Slow-Binding Inhibition of Mushroom (*Agaricus bisporus*) Tyrosinase Isoforms by Tropolone

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A kinetic study of the inhibition of mushroom tyrosinase by tropolone has been made. Three tyrosinase isoforms were used: two commercial tyrosinases from Fluka and Sigma (isoelectric points of 4.3 and 4.1, respectively) and one purified isoform from mushroom strain U1 (isoelectric point of 4.5). Tropolone is a slow-binding inhibitor of these mushroom tyrosinase isoforms. Increasing tropolone concentrations provoked a progressive decrease in both the initial velocity and the final (inhibited) steady-state rate in the progress curves of product accumulation. A rapid formation of an enzyme−inhibitor complex, which further undergoes a slow reversible reaction, could take place since the inhibition of the different isoforms was partially reversed by the addition of $CuSO_4$. The kinetic parameters that described the inhibition by tropolone were evaluated by nonlinear regression fits. Incubation experiments of the different isoforms with tropolone demonstrated that this inhibitor only could bind to the "*oxy*" form of tyrosinase which justifies a mechanism previously proposed to explain the inhibition of tyrosinase by slow-binding inhibitors.

Keywords: *Agaricus; inhibition; mushroom; slow-binding; tropolone; tyrosinase*

INTRODUCTION

Tyrosinase or polyphenol oxidase (EC 1.14.18.1, PPO) is a copper-containing enzyme which plays an important role in processes such as vertebrate pigmentation and browning of fruits and vegetables. This enzyme, in the presence of molecular 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) which nonenzymatically polymerize to render melanins. This browning can cause deleterious changes in the organoleptic properties of food product with loss of fruit and vegetable qualities (Prota, 1988; Martínez and Whitaker, 1995; Sánchez-Ferrer et al., 1995).

The active site of tyrosinase consists of two copper atoms and three states: "*met*" (E_{met}), "*oxy*" (E_{oxy}) and "*deoxy*" (E_{deox}) (Jolley et al., 1974; Lerch, 1981). Structural models for the active site of these three forms of tyrosinase have been proposed (Solomon et al., 1996). Recently, combined kinetic and structural reaction mechanisms have been reported for tyrosinase from different sources (Espín et al., 1998a−d).

Many studies on the inhibition of this enzyme have been carried out (Kahn and Andrawis, 1985a; Valero et al., 1991; Cabanes et al., 1994; Jiménez and García-Carmona, 1997; Espín et al., 1998e). Among all the inhibitors assayed up to date, tropolone (2-hydroxy-2,4,6-cycloheptatriene) was one of the most potent. It is structurally analogous to *o*-diphenolic substrates of PPO, as well as an effective copper chelator (Kahn and Andrawis, 1985a). The use of tropolone as peroxidase substrate has also been previously reported (Kahn and Andrawis, 1985b). These authors described the inhibition of tyrosinase by tropolone as a mixed-type inhibition when commercial mushroom tyrosinase from Sigma was used.

Slow-binding inhibition is characterized by the nonimmediate response of the enzymatic reaction to the presence of a competitive inhibitor (Sculley et al., 1996). The equilibria between enzyme, inhibitor, and enzyme−inhibitor complexes occur slowly on the steady-state time scale, varying from seconds to minutes (Morrison and Walsh, 1988). There are many significant examples of this type of inhibition such as the inhibition of lactate oxidase from *Mycobacterium* by oxalate, prostaglandine cyclooxygenase by nonstereoidal antiinflammatory agents, glutamine synthetase by methionine sufoximine, DNA polymerase from herpes simplex virus by "Aciclovir", etc. (Morrison and Walsh, 1988).

Slow-binding inhibition has been previously reported in the inhibition of tyrosinases from different sources (frog epidermis, grapes, and mushrooms) by several inhibitors (*m*-coumaric acid, tropolone, L-mimosine, kojic acid, and 4-substitued resorcinols) (Cabanes et al., 1984, 1987, 1994; Valero et al., 1991; Jiménez and García-Carmona, 1997). However, the slow-binding inhibition of isolated mushroom tyrosinase isoforms by tropolone has never been reported. Moreover, the previous reports used crude extracts as the source of enzyme with possible mixed kinetics due to the existence of several isoforms.

The aim of the work presented here is to study the inhibition of different tyrosinase isoforms (different isoelectric point) by tropolone. Three fully active isoforms (two commercial mushroom tyrosinases and another one from mushroom strain U1) are used. The kinetic parameters which describe this time-dependent inhibition are calculated according to the kinetic analysis previously proposed for this type of inhibition (Morrison, 1982; Cabanes et al., 1987; Morrison and Walsh,

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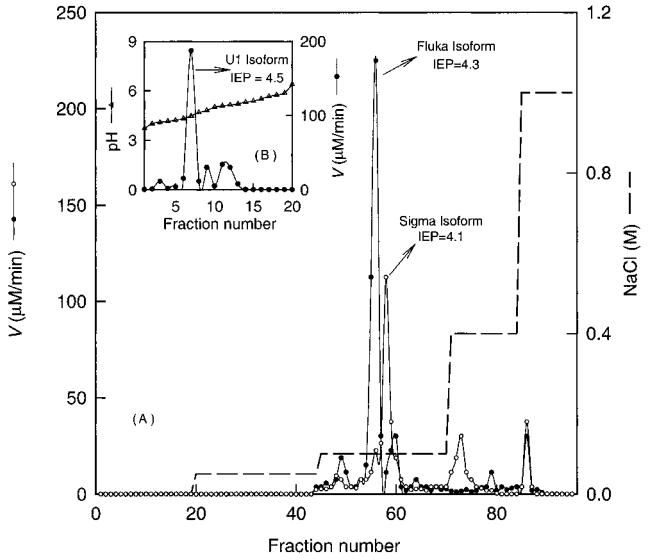


Figure 1. (A) Anionic exchange chromatography with the elution profile of Fluka (major isoform with IEP = 4.3) and Sigma (major isoform with IEP = 4.1) tyrosinases. (B) Preparative isoelectric focusing with U1 isoforms (major isoform with IEP = 4.5). See Materials and Methods for details.

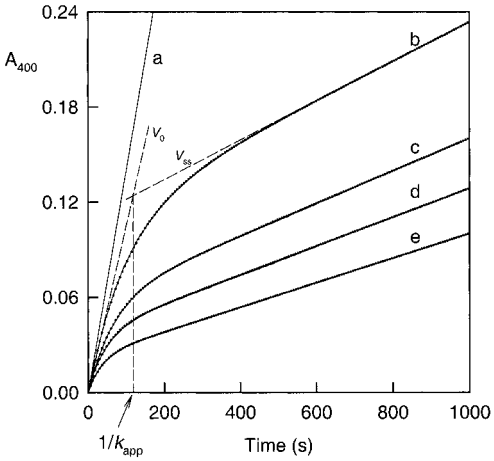


Figure 2. Spectrophotometric recordings of the progress curves for the inhibition of mushroom tyrosinase by tropolone. (—) Experimental recordings. (···) Nonlinear regression fits of experimental progress curves to eq 1. (- -) Linear regression fits to the initial and final portions of the progress curves to determine the initial estimation of V_0, V_{ss}, and k_{app}. Conditions were: PB 50 mM, pH 6.8, TBC 2 mM, 0.05 μg/mL mushroom tyrosinase from Fluka. Tropolone concentrations were as follows: (a) 0 μM; (b) 2 μM; (c) 4 μM; (d) 6 μM; (e) 8 μM.

1988; Sculley et al., 1996). Preincubation of tyrosinase isoforms with the inhibitor demonstrates that tropolone only can bind the E_{oxy} form of tyrosinase. This work corroborates and completes previous studies to explain the slow-binding inhibition of tyrosinase from different sources by several inhibitors (Cabanes et al., 1984, 1987, 1994; Valero et al., 1991; Jiménez and García-Carmona, 1997).

MATERIALS AND METHODS

Reagents. 4-*tert*-Butylcatechol (TBC) and tropolone were purchased from Sigma (Holland). Ampholites for preparative isoelectric focusing were obtained from Bio-Rad (Hercules, CA). All other reagents were of analytical grade and were also supplied by Sigma. Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout this research.

Preparation of Commercial Tyrosinases. Fluka (1200 units/mg) and Sigma (3900 units/mg) tyrosinases were purified by using anion exchanger column (length 75 cm; diameter 5 cm) with DEAE-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden). The column was equilibrated with buffer BIS−TRIS 20 mM pH 6. A stepwise gradient of increasing sodium chloride (NaCl) concentrations was applied (3 mL/min). Two isoforms with IEP of 4.3 for Fluka tyrosinase and 4.1 for Sigma tyrosinase were isolated (Figure 1A). Fractions with these isoforms, dialyzed and concentrated by using an ultrafiltration cell (Amicon, Beverly, MA), were used as source of commercial enzyme. Commercial tyrosinases can differ substantially in their isoform pattern and protein content, even among different batches. This could be one of the reasons that different results are observed in the literature. Therefore, a further purification of these commercial preparations is strongly recommended (Kumar and Flurkey, 1991).

"Isoenzymes" are, by definition, enzyme forms that originate from different genes. In this respect, the situation in mushrooms remains unclear because only two genes encoding tyrosinases have been identified, and the occurrence of multiple isoforms can only be explained by post-translational modifications (Wichers et al., 1995). For this reason we use the term "isoform" throughout this study.

Preparation of Tyrosinase Isoform from Strain U1. Boxes with U1 spawned compost and casing soil from the Mushroom Experimental Station (Horst, The Netherlands) were transported after bud initiation to a climate room at ATO-DLO (18 °C, 80% relative humidity). Mushrooms were harvested at stage 5 and frozen in liquid nitrogen immediately after picking. The developmental stage was determined according to Hammond and Nichols (1976). The freeze-dried mushrooms were ground under liquid nitrogen to a fine powder with mortar and pestle. The powder was rehydrated with PB 10 mM containing 10 mM ascorbic acid and mixed thoroughly on a vortex shaker. This homogenate was then extracted for 25 min on ice and centrifuged at 12000*g* for 10 min.

After centrifugation, the supernatant was dialyzed against ultrapure water for 1 day at 4 °C by using a dialysis tube with 12 kDa cutoff. This sample was applied to a Rotofor preparative isoelectric focusing system (Bio-Rad). The cell was loaded with 3.4 mL of Bio-Lyte ampholites, pH range 4−6, and 0.55 mL of Tween 20 in 45 mL of ultrapure water. Focusing conditions were controlled by limiting the power to 12 W (maximum 2000 V and 20 mA). The sample was focused for 4 h. Several isoforms were detected but one was the most abundant (approximately 80%) with an isoelectric point of 4.5 (Figure 1B). After dialysis and concentration, this fraction was used as enzyme source.

Enzymatic Assays. Tyrosinase activity was determined at 400 nm by measuring the accumulation of 4-*tert*-butylbenzo-1,2-quinone (TBQ). This *o*-quinone was highly stable at every pH assayed during the activity measurements (Waite, 1976; Ros et al., 1994a; Escribano et al., 1997; Jiménez and García-Carmona, 1997). One unit of tyrosinase was defined as the amount of the enzyme that produces 1 μmol of TBQ per minute. The final assay volume was 1 mL.

The spectrophotometric assays were recorded in an ultraviolet−visible Perkin-Elmer Lambda-2 spectrophotometer (Überlingen, Germany); on-line interfaced to a Pentium-100 microcomputer (Ede, The Netherlands). Temperature was controlled at 25 °C with a circulating bath with heater/cooler and checked using a precision of ±0.1 °C.

Inactivation Experiments. Inactivation experiments were carried out under two sets of assay conditions: reactions started by adding the enzyme as the last component in the reaction medium and reactions in which the substrate was added as the last component. The first condition (reaction started with the enzyme) corresponds with the standard assay to measure product accumulation (Figures 2−6). This approach was carried out to check the continuous effect of tropolone on the enzyme activity. The second condition (reaction started with the substrate) (Figure 7) involves a preincubation of the enzyme with the assay medium. This assay condition allowed us to check the possible importance of the intermediate species

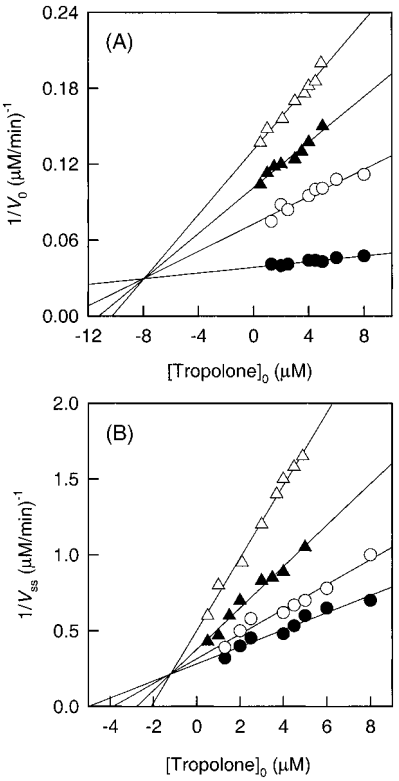


Figure 3. Dixon plots of the effect of tropolone on V_0 (A) and V_{ss} (B) of the diphenolase activity of mushroom tyrosinase. Conditions were as follows: PB 50 mM, pH 6.8, 0.03 μg/mL mushroom tyrosinase from Sigma. TBC concentrations were as follows: (△) 0.3 mM; (▲) 0.6 mM; (○) 1 mM; (●) 2 mM.

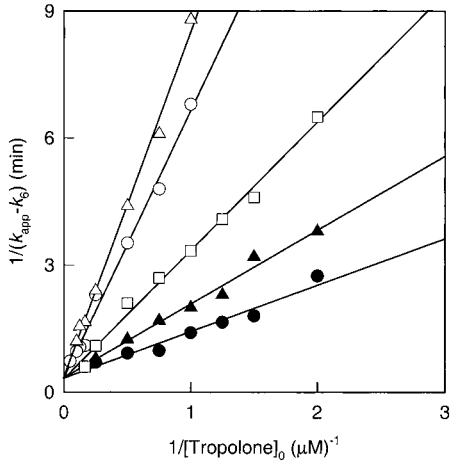


Figure 4. Plot of $1/(k_{app} - k_6)$ vs $1/[I]_0$ for determining the value of k_5 in the inhibition of mushroom tyrosinase by tropolone. Conditions were as in Figure 3 but with 0.6 μg/mL mushroom U1 tyrosinase. TBC concentrations were as follows: (△) 2 mM; (○) 1 mM; (□) 0.8 mM; (▲) 0.6 mM; (●) 0.3 mM.

in the reaction mechanism of tyrosinase (not present in the absence of substrate) in the inhibition by tropolone. The different tyrosinase isoforms were incubated at different times with tropolone, and the reaction was started by adding the substrate (Figure 7).

Kinetic Data Analysis. Triplicate measurements with 500 data points per instrumental recording (progress curves of product accumulation) were obtained. Data fittings were carried out by nonlinear regression (Leatherbarrow, 1990) using an improved Gauss−Newton algorithm (Marquardt, 1963) implemented in the Sigma Plot 2.01 program for Windows (Jandel Scientific, 1994). Kinetic parameters were calculated by making overall nonlinear regression fits of the

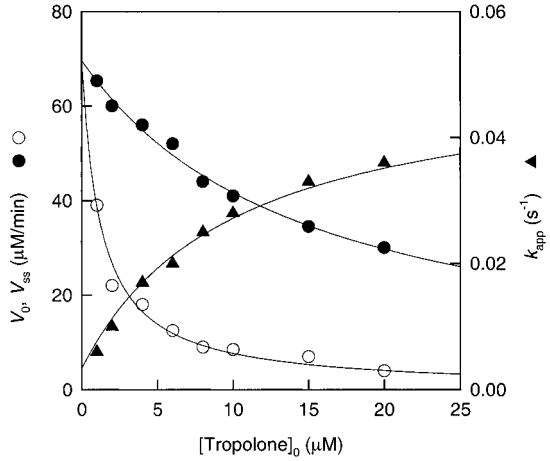


Figure 5. Dependencies of k_{app} (▲), V_0 (●), and V_{ss} (○) on tropolone concentration. Symbols are the result for the overall fit of the progress curves to eq 1 by nonlinear regression. (—) Nonlinear regression fits of these fitted data to eq 4 for k_{app}, eq 6a for V_0 and eq 6b for V_{ss}. Conditions were as follows: PB 50 mM, pH 6.8, TBC 1.2 mM, and 0.6 μg/mL mushroom U1 tyrosinase.

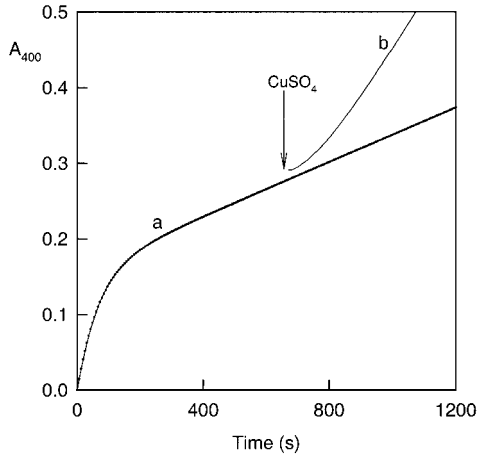


Figure 6. Effect of addition of $CuSO_4$ on inhibition of tyrosinase by tropolone. Conditions were as follows: PB 50 mM, pH 6.8, TBC 3 mM, tropolone 4 μM, and 0.6 μg/mL U1 tyrosinase. When the steady state was reached, 0.2 mM $CuSO_4$ was added to the reaction medium. 50% of initial activity was restored.

experimental progress curves of product accumulation to the integrated form of Frieden's equation for a first-order process (Frieden, 1970):

$$P = V_{ss}t + (V_0 - V_{ss})(1 - e^{-k_{app}t})/k_{app} + C \qquad (1)$$

where V_0 is the initial velocity, V_{ss} is the inhibited steady-state rate, and k_{app} is the apparent first-order rate constant for the establishment of the equilibrium between E−I and E−I*. The term C was included to correct any possible deviation of the baseline. The coefficient of variation (CV) for every nonlinear regression fit of the experimental progress curves to the above equation was always less than 1%.

Initial estimations of V_0 and V_{ss} were calculated by linear regression fittings from the first and last portions of the progress curves, respectively. The initial estimation of k_{app} was calculated from the extrapolation to the axis time of the intersection point of the linear regressions of the first and last portions of the progress curves (Figure 2).

Other Methods. Protein content was determined by using the method of Bradford (Bradford, 1976) using bovine serum albumin as the standard.

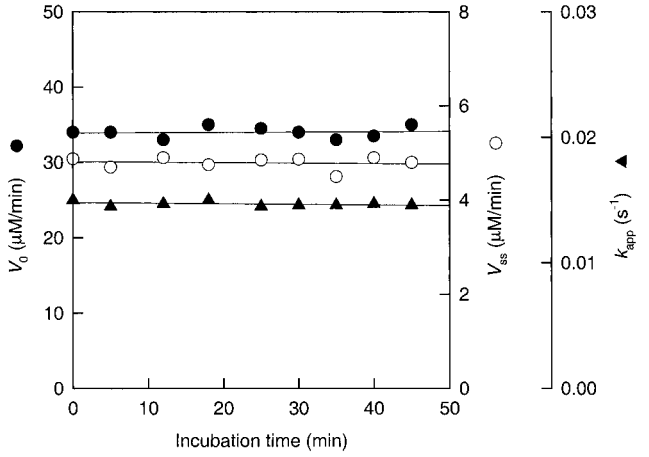
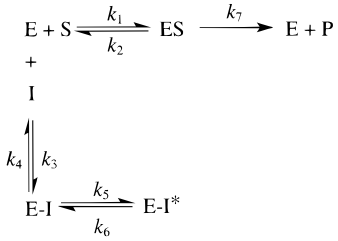


Figure 7. Effect of preincubation of tyrosinase with tropolone on V_0, V_{ss}, and k_{app}. Conditions were as follows: PB 50 mM, pH 6.8, TBC 1 mM, tropolone 2 μM, and 0.05 μg/mL mushroom tyrosinase from Fluka.

Scheme 1. Mechanism Proposed To Illustrate the Slow-Binding Inhibition (Morrison, 1982)



RESULTS AND DISCUSSION

Progress Curves for Reactions Started with Enzyme. The progress curves of product accumulation in the slow-binding inhibition are characterized by an initial burst phase followed by a slower steady-state rate (Morrison and Walsh, 1988). There is a general mechanism which assumes the formation of an E−I complex that undergoes slow and favorable isomerization to an E−I* complex (Sculley et al., 1996) (Scheme 1).

Diphenolase activity of tyrosinase in the absence of inhibitor was characterized by a steady-state rate from the start of the reaction (Figure 2, curve a) (Rodríguez-López et al., 1992; Ros et al., 1994b; Espín et al., 1995, 1997a,b, 1998a−e). However, tropolone inhibited both commercial and U1 mushroom tyrosinase isoforms in a nonclassical manner. The initial velocity (V_0) of the reaction decreased to reach an inhibited, constant, steady-state rate (V_{ss}). Both, V_0 and V_{ss} decreased, and the apparent rate constant (k_{app}) increased when tropolone concentrations were increased (Figure 2, curves b−e). Therefore, the prolonged transient phase observed for the inhibition of mushroom tyrosinases by tropolone presents the characteristics of a slow-binding inhibitor (Morrison, 1982; Cabanes et al., 1987, 1994; Valero et al., 1991; Sculley et al., 1996; Jiménez and García-Carmona, 1997).

The overall fit by nonlinear regression of the experimental recordings to the eq 1 yielded the different V_0, V_{ss}, and k_{app} values. The Dixon plots ($1/V_0$ vs $1/[I]_0$ and $1/V_{ss}$ vs $[I]_0$) permitted us to calculate the apparent constant for E_{oxy}−I complex dissociation (K_I) and the apparent constant for E_{oxy}I* dissociation (K_I^*) (Figure 3; Table 1). The slow transition constant, k_6, (Scheme 1; Table 1) can be calculated from the equation

Table 1. Values of the Kinetic Constants K_I, K_I^*, k_5, and k_6 Which Characterize the Slow-Binding Inhibition of Different Tyrosinase Isoforms by Tropolone

source of mushroom tyrosinase	K_I (μM)	K_I^* (μM)	k_6 (s^{-1})	k_5 (s^{-1})	k_5/k_6
Sigma (IEP = 4.1)	8	1.4	0.0044	0.021	4.8
Fluka (IEP = 4.3)	10.1	1.5	0.0022	0.012	5.4
strain U1 (IEP = 4.5)	11.5	0.8	0.0034	0.045	13.2

$$k_6 = \frac{V_{ss} k_{app}}{V_0} \tag{2}$$

(Morrison, 1982; Morrison and Walsh, 1988). The kinetic constant which rules the formation of the E−I* complex (k_5) can be determined by means of a plot of $1/(k_{app} - k_6)$ versus $1/[I]_0$ according to the following equation (Cabanes et al., 1987):

$$k_{app} = \frac{k_5[I]}{K_I(1 + ([S]/K_m)) + [I]} + k_6 \tag{3}$$

A series of straight lines intersecting at a point on the ordinate axis equal to $1/k_5$ was obtained (Figure 4). From this plot the value of k_5 was calculated for the different tyrosinase isoforms (Table 1). An alternative method for determining the value of k_5 is to fit the data for the variation of k_{app} as a function of tropolone concentrations (Morrison, 1982; Morrison and Walsh, 1988) (Figure 5)

$$k_{app} = k_5\left[\frac{1 + \dfrac{[I]_0}{K_I^*(1 + [S]_0/K_m)}}{1 + \dfrac{[I]_0}{K_I(1 + [S]_0/K_m)}}\right] \tag{4}$$

where $[S]_0$ is the initial substrate concentration, $[I]_0$ is the initial inhibitor concentration, K_m is the Michaelis constant for S, and K_I and K_I^* are the dissociation constants for the E−I and E−I* complexes, respectively (Scheme 1). This equation describes a hyperbola with limiting values of k_6 and $k_5 + k_6$ at zero and infinite concentrations of inhibitor, respectively. The value of k_5 can also be determined by the following relationship (Table 1):

$$\frac{k_5}{k_6} = \frac{K_I}{K_I^*} - 1 \tag{5}$$

The variation of the initial rate (V_0) and the final steady-state rate (V_{ss}) are defined by the general equation for classical competitive inhibition:

$$V_0 = \frac{V_{max}[S]_0}{K_m\left(1 + \dfrac{[I]_0}{K_I}\right) + [S]_0} \tag{6a}$$

$$V_{ss} = \frac{V_{max}[S]_0}{K_m\left(1 + \dfrac{[I]}{K_I^*}\right) + [S]_0} \tag{6b}$$

The different values of V_0 and V_{ss} were fitted to both equations to describe their dependencies on tropolone concentrations (Figure 5).

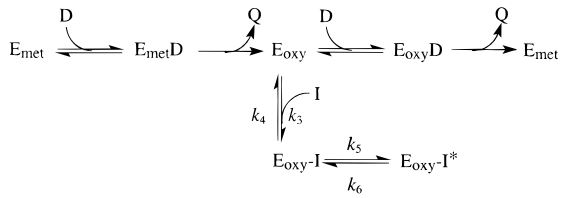
It is noteworthy that Kahn and Andrawis (1985a) used a commercial tyrosinase from Sigma for the inhibition of mushroom tyrosinase by tropolone. However, they did not report time-dependent inhibition but mixed-type inhibition. Several possible explanations can justify their results. One of them is that, taking into account the heterogeneity of commercial tyrosinases (Kumar and Flurkey, 1991), the inhibition for their enzyme preparation by tropolone was not time-dependent. A very likely alternative explanation could be that these authors measured the diphenolase activity of tyrosinase in the presence of tropolone in short kinetic assays (which is very reasonable because a straight line from zero time is expected), and then further evolution into to the inhibited steady-state rate would not be detected. The final possible explanation is the use of a high enzyme concentration. In this case the rate of catalysis is high in relation to the rate of isomerization of the E−I complex, and then possible substrate depletion is likely to occur before the steady-state equilibrium between E−I and E−I* is established. Under these assay conditions an apparent classical inhibition can be observed. The inhibition constant would be that for K_I. To avoid possible misunderstandings in the interpretation of the type of inhibition, lower enzyme concentrations and longer kinetic assays should be used (Morrison and Walsh, 1988).

The activity of the isoforms assayed could be partially restored by adding $CuSO_4$ after the system reached the steady-state rate (Figure 6). This is explained because tropolone is a well-known copper chelator (Kahn and Andrawis, 1985a). However, higher $CuSO_4$ concentrations could even inhibit enzyme activity (results not shown) (Kahn and Andrawis, 1985a). Moreover, this experiment demonstrated that tropolone is not a tight-binding inhibitor which would require more drastic methods to demonstrate the reversibility of the inhibition.

Progress Curves for Reactions Started with Substrate. Preincubation of enzyme with inhibitor in slow-binding inhibition assays provokes progress curves with an initial velocity which is less than the steady-state velocity. Therefore, instead of a burst phase, we should observe a lag period (Morrison and Walsh, 1988). However, preincubation experiments of tyrosinase isoforms with tropolone did not change the original shape of the progress curves when the reaction was started by addition of enzyme (results not shown). Equal curves were obtained after preincubation of tyrosinase isoforms with different tropolone concentrations for various time periods. The kinetic parameters V_0, V_{ss}, and k_{app} which characterize the system remained constant (Figure 7). This behavior can be justified by a previously proposed reaction mechanism which explains the time-dependent inhibition of tyrosinase from several sources by several inhibitors (Scheme 2) (Cabanes et al., 1984, 1987, 1994; Valero et al., 1991; Jiménez and García-Carmona, 1997). This mechanism states that tropolone only can bind to the E_{oxy} form of the enzyme. This *oxy* form of the enzyme is an intermediate in the catalytic turnover, and therefore the presence of substrate is necessary before slow binding of the inhibitor to the enzyme can occur.

The lowest dissociation constant at a time equal to zero (K_I) was found for the inhibition of Sigma tyrosinase by tropolone (K_I = 8 μM) (Table 1). The lowest overall dissociation constant (K_I^*), which is the constant that really indicates the capacity of the slow-binding inhibitor, was found for the inhibition of U1 tyrosinase (K_I^* = 0.8 μM). Therefore, the efficiency of a slow-binding inhibitor only can be compared to another one when exactly the same source of tyrosinase with a similar purification level is used. Thus, we could say that tropolone is more potent slow-binding inhibitor than 4-hexylresorcinol (K_I = 5 μM, K_I^* = 2.4 μM) (Jiménez and García-Carmona, 1997) and approximately with the same inhibition capacity as kojic acid (K_I = 2.75 μM, K_I^* = 0.62 μM) (Cabanes et al., 1994). The fastest time-dependent inhibitor appears to be tropolone in the inhibition of Fluka tyrosinase by tropolone, with $k_5 = 0.012$ s^{-1} (Table 1) if it is compared to $k_5 = 0.062$ s^{-1} for 4-hexylresorcinol and 0.06 s^{-1} for kojic acid (Jiménez and García-Carmona, 1997). However, this comparison is doubtful due to the heterogeneity of commercial preparations of tyrosinase (Table 1) (Kumar and Flurkey, 1991).

Scheme 2. Proposed Reaction Mechanism by Cabanes et al. (1987) To Justify the Slow-Binding Inhibition of Frog Epidermis Tyrosinase by L-Mimosine[a]



[a] D, *o*-diphenolic substrate; E_{met}, *met*tyrosinase; E_{oxy}, *oxy*-tyrosinase; Q, reaction product (*o*-quinone). Only the steps which rule the formation of both E_{oxy}−I and E_{oxy}−I* complexes have been noted to keep homology with Scheme 1.

According to previous reports (Jiménez and García-Carmona, 1997) it is very likely that the values of K_I, k_5, and k_6 are also the same for the monophenolase activity of tyrosinase. The presence of the lag period in this activity (Rodríguez-López et al., 1992; Ros et al., 1994b; Espín et al., 1995, 1997a,b, 1998c) renders the determination of the kinetic constant in this type of inhibition impossible. However, taking into account that the E_{oxy} and E_{met} forms of the enzyme are common intermediates in both diphenolase and monophenolase activities, it is logical to presume that K_I, k_5, and k_6 are equal for both activities. Therefore, in the case of tyrosinase, these constants only describe the interaction between the inhibitor and the E_{oxy} form of the enzyme (Jiménez and García-Carmona, 1997).

It can be concluded that tropolone is a slow-binding inhibitor of mushroom tyrosinase (Figures 2−7). There are some reasons why it has taken so long to determine the slow-binding inhibition of tyrosinase by tropolone despite the extensive studies that have been made. For instance, it is strongly recommended to use different enzyme concentrations for studying possible slow binding inhibitors. These enzyme concentrations can differ notably from those used in the studies on classical inhibitors. It is even possible that more "classical" inhibitors are actually slow binding inhibitors, not yet recognized as such. Moreover, wrong rate determinations and therefore incorrect kinetic parameters can be obtained if the product accumulation is recorded over only a short time period. This can be also observed in the common practice of incubation of enzyme and

inhibitor to measure residual activity. Again, in this type of inhibition, if the assay is too short, only initial velocities will be obtained. Moreover, it is not possible to generalize this type of inhibition for tyrosinase enzyme that can be found in many sources with high heterogeneity.

It is very interesting to emphasize the use of slow binding inhibitors for preventing the enzymatic browning caused by tyrosinase. The ratio k_5/k_6 (Table 1) is an index of the accumulation of the complex E−I* and the energetics of its formation. The values of this ratio, which can vary from 1 to 10^6, have been tabulated for many slow-binding inhibitors (Morrison and Walsh, 1988). A high value for this ratio is a measure of the stability of the complex E−I*, and more likely the inhibitor can be useful in vivo lifetime. One of the most common problems in the in vivo use of many classical inhibitors is that the inhibition of the enzyme is overcome by the high availability of substrate. However, this has not any effect on the isomerization of E−I* to E−I and hence reversal of inhibition (Morrison and Walsh, 1988). Therefore, the design of slow-binding inhibitors to prevent enzymatic browning in fruits and vegetables is strongly encouraged.

The comparison of the kinetic parameters that characterize this type of inhibition should only be made with the same tyrosinase isoform for different slow-binding inhibitors. The study presented here complete those previously reported for this complex type of inhibition for several compounds (4-substituted resorcinols, L-mimosine, kojic acid and tropolone) that are structurally analogues to phenolic substrates of several tyrosinases (Cabanes et al., 1987, 1994; Valero et al., 1991; Jiménez and García-Carmona, 1997).

ABBREVIATIONS USED

ATO-DLO, Agrotechnological Research Institute; DEAE, diethylaminoethane; E_{deoxy}, *deoxy*tyrosinase; E_{met}, *met*tyrosinase; E_{oxy}, *oxy*tyrosinase; I, inhibitor (tropolone); k_{app}, apparent constant for the evolution of the first-order process from V_0 to V_{ss}; k_5, constant which rules the transition from E_{oxy}−I to E_{oxy}−I*; k_6, constant which rules the slow transition from E_{oxy}−I* to E_{oxy}−I; K_I, dissociation constant at $t = 0$ (apparent constant for E_{oxy}−I complex dissociation); K_I^*, overall dissociation constant (apparent constant for E_{oxy}−I* dissociation); kojic acid, 5-hydroxy-2-(hydroxymethyl)-4-pyrone; L-mimosine, β-[(*N*-3-hydroxy-4-oxopyridone)]-α-aminopropionic acid; IEP, isoelectric point; PB, sodium phosphate buffer; S, substrate (TBC); TBC; 4-*tert*-butylcatechol; tropolone, 2-hydroxy-2,4,6-cycloheptatriene; V_0, initial velocity; V_{max}, maximum rate; V_{ss}, (inhibited) steady-state rate.

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LITERATURE CITED

Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* **1983**, *210*, 727−735.

Cabanes, J.; García-Carmona, F.; García-Carmona, F.; Iborra, J. L.; Lozano, J. A. Kinetic study on the slow inhibition of frog epidermis tyrosinase by *m*-coumaric acid. *Biochim. Biophys. Acta* **1984**, *790*, 101−107.

Cabanes, J.; García-Cánovas, F.; Tudela, J.; Lozano, J. A.; García-Carmona, F. L-Mimosine, a slow-binding inhibitor of mushroom tyrosinase. *Phytochemistry* **1987**, *26*, 917−919.

Cabanes, J.; Chazarra, S.; Garcia-Carmona, F. Kojic acid, a cosmetic skin whitening agent, is a slow-binding inhibitor of catecholase activity of tyrosinase. *J. Pharm. Pharmacol.* **1994**, *46*, 982−5.

Escribano, J.; Cabanes, J.; García-Carmona, F. Characterisation of latent polyphenol oxidase in table beet: effect of sodium dodecyl sulfate. *J. Sci. Food Agric.* **1997**, *73*, 34−38.

Espín, J. C.; Morales, M.; Varón, R.; Tudela, J.; García-Cánovas, F. Monophenolase activity of polyphenol oxidase from Verdedoncella apple. *J. Agric. Food Chem.* **1995**, *43*, 2807−2812.

Espín, J. C.; Varón, R.; Tudela, J.; García-Cánovas, F. Kinetic study of the oxidation of 4-hydroxyanisole catalyzed by tyrosinase. *Biochem. Mol. Biol. Int.* **1997a**, *41*, 1265−1276.

Espín, J. C.; Trujano, M. F.; Tudela, J.; García-Cánovas, F. Monophenolase activity of polyphenol oxidase from Haas avocado. *J. Agric. Food Chem.* **1997b**, *45*, 1091−1096.

Espín, J. C.; García-Ruiz, P. A.; Tudela, J.; García-Cánovas, F. Study of the stereospecificity in pear and strawberry polyphenol oxidases. *J. Agric. Food. Chem.* **1998a**, *46*, 2469−2473.

Espín, J. C.; García-Ruiz, P. A.; Tudela, J.; García-Cánovas, F. Study of stereospecificity in mushroom tyrosinase. *Biochem. J.* **1998b**, *331*, 547−551.

Espín, J. C.; García-Ruiz, P. A.; Tudela, J.; Varón, R.; García-Cánovas, F. Monophenolase and diphenolase reaction mechanisms of apple and pear polyphenol oxidases. *J. Agric. Food Chem.* **1998c**, *46*, 2968−2975.

Espín, J. C.; Tudela, J.; García-Cánovas, F. 4-Hydroxyanisole: the most suitable monophenolic substrate for determining spectrophotometrically the monophenolase activity of polyphenol oxidase from fruits and vegetables. *Anal. Biochem.* **1998d**, *259*, 118−126.

Espín, J. C.; Jolivet, S.; Wichers, H. J. Inhibition of mushroom polyphenol oxidase by agaritine. *J. Agric. Food Chem.* **1998e**, *46*, 2976−2980.

Frieden, C. Kinetic aspects of regulation of metabolic processes. The hysteretic enzyme concept. *J. Biol. Chem.* **1970**, *245*, 5788−5799.

Hammond, B. W.; Nichols, R. Carbohydrate metabolism in *Agaricus bisporus* (Lange) Sing.: changes in soluble carbohydrates during growth of mycelium and sporophore. *J. Gen. Microbiol.* **1976**, *93*, 309−320.

Jandel Scientific. *Sigma Plot 2.01 for Windows*; . Jandel Scientific: Corte Madera, 1994.

Jiménez, M.; García-Carmona, F. 4-Substituted resorcinols (sulfite alternatives) as slow-binding inhibitors of tyrosinase catecholase activity. *J. Agric. Food Chem.* **1997**, *45*, 2061−2065.

Jolley, R. L., Jr.; Evans, L. H.; Makino, N.; Mason, H. S. Oxytyrosinase. *J. Biol. Chem.* **1974**, *249*, 335−345.

Kahn, V.; Andrawis, A. Inhibition of mushroom tyrosinase by tropolone. *Phytochemistry* **1985a**, *24*, 905−908.

Kahn, V.; Andrawis, A. Tropolone as substrate for horseradish peroxidase. *Phytochemistry* **1985b**, *24*, 909−913.

Kumar, M.; Flurkey, W. H. Activity, isoenzymes and purity of mushroom tyrosinase in commercial preparations. *Phytochemistry* **1991**, *30*, 3899−3902.

Leatherbarrow, R. J. Using linear and nonlinear regression to fit biochemical data. *Trends Biochem. Sci.* **1990**, *15*, 455−458.

Lerch, K. *Metal Ions in Biological Systems*; Sigel, H., Ed.; Marcel Dekker: New York, 1981; Vol. 13, pp 143−186.

Marquardt, D. An algorithm for least-squares estimation of nonlinear parameters. *J. Soc. Ind. Appl. Math.* **1963**, *11*, 431−441.

Martínez, M. V.; Whitaker, J. R. The biochemistry and control of enzymatic browning. *Trends Food Sci. Technol.* **1995**, *6*, 195−200.

Morrison, J. F. The slow-binding and slow, thigh-binding inhibition of enzyme catalysed reactions. *Trends Biochem. Sci.* **1982**, *7*, 102−105.

Morrison, J. F.; Walsh, C. T. The behavior and significance of slow-binding enzyme inhibitors. In *Advances in Enzymology*; Meister, A., Ed.; Wiley-Interscience: New York, 1988; Vol. 61, pp 201−301.

Prota, G. Progress in the chemistry of melanin and related metabolites. *Med. Res. Rev.* **1988**, *8*, 525−556.

Rodríguez-López, J. N.; Tudela, J.; Varón, R.; García-Carmona, F.; García-Cánovas, F. Analysis of a kinetic model for melanin biosynthesis pathway. *J. Biol. Chem.* **1992**, *267*, 3801−3810.

Ros, J. R., Rodríguez-López, J. N. and García-Cánovas, F. Kinetics study of the oxidation of 4-*tert*-butylphenol by tyrosinase. *Eur. J. Biochem.* **1994a**, *222*, 449−452.

Ros, J. R.; Rodríguez-López, J. N.; García-Cánovas, F. Tyrosinase: kinetic analysis of the transient phase and the steady state. *Biochim. Biophys. Acta* **1994b**, *1204*, 33−42.

Sánchez-Ferrer, A.; Rodríguez-López, J. N.; García-Cánovas, F.; García-Carmona, F. Tyrosinase: A comprehensive review of its mechanism. *Biochim. Biophys. Acta* **1995**, *1247*, 1−11.

Sculley, M. J.; Morrison, J. F.; Cleland, W. W. Slow-binding inhibition: the general case. *Biochim. Biophys. Acta* **1996**, *1298*, 78−86.

Solomon, E. I.; Sundaram, U. M.; Machonkin, T. E. Multicopper oxidases and oxygenases. *Chem. Rev.* **1996**, *96*, 2563−2605.

Valero, E.; García-Moreno, M.; Varón, R.; García-Carmona, F. Time-dependent inhibition of grape polyphenol oxidase by tropolone. *J. Agric. Food Chem.* **1991**, *39*, 1043−1046.

Waite, J. H. Calculating extinction coefficients for enzymatically produced *o*-quinones. *Anal. Biochem.* **1976**, *75*, 211−218.

Wichers, H. J.; van den Bosch, T.; Gerritsen, I. A. M.; Oyevaar, J.; Ebbelaar, M.; Recourt, K.; Kerrigan, R. Enzymology and molecular biology of *Agaricus bisporus* tyrosinase. *Mushroom Sci.* **1995,** *14*, 723−728.

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