBTH). This nucleophile traps the enzyme-generated *o*-quinones to render a stable and soluble MBTH–*o*-quinone adduct of high molar absorption coefficient [21,23–31]. NMR studies on these chiral substrates were also made, to estimate their nucleophilic power as predictors of their catalytic efficiency with tyrosinase. The results obtained for the enzymic activity were compared with the NMR predictions and were found to fit to a previously proposed reaction mechanism for tyrosinase from different fruits and vegetables [19,24,26, 28–30,38].

EXPERIMENTAL

Materials

L-Tyrosine, D,L-tyrosine, D-tyrosine, L-α-methyltyrosine, D,L-α-methyltyrosine, L-dopa, D,L-dopa, D-dopa, L-α-methyldopa, D,L-α-methyldopa, L-isoprenaline, D,L-isoprenaline, D-isoprenaline and MBTH were purchased from Sigma (Madrid, Spain). All other chemicals were of analytical grade and supplied by Fluka

Abbreviations used: δ_3, chemical displacement value for C-3 (*meta*); δ_4, chemical displacement value for C-4 (*para*); DMF, *N,N′*-dimethylformamide; MBTH, 3-methyl-2-benzothiazolinone hydrazone; V_{ss}, steady-state rate.

[1] To whom correspondence should be addressed (e-mail canovasf@fcu.um.es).

(Madrid, Spain). Stock solutions of the phenolic substrates were prepared in 0.15 mM phosphoric acid to prevent autoxidation. The acidic character of MBTH required the use of 50 mM buffer in the assay medium. To dissolve the MBTH–quinone adducts, 2% (v/v) *N*,*N*′-dimethylformamide (DMF) was added to the assay medium [21,23–31]. Milli-Q system (Millipore Corp.) ultrapure water was used throughout this research.

Enzyme source

Mushroom tyrosinase (8300 units/mg) was purchased from Sigma (St. Louis, MO, U.S.A.) and purified by the procedure of Duckworth and Coleman [39]. Protein concentration was determined by the method of Bradford [40]. The enzyme concentration was calculated by assuming a molecular mass of 120 kDa.

Spectrophotometric assays

Kinetic assays were performed by measuring the appearance of the product in the reaction medium in a UV–visible Perkin Elmer Lambda-2 spectrophotometer, interfaced on-line with an IBM PC 486 DX microcomputer. Temperature was controlled at 25 °C by using a Haake D1G circulating-water bath with a heater/cooler and checked using a Cole-Parmer digital thermometer with a precision of ±0.1 °C. Reference cuvettes contained all the components except the substrate in a final volume of 1 ml.

Monophenolase and diphenolase activities of mushroom tyrosine were determined spectrophotometrically by using MBTH, a potent nucleophile through its amino group, which attacks the enzyme-generated *o*-quinone in position 6 [21,23–31], trapping it to render a soluble and stable MBTH–quinone adduct of high molar absorption coefficient. The stability of the MBTH–quinone adducts and the rapid assay times provide a reliable assay method for determining the monophenolase and diphenolase activities of tyrosinase from several sources [21,23–30].

Kinetic data analysis

The K_m and V_{max} values for different substrates were calculated from triplicate measurements of the steady-state rate, V_{ss}, for each initial substrate concentration, $[S]_0$. The reciprocals of the variances of V_{ss} were used as weighting factors in the non-linear regression fitting of V_{ss}–$[S]_0$ data to the Michaelis equation [41,42]. The fitting was performed by using Marquardt's algorithm [43] implemented in the SIGMA PLOT 2.01 program for Windows™ [44]. Initial estimates of K_m and V_{max} were obtained from the Hanes–Woolf equation, a linear transformation of the Michaelis equation [41].

NMR assays

^{13}C NMR spectra of several substrates were obtained in a Varian Unity spectrometer at 300 MHz. The spectra were obtained at the optimum pH of mushroom tyrosinase by using ^2H_2O as solvent for the substrates. Chemical displacement (δ) values were measured relative to those for tetramethylsilane ($\delta = 0$). The maximum line width accepted in the NMR spectra was 0.06 Hz. Therefore the maximum error for each peak of the spectrum was ±0.03 p.p.m.

RESULTS AND DISCUSSION

Kinetic assays

V_{max} and K_m values were obtained for each series of isomers. Whereas V_{max} values were the same, the K_m values were different for each series of isomers at the same enzyme concentration. The sequence of K_m values was: D-isomers > D,L-isomers > L-isomers, the same sequence of results as that obtained for monophenols and *o*-diphenols (Tables 1 and 2). It should be noted that the V_{max} values decreased and K_m values increased when the molecular mass of the substituent side chain of the monophenols or *o*-diphenols increased (Tables 1 and 2).

NMR assays

Because monophenols or *o*-diphenols with an electron-withdrawing substituent are poorly oxidized [13], a side chain of high electron donor capacity will facilitate the hydroxylation and oxidation of monophenols and *o*-diphenols respectively. To study the electron donor capacity of the side chain, the different isomers for monophenols and *o*-diphenols were studied by means of NMR assays. In such a study, the highest electron donor capacity corresponds to the highest electronic charge and the lowest δ values for C-4 (*para*) in monophenols and for C-3 (*meta*) and C-4 in *o*-diphenols. The δ_4 values for monophenols and δ_3 and δ_4 values (where 3 and 4 refer to C-3 and C-4 respectively) for *o*-diphenols are shown in Table 3. The δ values were similar for the series (L-, D,L- and D-isomers) of each monophenol and *o*-diphenol.

From the results of the NMR studies (Table 3) it can be seen that similar δ_4 values for each isomer of monophenol and similar δ_3 and δ_4 values for each isomer of *o*-diphenol predicted the same nucleophilic power of the oxygen from the aromatic hydroxy group to attack the copper atoms of the enzyme's active site. Therefore the V_{max} values must be the same (Tables 1 and 2).

These results also indicated that the reactivity of each series of isomers was the same. This NMR study supported the same sequence of V_{max} values as was obtained spectrophotometrically. If the electronic density (reactivity) is exactly the same (Table 3), then the nucleophilic power of the oxygen from the hydroxyl group to attack the copper atoms of the active site of the enzyme must be the same. Therefore the transformation rate (V_{max}) of the enzyme on the several isomers of each series of substrates is also the same (Tables 1 and 2).

The sequence of K_m values obtained for the different enantiomers (L- < D,L- < D-) could be explained by the effect of the spatial orientation of the side chain, which would affect one of several rate constants in the K_m expression for the monophenolase and diphenolase activities of tyrosinase [19,38].

Diphenolase activity

The diphenolase reaction mechanism of tyrosinase (Scheme 1) involves several rate constants for the analytical expressions of V^D_{max} and K^D_m [19,38], where D indicates *o*-diphenols:

$$V^D_{max} = \frac{k_3 k_7 [E]_0}{(k_3 + k_7)} \quad (1)$$

$$K^D_m = \frac{k_2 k_3 (k_{-6} + k_7) + k_6 k_7 (k_{-2} + k_3)}{k_2 k_6 (k_3 + k_7)} \quad (2)$$

The transformation steps ruled by k_3 and k_7 could be separated into two sub-steps: first the nucleophilic attack of the oxygen

Table 1 Kinetic constants for the monophenolase activity of mushroom tyrosinase on several monophenolic enantiomorphs

Conditions were: 50 mM phosphate buffer, pH 6.8, 2% (v/v) DMF, saturating MBTH concentration, differing monophenol concentrations and 3.8 nM mushroom tyrosinases. Results are means ± S.D. for three separate experiments.

Monophenol	V_{max}^{M} (μM/min)	k_{cat}^{M} (s^{-1})	K_{m}^{M} (mM)	V_{max}^{M}/K_{m}^{M} (min^{-1})
L-Tyrosine	1.80 ± 0.05	7.9 ± 0.2	0.27 ± 0.01	$6.7 \times 10^{-3} \pm 4.5 \times 10^{-4}$
D,L-Tyrosine	1.85 ± 0.06	8.1 ± 0.3	0.90 ± 0.01	$2.0 \times 10^{-3} \pm 8.7 \times 10^{-5}$
D-Tyrosine	1.82 ± 0.06	8.0 ± 0.3	1.86 ± 0.02	$9.8 \times 10^{-4} \pm 4.3 \times 10^{-5}$
L-α-Methyltyrosine	0.13 ± 0.01	0.6 ± 0.05	1.20 ± 0.01	$1.1 \times 10^{-4} \pm 9.4 \times 10^{-6}$
D,L-α-Methyltyrosine	0.13 ± 0.01	0.6 ± 0.05	1.45 ± 0.01	$9.0 \times 10^{-5} \pm 7.5 \times 10^{-6}$

Table 2 Kinetic constants for the diphenolase activity of mushroom tyrosinase on several *o*-diphenolic enantiomorphs

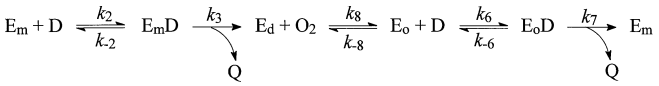
Conditions were the same as described in Table 1. Results are means ± S.D. for three separate experiments.

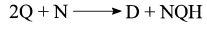
o-Diphenol	V_{max}^{D} (μM/min)	k_{cat}^{D} (s^{-1})	K_{m}^{D} (mM)	V_{max}^{D}/K_{m}^{D} (min^{-1})
L-Dopa	24.5 ± 1.0	107.4 ± 4.4	0.8 ± 0.04	$3.1 \times 10^{-2} \pm 2.8 \times 10^{-3}$
D,L-Dopa	24.4 ± 1.0	107.0 ± 4.4	1.4 ± 0.06	$1.7 \times 10^{-2} \pm 1.4 \times 10^{-3}$
D-Dopa	24.5 ± 1.0	107.4 ± 4.4	4.5 ± 0.15	$5.4 \times 10^{-3} \pm 4.0 \times 10^{-4}$
L-α-Methyldopa	10.1 ± 0.5	44.3 ± 2.2	6.8 ± 0.28	$1.5 \times 10^{-3} \pm 1.4 \times 10^{-4}$
D,L-α-Methyldopa	10.1 ± 0.5	44.3 ± 2.2	8.0 ± 0.41	$1.3 \times 10^{-3} \pm 1.3 \times 10^{-4}$
L-Isoprenaline	6.7 ± 0.3	29.4 ± 1.2	6.6 ± 0.31	$1.0 \times 10^{-3} \pm 9.2 \times 10^{-5}$
D,L-Isoprenaline	6.7 ± 0.3	29.4 ± 1.2	9.7 ± 0.61	$6.9 \times 10^{-4} \pm 7.4 \times 10^{-5}$
D-Isoprenaline	6.8 ± 0.3	29.8 ± 1.4	18.1 ± 0.91	$3.7 \times 10^{-4} \pm 3.5 \times 10^{-5}$

Table 3 δ values for C-3 and C-4 of several phenolic compounds at pH 6.8

Conditions were as detailed in the Experimental section.

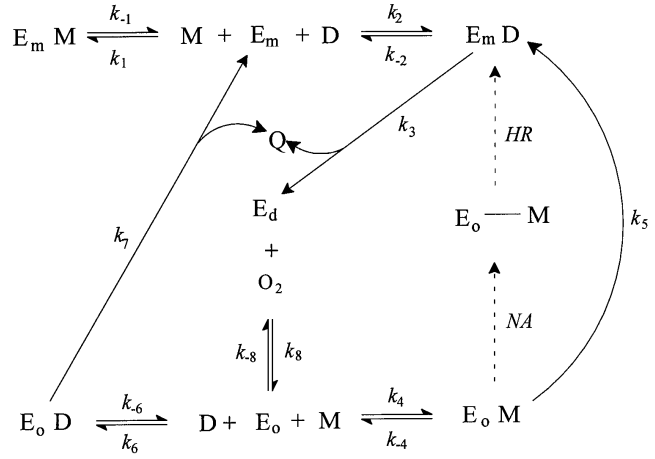
Substrate	δ_3 (p.p.m.)	δ_4 (p.p.m.)
L-α-Methyltyrosine	–	159.10
D,L-α-Methyltyrosine	–	159.10
L-Tyrosine	–	158.88
D,L-Tyrosine	–	158.88
D-Tyrosine	–	158.89
L-Dopa	146.92	146.05
D,L-Dopa	146.92	146.06
D-Dopa	146.92	146.06
L-α-Methyldopa	146.74	146.18
D,L-α-Methyldopa	146.73	146.18
L-Isoprenaline	146.88	146.83
D,L-Isoprenaline	146.87	146.83
D-Isoprenaline	146.87	146.83

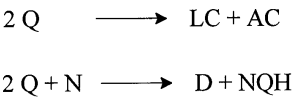




Scheme 1 Kinetic reaction mechanism for the diphenolase activity of tyrosinase

Abbreviations: E_m, *met*-tyrosinase or oxidized form of tyrosinase with Cu^{2+}–Cu^{2+} in the active site; E_d, reduced form of tyrosinase with Cu^{+}–Cu^{+} in the active site; E_o, *oxy*-tyrosinase (E_dO_2 or $E_mO_2^{2-}$); D, *o*-diphenol; Q, *o*-quinone; N, nucleophile (MBTH); NQH, MBTH–quinone adduct.





Scheme 2 Reaction mechanism for the monophenolase and diphenolase activities of tyrosinase coupled to non-enzymic reactions from *o*-quinone

Abbreviations: E_m, *met*-tyrosinase or oxidized form of tyrosinase with Cu^{2+}–Cu^{2+} in the active site; E_d, reduced form of tyrosinase with Cu^{+}–Cu^{+} in the active site; E_o, *oxy*-tyrosinase (E_dO_2 or $E_mO_2^{2-}$); M, monophenol; D, *o*-diphenol; Q, *o*-quinone; LC, leucochrome; AC, aminechrome; N, nucleophile (MBTH); NQH, MBTH–quinone adduct; NA, nucleophilic attack; HR, hydroxylation reaction [19,23,25,28–30,38].

from the hydroxy group of the aromatic ring on the copper atoms of the active site, and then oxidoreduction. It is of note that the δ_4 values increased with the increasing size of the side

substituent. This implies a decrease in the nucleophilic power and therefore a decrease in the V^{D}_{max} value to be expected (Table 2). Because the side substituents of the different isomers of each series assayed were the same, the V^{D}_{max} values were also the same (Table 3).

Moreover, in accordance with eqn. (2), an increase in the size of the side substituent provoked an increase in the K^{D}_{m} values. This could be explained by a decreased k_2 and k_6 values and increased k_{-2} and k_{-6} values. When the different isomers of each series were assayed, the spatial orientation of the side chain of D-isomers provoked the same effect.

Therefore, as regards the diphenolase activity of mushroom tyrosinase, these results suggest that this enzyme shows stereospecificity in its affinity towards the substrates (K^{D}_{m}), but does not show stereospecificity in the transformation reaction of these substrates (V^{D}_{max}).

Monophenolase activity

In the monophenolase reaction mechanism of tyrosinase (Scheme 2) the analytical expressions for V^{M}_{max} and K^{M}_{m} (where M indicates monophenols) involve more rate constants than those for the diphenolase activity [19,38]:

$$V^{M}_{max} = \frac{2K_1 k_2 k_3 k_6 k_7 k_8 k_5 (k_{-4}+k_5)(k_{-4}+k_5) k_6 [E]_0}{K_1 k_2 k_6 k_8 (k_3+3k_7)(k_{-4}+k_5) k_5 k_6 (k_{-4}+k_5) + [(K_1 k_2 k_3 k_4 k_8 (k_{-6}+k_7) + (k_6 k_7 k_8 (3k_{-2}k_3)(k_{-4}+k_5)] k_7 (k_{-6}+k_7) k_4} \quad (3)$$

$$K^{M}_{m} = \frac{2k_7 (k_{-6}+k_7) k_4 K_1 k_8 (k_{-4}+k_5)[k_2 k_3 (k_{-6}+k_7) + k_6 k_7 (3k_{-2}+k_3)]}{K_1 k_2 k_6 k_8 (k_3+3k_7)(k_{-4}+k_5) k_5 (k_{-4}+k_5) k_6 + [K_1 k_2 k_3 k_4 k_8 (k_{-6}+k_7) + k_6 k_7 k_8 (3k_{-2}+k_3)(k_{-4}+k_5)] 2k_7 (k_{-6}+k_7) k_4} \quad (4)$$

The effect of the side substituent is similar to that which occurs in the diphenolase activity. When δ_4 increased (Table 3), the nucleophilic power of the oxygen from the hydroxy group on C-4 decreased.

The step ruled by k_5 could be separated into two sub-steps, a nucleophilic attack and a hydroxylation reaction. The transformation reaction rate decreased when the side substituent increased in size (Table 1). However, the size of the side substituent of different isomers of each series was the same, so the V^{M}_{max} values were the same (Table 1).

The monophenolase reaction mechanism of tyrosinase is coupled to diphenolase activity (Scheme 2) [19,38]. δ_4 values were much lower for *o*-diphenols than for monophenols (Table 3). This indicated that the limiting step in the reaction mechanism of tyrosinase could be the step ruled by k_5. These results agree with previous findings discussed elsewhere [38].

Regarding the K^{M}_{m} values, the behaviour was the same as that previously found for *o*-diphenols. It is of note that the hydroxylation constant (k_5) is in direct proportion to V^{M}_{max} (eqn. 3). In the K^{M}_{m} expression, the hydroxylation constant is not in direct proportion (eqn. 4), so it is concluded that the effect of k_5 on K^{M}_{m} is not direct. Moreover, the expressions for K^{M}_{m} and V^{M}_{max} are complex and variations in k_2, k_6, k_4 and K_1 could lead to an increased K^{M}_{m} value.

Furthermore the spectrophotometric recordings of the monophenolase activity of mushroom tyrosinase with L-, D,L- and D-tyrosine (Figure 1) showed different lag periods (τ). The largest τ corresponded to the isomer whose K^{M}_{m} was the lowest, and the smallest τ corresponded to the isomer whose K^{M}_{m} was the highest (Table 1). That the substrate with the highest catalytic power showed a longer lag period might seem contradictory, but the proposed reaction mechanism [19,38] (Scheme 2) would explain this. If the K^{M}_{m} for the monophenol is low (high affinity of the enzyme for the substrate), then the E_m form, which is inactive on monophenols (Scheme 2), is more saturated by the monophenol (M) and therefore the step from E_m to E_d, which is governed by k_2, is delayed because there is less free enzyme, E_m, and because there is more enzyme in the dead-end complex $E_m M$. This phenomenon can also be appreciated when the effect of monophenol concentration is studied. In this case, τ increases with increasing monophenol concentration [19,24,26,28–30,38].

To sum up, previous studies have reported that electron-withdrawing substituents in the aromatic ring of phenolic compounds cause poor tyrosinase-catalysed oxidation. A high electron donor capacity of the substituent will increase this tyrosinase-catalysed reaction. If the electron donor capacity is the same (similar δ_4 values for monophenols, and similar δ_3 and δ_4 values for *o*-diphenols) for several substrates (enantiomers of monophenols and *o*-diphenols), the oxidation rate will also be the same. On the basis of the kinetic (Tables 1 and 2) and NMR (Table 3) assays, mushroom tyrosinase did not show stereospecificity in the transformation reaction rate (V_{max}) of several isomers of monophenols and *o*-diphenols. From the sequence of K_m values obtained, mushroom tyrosinase showed stereospecificity in its affinity towards these chiral substrates (Tables 1 and 2). Moreover the decrease in size of the side substituent in the aromatic ring increased the affinity properties ($1/K_m$), velocity (V_{max}) and catalytic power (V_{max}/K_m) of tyrosinase towards monophenolic and *o*-diphenolic substrates.

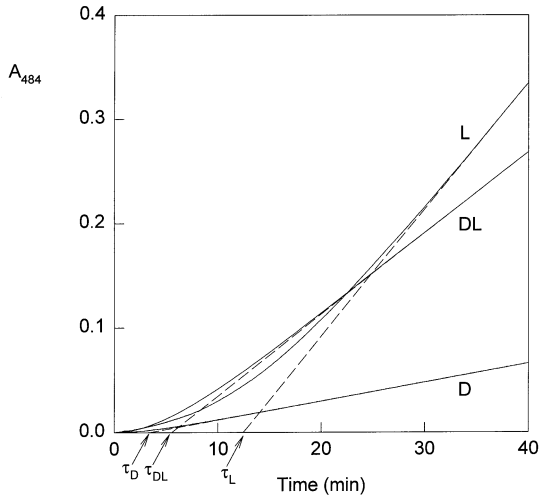


Figure 1 Spectrophotometric recordings of the monophenolase activity of mushroom tyrosinase: dependence of the lag period on monophenolic isomers

Conditions were: 50 mM phosphate buffer, pH 6.8, 2% (v/v) DMF, saturating MBTH concentration, 3.8 nM mushroom tyrosinase and 0.3 mM L-tyrosine (L), 0.3 mM D,L-tyrosine (DL) or 0.3 mM D-tyrosine (D).

This work was partly supported by 'Comisión Interministerial de Ciencia y Tecnología' (C.I.C.Y.T.), project ALI96-1111-C04-01. J.C.E. has a fellowship from the Programa Nacional de Formación del Personal Investigador, Ministerio de Educación y Ciencia (Spain), reference AP93 34785457.

REFERENCES

1 Prota, G. (1988) Med. Res. **8**, 525–556
2 Robb, D. A. (1984) in Copper Proteins and Copper Enzymes, (Lontie, R., ed.), vol. 2, pp. 207–240, CRC Press, Boca Raton, FL
3 Mason, H. S. (1956) Nature (London) **177**, 79–81
4 Jolley, Jr., R. L., Evans, L. H. and Mason, H. S. (1972) Biochem. Biophys. Res. Commun. **46**, 878–884
5 Schoot-Uiterkamp, A. J. and Mason, H. S. (1973) Proc. Natl. Acad. Sci. U.S.A. **70**, 993–996
6 Makino, N. and Mason, H. S. (1973) J. Biol. Chem. **248**, 5731–5735
7 Schoot-Uiterkamp, A. J. M., Evans, L. H., Jolley, R. L. and Mason, H. S. (1976) Biochim. Biophys. Acta **453**, 200–204
8 Lerch, K. (1981) in Metal Ions in Biological Systems (Sigel, H., ed.), pp. 143–186, Marcel Dekker, New York
9 Himmelwright, R. S., Eickman, N. C., Lu Bien, C. D., Lerch, K. and Solomon, E. I. (1980) J. Am. Chem. Soc. **102**, 7339–7344
10 Solomon, E. I. (1981) in Copper Proteins (Spiro, T. G., ed.), vol. 3, pp. 41–108, Wiley-Interscience, New York
11 Wilcox, D. E., Porras, A. G., Hwnag, Y. T., Lerch, K., Winkler, M. E. and Solomon, E. I. (1985) J. Am. Chem. Soc. **107**, 4015–4027
12 Solomon, E. I. and Lowery, M. D. (1993) Science **259**, 1575–1581
13 Solomon, E. I., Sundaram, U. M. and Machonkin, T. E. (1996) Chem. Rev. **96**, 2563–2605
14 Vámos-Vigyázó, L. (1981) CRC Crit. Rev. Food Sci. Nutr. **15**, 49–127
15 García-Cánovas, F., García-Carmona, F., Vera, J., Iborra, J. L. and Lozano, J. A. (1982) J. Biol. Chem. **257**, 8738–8744
16 García-Carmona, F., García-Cánovas, F., Iborra, J. L. and Lozano, J. A. (1982) Biochim. Biophys. Acta **717**, 124–131
17 Tudela, J., García-Cánovas, F., Varón, R., Jiménez, M., García-Carmona, F. and Lozano, J. A. (1987) J. Enzyme Inhib. **2**, 47–56
18 Rodríguez-López, J. N., Tudela, J., Varón, R. and García-Cánovas, F. (1991) Biochim. Biophys. Acta **1076**, 379–386
19 Rodríguez-López, J. N., Tudela, J., Varón, R., García-Carmona, F. and García-Cánovas, F. (1992) J. Biol. Chem. **267**, 3801–3810
20 García-Morena, M., Varón, R., Sánchez, A., Tudela, J. and García-Cánovas, F. (1994) Biochim. Biophys. Acta **1205**, 282–288
21 Rodríguez-López, J. N., Escribano, J. and García-Cánovas, F. (1994) Anal. Biochem. **216**, 205–212
22 Sánchez-Ferrer, A., Rodríguez-López, J. N., García-Cánovas, F. and García-Carmona, F. (1995) Biochim. Biophys. Acta **1247**, 1–11
23 Espín, J. C., Morales, M., Varón, R., Tudela, J. and García-Cánovas, F. (1995) Anal. Biochem. **231**, 237–246
24 Espín, J. C., Morales, M., Varón, R., Tudela, J. and García-Cánovas, F. (1995) J. Agric. Food Chem. **43**, 2807–2812
25 Espín, J. C., Morales, M., Varón, R., Tudela, J. and García-Cánovas, F. (1996) J. Food Sci. **61**, 1177–1182
26 Espín, J. C., Morales, M., Varón, R., Tudela, J. and García-Cánovas, F. (1997) Phytochemistry **44**, 17–22
27 Espín, J. C., Morales, M., García-Ruiz, P. A., Tudela, J. and García-Cánovas, F. (1997) J. Agric. Food Chem. **45**, 1084–1090
28 Espín, J. C., Trujano, M. F., Tudela, J. and García-Cánovas, F. (1997) J. Agric. Food Chem. **45**, 1091–1096
29 Espín, J. C., Ochoa, M., Tudela, J. and García-Cánovas, F. (1997) Phytochemistry **45**, 667–670
30 Espín, J. C., Varón, R., Tudela, J. and García-Cánovas, F. (1997) Biochem. Mol. Biol. Int. **41**, 1265–1276
31 Winder, A. J. and Harris, H. (1991) Eur. J. Biochem. **198**, 317–326
32 Sonesson, B., Eide, S., Rorsman, H. and Rosengren, E. (1995) Acta Derm. Venereol. **75**, 283–286
33 Khan, V. and Pomerantz, S. H. (1980) Phytochemistry **19**, 379–385
34 Pomerantz, S. H. and Murthy, V. V. (1974) Arch. Biochem. Biophys. **160**, 73–77
35 Pomerantz, S. H. (1963) J. Biol. Chem. **238**, 2351–2357
36 Yoshida, H., Tanaka, Y. and Nakayama, K. (1974) Agric. Biol. Chem. **38**, 627–632
37 Ferrari, R. P., Laurenti, E., Casella, L. and Poli, S. (1993) Spectrochim. Acta **49**, 1261–1267
38 Ros, J. R., Rodríguez-López, J. N. and García-Cánovas, F. (1994) Biochim. Biophys. Acta **1204**, 33–42
39 Duckworth, H. W. and Coleman, J. E. (1970) J. Biol. Chem. **245**, 1613–1625
40 Bradford, M. (1976) Anal. Biochem. **72**, 248–254
41 Wilkinson, G. N. (1961) Biochem. J. **80**, 324–332
42 Endrenyi, L. (1981) Kinetic Data Analysis: Design and Analysis of Enzyme and Pharmacokinetics Experiments, Plenum Press, New York
43 Marquardt, D. (1963) J. Sci. Ind. Appl. Math. **11**, 431–444
44 Jandel Scientific (1994) Sigma Plot 2.01 for Windows™, Jandel Scientific, Corte Madera

Received 22 September 1997/5 January 1998; accepted 20 January 1998