4-Substituted Resorcinols (Sulfite Alternatives) as Slow-Binding Inhibitors of Tyrosinase Catecholase Activity

Mercedes Jiménez and Francisco García-Carmona*

Departamento de Bioquímica y Biología Molecular A, Unidad Docente de Biología, Facultad de Veterinaria, Universidad de Murcia, E-30080 Murcia, Spain

A kinetic study of the inhibition of mushroom tyrosinase catecholase activity by 4-substituted resorcinols has been made. The results obtained show that 4-substituted resorcinols inhibit tyrosinase in a nonclassical manner and that the inhibition is characterized by a long transient phase. Progress curves of enzymatic reaction in the presence of inhibitor show a progressive decrease in initial velocity followed by a constant steady-state rate. Both the initial and the constant rates decreased with increasing concentrations of inhibitor. Kinetic data obtained correspond to those for a postulated mechanism involving rapid formation of an enzyme−inhibitor complex that subsequently undergoes a relatively slow reversible reaction. The kinetic parameters characterizing this time-dependent inhibition were evaluated by means of nonlinear regression of the product accumulation curves.

Keywords: *Tyrosinase; 4-hexylresorcinol; slow-binding inhibitors*

INTRODUCTION

Browning of raw fruits, vegetables, and beverages is a major problem in the food industry and is believed to be one of the main causes of quality loss during postharvest handling and processing. The mechanism of browning in food is well characterized and is mainly enzymatic in origin (McEvily et al., 1992). The formation of pigments via enzymatic browning is initiated by the enzyme tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase (PPO).

Tyrosinase is a copper-containing monooxygenase widely distributed in nature that is also responsible for melanization in animals. Chemical and spectroscopic studies of tyrosinase have shown that its coupled binuclear copper active site can exist in three different forms during the mechanism reaction: met, oxy, and deoxy (Lerch, 1981; Rob, 1981). The enzyme catalyzes two distinct reactions involving molecular oxygen: the hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the oxidation of the *o*-diphenols to *o*-quinones (catecholase activity). The quinones are highly reactive compounds and can polymerize spontaneously to form high molecular weight compounds or brown pigments (melanins) or can react with amino acids and proteins that enhance the brown color produced (Vámos-Vigyázó, 1981).

Browning can cause deleterious changes in the appearance and organoleptic properties of the food product, resulting in shorter shelf life and decreased market value. Because of the undesirable effects of enzymatic browning on fruits and vegetables, a considerable number of inhibitors of tyrosinase are known. A method widely used in the food and beverage industries to control browning is the addition of reducing agents (sulfites, ascorbic acid, etc.) which chemically reduce the *o*-quinones to the less reactive colorless diphenols. However, these compounds can have adverse health effects and can also react with other components in the food system, resulting in unwanted effects (McEvily et al., 1992).

Another important group of browning inhibitors is constituted by compounds structurally analogous to phenolic substrates, which generally show competitive inhibition with respect to these substrates, although this can vary depending on enzyme source and the substrate used (Mayer and Harel, 1979; Khan and Andrawis, 1985). Among this group, L-mimosine (Cabanes et al., 1987b), tropolone (Valero et al., 1991), and kojic acid (Cabanes et al., 1994) have been described as competitive, slow-binding inhibitors, according to the classification of reversible enzyme inhibitors established by Morrison (1982). Substituted resorcinols, which are also structurally related to phenolic substrates (Figure 1), have recently been recognized as polyphenol oxidase inhibitors. Although resorcinol is a poor PPO inhibitor, substitutions in the 4-position yield decreased IC_{50} values, the only kinetic parameter found in the bibliography. Among the 4-substituted resorcinols, the highest inhibition was obtained with hydrophobic substituents in the 4-position, such as 4-hexyl- and 4-dodecylresorcinols (McEvily et al., 1992). 4-Hexylresorcinol is claimed to be the most effective inhibitor for use in the food industry since it is water soluble, stable, nontoxic, nonmutagenic, and noncarcinogenic, and it has been recognized as safe for use in the prevention of shrimp melanosis (Iyengar et al., 1991; McEvily et al., 1991) and for browning control of fresh and hot-air-dried apple slices as well as potatoes, avocados, and apple and grape juices (Frankos et al., 1991).

Although the great potential of 4-substituted resorcinols as PPO inhibitors has been demonstrated, their kinetic behavior has not been investigated. The aims of the present paper are, therefore, to carry out a kinetic study of the inhibition of the catecholase activity of tyrosinase by 4-substituted resorcinols and to evaluate the kinetic parameters and constants characterizing the system.

* Author to whom correspondence should be addressed (e-mail, mja@gaia.fcu.um.es; fax, 07-3468-364147).

S0021-8561(96)00810-2 CCC: $14.00 © 1997 American Chemical Society

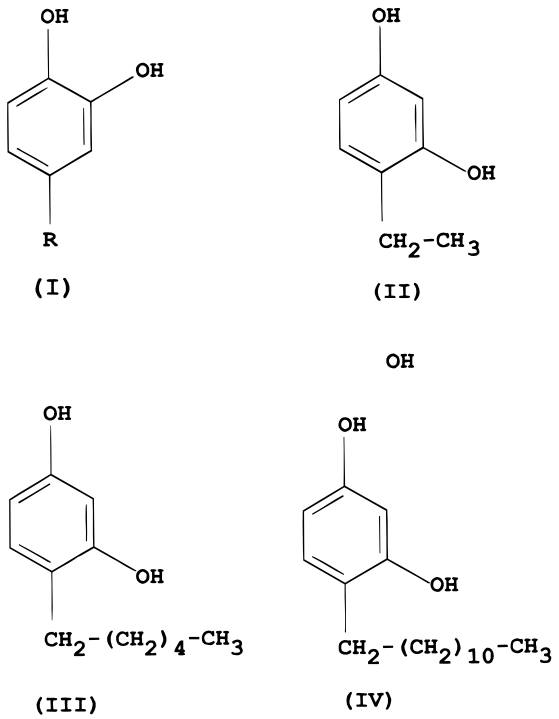

Figure 1. Structures of a normal *o*-diphenolic substrate of polyphenol oxidase (catechols) (**I**) and 4-substituted resorcinols: ethylresorcinol (**II**), hexylresorcinol (**III**), and dodecylresorcinol (**IV**).

MATERIALS AND METHODS

Reagents. Mushroom tyrosinase (3870 units/mg) was purchased from Sigma Quimica (Madrid, Spain). 4-*tert*-Butylcatechol, 4-dodecylresorcinol, and 4-ethylresorcinol were obtained from Aldrich (Madrid, Spain). All other chemicals were of analytical grade and supplied by Merck (Barcelona, Spain). All buffers were prepared with water purified by a MilliQ water purification system (Millipore Iberica, Madrid, Spain).

Enzyme Assays. Catecholase activity was followed by spectrophotometric measurement of 4-*tert*-butyl-*o*-benzoquinone at 400 nm ($\epsilon = 1682\ M^{-1}\ cm^{-1}$) (Sánchez-Ferrer et al., 1993) using a Uvikon 940 spectrophotometer. 4-*tert*-Butyl-*o*-benzoquinone was checked in the assay conditions and was stable for a longer period than those used in the activity measurements. Temperature was controlled at 25 °C using a Haake D1G circulating bath with a heater/cooler and checked using a Cole-Palmer digital thermometer with a precission of ±0.1 °C. Unless otherwise stated, the reaction media (1.0 mL), at 25 °C, contained 4.95 mM 4-*tert*-butylcatechol, 50 mM sodium phosphate buffer (pH 6.5), inhibitor at the indicated concentration, and mushroom tyrosinase (0.39 μg/mL). One unit of enzymatic activity is defined as the amount of enzyme that produces 1 μmol of 4-*tert*-butyl-*o*-benzoquinone/min.

The progress curves (product formation as a function of time) were fitted by nonlinear regression using Marquardt's algorithm (Marquardt, 1963), to the integrated form of Frieden's equation (Frieden, 1970):

$$P = v_s t + (v_o - v_s)(1 - e^{-k_{app}t})/k_{app} + c_4 \qquad (1)$$

where v_o, v_s, and k_{app} represent, respectively, the initial and the steady-state rates and the apparent first-order rate constant; the parameter c_4 was included to correct any possible deviation from the initial absorption value.

Other Methods. Protein concentration was determined according to the dye-binding method of Bradford (Bradford, 1976) using a bovine serum albumin as a standard.

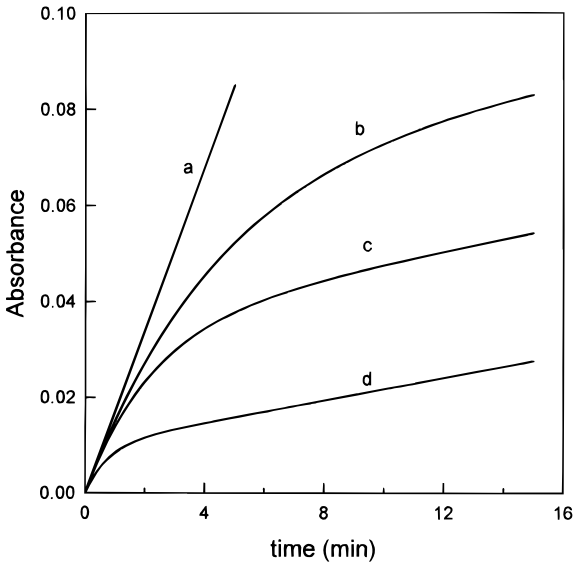

Figure 2. Progress curves for the inhibition of mushroom tyrosinase by 4-hexylresorcinol. The reaction medium contained 0.75 mM 4-*tert*-butylcatechol in 50 mM phosphate buffer, pH 6.5. The reaction was started by the addition of the enzyme (0.39 μg/mL). 4-Hexylresorcinol concentrations were (a) 0, (b) 0.5, (c) 1, and (d) 3.6 μM.

RESULTS AND DISCUSSION

When catecholase activity of mushroom tyrosinase was assayed by using 4-*tert*-butylcatechol as substrate, the reaction immediately reached a steady-state rate (Figure 2, curve a); the absorbance changes were linear up to at least 0.3−0.4 absorbance unit. However, when the reaction was started by addition of the enzyme in the presence of 4-hexylresorcinol, a biphasic response was observed, with a progressive decrease in the initial velocity followed by a constant rate; both the initial and constant rates decreased as the inhibitor concentration increased (Figure 2, curves b−d). Similar results were obtained when 4-ethylresorcinol or 4-dodecylresorcinol was used as substrates (data not shown).

These results indicate that the inhibition produced by 4-substituted resorcinols is expressed slowly and that they behave as slow-binding inhibitors, according to the classification given by Morrison and Walsh (1989). This inhibition is not necessarily tight-binding or stoichiometric since the inhibitor concentration in the progress curve experiments was always much higher than the enzyme concentration (Williams and Morrison, 1979). This time-dependent inhibition of tyrosinase has also been found by using structurally analogous compounds, such as *m*-coumaric acid, L-mimosine, tropolone, and kojic acid, and frog epidermis, grape, and mushroom as sources of enzyme (Cabanes et al., 1984, 1987b, 1994; Valero et al., 1991).

The progress curves obtained can be described by the integrated form of Frieden's equation (Frieden, 1970) for a first-order process:

$$P = v_s t + (v_o - v_s)(1 - e^{-k_{app}t})/k_{app} \qquad (2)$$

where v_o, v_s, and k_{app} represent, respectively, the initial rate, the steady-state rate, and the apparent first-order rate constant, whose meaning depends on the mechanism under study. The experimentally obtained progress curves can therefore be analyzed by making an overall fit of the experimental data to eq 2 by nonlinear regression (Morrison, 1982).

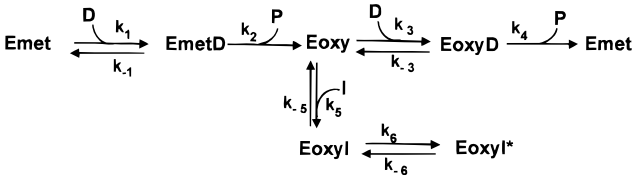

Figure 3. Mechanism proposed by Cabanes et al. (1987a) for the slow inhibition of frog epidermis tyrosinase by L-mimosine: Emet, met form of tyrosinase; Eoxy, oxy form of tyrosinase; D, *o*-diphenolic substrate; EoxyI*, enzyme−inhibitor complex formed by slow isomerization of EoxyI complex.

To investigate the inhibition of tyrosinase by 4-substituted resorcinols, experiments were performed in which the enzyme was preincubated with different inhibitor concentrations at various times, at the end of which times the reaction was started by the addition of the substrate; progress curves similar to those in Figure 2 were obtained (data not shown). This result indicates that the inhibitor does not bind to the free form of the enzyme (met form) and can only be interpreted by taking into account the internal mechanism of the catecholase activity of tyrosinase (Figure 3) (Galindo et al., 1983; Cabanes et al., 1987a; Sánchez-Ferrer et al., 1995). This scheme proposes the successive binding of two diphenolic substrates to complete the catalytic cycle with the appearance of an enzymatic oxy form, which has a greater affinity for the substrate than the met form. The oxy form is an obligatory intermediate in the catalytic turnover, and thus, the presence of the substrate (and therefore of the catalytic activity) is necessary for the slow binding of inhibitor to the enzyme to be observed. For this reason 4-substituted resorcinols cannot be considered as classical competitive inhibitors and are also different from other previously described slow-binding inhibitors (Belda et al., 1983), since 4-substituted resorcinols require an enzymatic turnover for their inhibitory effect to be exhibited.

Figure 4 shows the effect of 4-hexylresorcinol on the initial (A) and steady-state (B) rates, respectively, at different concentrations of substrate. Initial velocities (v_o) decreased with inhibitor concentration, indicating, according to Morrison and Walsh (1989), that an enzyme−inhibitor complex is rapidly formed with an apparent dissociation constant (K_I) for the EoxyI complex of 5 μM. The steady-state rates also decreased as 4-hexylresorcinol concentration rose, and when Dixon plots were made for the steady-state rates at different 4-*tert*-butylcatechol concentrations (Figure 4B), a value of 2.4 μM was determined for the apparent dissociation constant (K'_I) for the EoxyI* complex. The above results show that the rapidly formed complex (EoxyI) subsequently undergoes a slow reversible isomerization to a second complex (EoxyI*). The more enzyme drawn into the second complex, the more pronounced the inhibition becomes.

All these effects of 4-hexylresorcinol on mushroom tyrosinase were similar to those obtained for the slow inhibition of tyrosinase by L-mimosine (Cabanes et al., 1987b), tropolone (Valero et al., 1991), and kojic acid (Cabanes et al., 1994). These results can be explained by the mechanism shown in Figure 3. According to the equations obtained by kinetic analysis of this mechanism (Cabanes et al., 1987a), the slow transition constant, k_{-6}, can be evaluated as follows:

$$v_s = \frac{k_{-6}}{k_{app}} v_o \qquad (3)$$

since v_s, k_{app}, and v_o can be obtained experimentally.

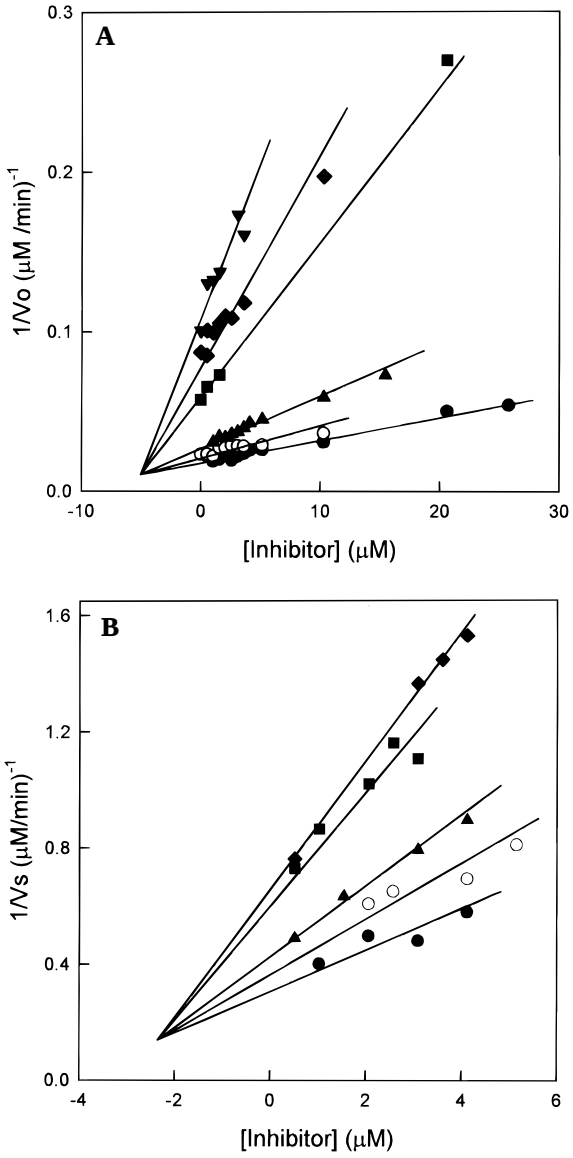

Figure 4. Dixon plots for the effect of 4-hexylresorcinol on initial (A) and steady-state (B) rates of the catecholase activity of mushroom tyrosinase. 4-*tert*-Butylcatechol concentrations used were (●) 4.95, (○) 3.75, (▲) 2.25, (■) 0.975, (◆) 0.75, and (▼) 0.5 mM. Enzyme concentration was 0.39 μg/mL.

Once k_{-6} and k_{app} are known, k_6 can be evaluated by means of a double-reciprocal plot of ($k_{app} - k_{-6}$) versus inhibitor concentration according to eq 4:

$$k_{app} = k_{-6} + \frac{k_6[I]}{k_I(1 + ([D])/K_M) + [I]} \qquad (4)$$

A series of straight lines intersecting at a point on the ordinate axis equal to $1/k_6$ was obtained (Figure 5). A value of 0.062 s^{-1} was calculated for k_6.

When 4-dodecylresorcinol and 4-ethylresorcinol were used as inhibitors, similar results to those shown for 4-hexylresorcinol were obtained. When the initial velocities and steady-state rates were plotted according to the Dixon equation, the values of K_I and K'_I were evaluated (Table 1); k_6 values were determined by plotting $1/(k_{app} - k_{-6})$ vs 1/[I], such as has been previously described for 4-hexylresorcinol. As can be seen from Table 1, K_I values for all the 4-substituted resorcinols assayed were always higher than those obtained for K'_I, indicating, according to the behavior of slow-

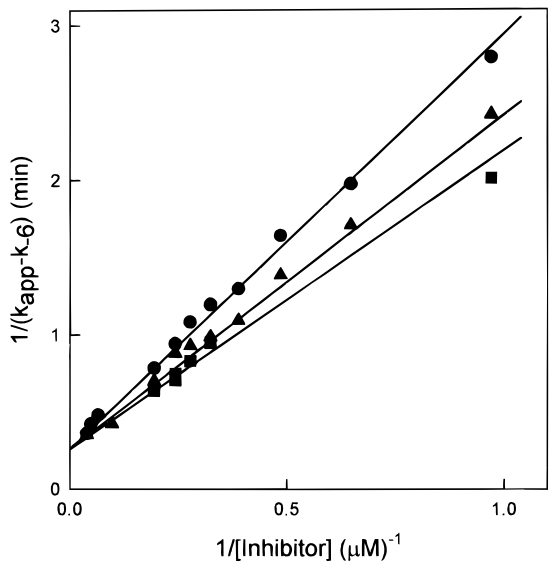

Figure 5. Graphical calculation of k_6 for the inhibition of mushroom tyrosinase by 4-hexylresorcinol. Experimental conditions were as in Figure 4. 4-*tert*-Butylcatechol concentrations were (●) 4.95, (▲) 2.25, and (■) 0.975 mM.

Table 1. Values of the Kinetic Constants K_I, K'_I, and k_6 for the Inhibition of Catecholase Activity of Mushroom Tyrosinase[a]

	K_I (μM)	K'_I (μM)	k_6 (s^{-1})
4-hexylresorcinol	5.0	2.4	0.062
4-dodecylresorcinol	13.2	0.9	0.085
4-ethylresorcinol	21.8	10	0.028
L-mimosine[b]	80.0	15	0.17
tropolone[c]	16.0	1.5	0.05
kojic acid[d]	2.75	0.62	0.06

[a] The values of K_I, K'_I, and k_6 for L-mimosine, tropolone, and kojic acid, shown in this table, have been taken from the bibliography. [b] Cabanes et al., 1987b. [c] Valero et al., 1991. [d] Cabanes et al., 1994.

binding inhibitors reported by Morrison and Walsh (1989), that inhibition of mushroom tyrosinase catecholase activity by 4-substituted resorcinols follows the scheme shown in Figure 3. Thus, an enzyme−inhibitor complex (EoxyI) is rapidly formed, which subsequently undergoes a slow reversible isomerization to a second complex (EoxyI*). This table also shows the values of K_I, K'_I, and k_6 for other slow-binding inhibitors such as mimosine (Cabanes et al., 1987b), tropolone (Valero et al., 1991), and kojic acid (Cabanes et al., 1994) using different tyrosinases. Among the 4-substituted resorcinols, the lowest value obtained for K_I (dissociation constant at $t = 0$) corresponded to 4-hexylresorcinol (5 μM), which was slightly higher than that obtained for kojic acid (2.75 μM). Thus, at $t = 0$, 4-hexylresorcinol and kojic acid are the two best tyrosinase inhibitors. However, K'_I, the overall dissociation constant, is the parameter which really indicates the strength of slow-binding inhibitors, and in this respect, Table 1 clearly shows that dodecylresorcinol is the best inhibitor among the 4-substituted resorcinols. This K'_I value (0.9 μM) was only slightly higher than that obtained for kojic acid (0.62 μM), which is one of the most powerful tyrosinase inhibitors. Thus, as shown in Table 1, dodecylresorcinol and kojic acid are the two best slow-binding inhibitors. The values of k_6 were very similar for both inhibitors, although the k_6 value for 4-dodecylresorcinol was slightly higher than for kojic acid (0.085 and 0.06 s^{-1}, respectively) suggesting that 4-dodecylresorcinol might be a faster time-dependent inhibitor than kojic acid.

We can conclude, therefore, that of all the 4-substituted resorcinols, dodecylresorcinol is the best inhibitor, being even better than kojic acid, since it does not manifest the antibiotics and insecticide effects shown by kojic acid (Saruno et al., 1979).

As regards cresolase activity, this hydroxylating activity of tyrosinase shows a complex kinetic response with a slow transition phase, which represents the accumulation of the *o*-diphenol necessary to maintain this activity. In agreement with the kinetic mechanism for tyrosinase (Cabanes et al., 1987a), only the oxy form of the three different forms of the enzyme acts on monophenol, by hydroxylating it to *o*-diphenol. Thus, the inhibition produced by 4-substituted resorcinols affects both catecholase and cresolase activity through the oxy form.

The presence of the lag period in the cresolase activity of tyrosinase means that it is impossible to quantify the kinetic constants of the interaction of 4-substituted resorcinols and the enzyme. However, since the enzymatic intermediates, oxy and met, are the same in both cresolase and catecholase activities, it might reasonably be assumed that K_I, k_6, and k_{-6} are the same for both activities, since these constants can only reflect the presence of the oxy form and the inhibitor (Figure 3).

The results obtained in this paper clearly show, therefore, that 4-substituted resorcinols can be classified as slow-binding competitive inhibitors of mushroom tyrosinase when 4-*tert*-butylcatechol is used as substrate. This is a complex response of tyrosinase to several compounds structurally analogous to phenolic substrates and can only be explained by taking into account the complex internal mechanism of this enzyme. The above might explain some of the different results appearing in the literature concerning the inhibition of tyrosinase by compounds structurally analogous to phenolic substrates since the expressed activity is not constant in time in the presence of the inhibitor. Thus, mistaken rate values and incorrect kinetic parameters are obtained if the product increase is only followed over a limited time. Therefore, IC_{50} is not a valid parameter for this kind of inhibitor since, due to the complex nature of this kind of inhibition, a complete kinetic study must be carried out in order to determine the kinetic parameters (K_I, K'_I, and k_6) which characterize the behavior of these inhibitors.

ABBREVIATIONS USED

PPO, polyphenol oxidase; L-mimosine, β-(*N*-3-hydroxy-4-oxopyridin-4-yl)-α-aminopropionic acid.

LITERATURE CITED

Belda, F.; García-Carmona, F.; García-Cánovas, F.; Gómez-Fernández, J. C.; Lozano, J. A. A kinetic study of the interaction between mitochondrial F1 adenosine triphosohatase and adenylylimido diphosphatase and guanylylliminodiphosphatase. *Biochem. J.* **1983**, *210*, 727−735.

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.* **1976**, *72*, 248−254.

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

Cabanes, J.; García-Cánovas, F.; Lozano, J. A.; García-Carmona, F. A kinetic study of the melanization pathway between L-tyrosine and dopachrome. *Biochim. Biophys. Acta* **1987a**, *923*, 187−195.

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

Cabanes, J.; Chazarra, S.; García-Carmona, F. Kojic acid, a cosmetic skin whitening agent, is a slow-binding inhibitor of catecholase activity of tyrosinase. *J Pharm. Pharmacol.* **1994**, *46*, 982−985.

Frankos, V. H.; Schmitt, D. F.; Haws, L. C.; McEvily, A. J.; Iyengar, R.; Miller, S. A. Generally recognized as safe (GRAS) evaluation of 4-hexylresorcinol for use as a processing aid for prevention of melanosis in shrimp. *Regul. Toxicol. Pharmacol.* **1991**, *14*, 202−212.

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

Galindo, J. D.; Pedreño, E., García-Carmona, F.; García-Cánovas, F.; Solano, F.; Lozano, J. A. Steady-state study of the mechanism of dopa-oxidase activity of tyrosinase. *Int. J. Biochem.* **1983**, *15*, 1455−1461.

Iyengar, R.; Bohmont, C. W.; McEvily, A. 4-Hexylresorcinol and prevention of shrimp blacspot: residual analysis. *J. Food Compos. Anal.* **1991**, *4*, 148−157.

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

Lerch, K. Copper monooxygenases: tyrosinase and dopamine-β-monooxygenase. In *Metal Ions in Biological Systems*; Sigel, H., Ed.; Marcel Dekker, Inc.: New York, 1981; pp 143−186.

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

Mayer, A. M.; Harel, E. Polyphenol oxidases in plants. *Phytochemistry* **1979**, *18*, 193−215.

McEvily, A. J.; Iyengar, R.; Otwell, W. S. Sulfite alternative prevents shrimp melanosis. *Food Technol.* **1991**, *45*, 80−86.

McEvily, J. A.; Iyengar, R.; Otwell, W. S. Inhibition of enzymatic browning in foods and beverages. *Crit. Rev. Food Sci. Nutr.* **1992**, *32*, 253−273.

Morrison, J. F. The slow-binding and slow, tight-binding inhibition of enzyme-catalysed reactions. *TIBS* **1982**, *7*, 102−105.

Morrison, J. F.; Walsh, C. T. The behavior and significance of slow-binding enzyme inhibitors. *Adv. Enzymol.* **1989**, 201−301.

Rob, D. A. Molecular properties of plant tyrosinases. In *Biochemistry of fruits and vegetables*; Friend, J., Rhodes, M., Eds.; Academic Press: London, 1981; pp 181−192.

Sánchez-Ferrer, A.; Laveda, F.; García-Carmona, F. Partial purification of soluble potato polyphenol oxidase by partitionng in an aqueous two-phase system. *J. Agric. Food Chem.* **1993**, *41*, 1219−1224.

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

Saruno, R.; Kato, F.; Ikeno, T. Kojic acid, a tyrosinase inhibitor from *Aspergillus albus*. *Agric. Biol. Chem.* **1979**, *43*, 1337−1338.

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.

Vámos-Vigyázó, L. Polyphenol oxidase and peroxidase in fruits and vegetables. *CRC Crit. Rev. Food Sci. Nutr.* **1981**, *15*, 49−127.

Williams, J. W.; Morrison, J. E. The kinetics of reversible tight-binding inhibition. *Methods Enzymol.* **1979**, *63*, 437−467.

Received for review October 24, 1996. Accepted February 4, 1997.[⊗] This work was supported by a grant from the Comisión Interministerial de Ciencia y Tecnología (Spain), Proyect TB95-1024.

JF960810N

[⊗] Abstract published in *Advance ACS Abstracts,* April 1, 1997.