Time-Dependent Inhibition of Grape Polyphenol Oxidase by Tropolone

Edelmira Valero,† Manuela García-Moreno,† Ramón Varón,† and Francisco García-Carmona*,‡

Departamento de Química, Escuela Universitaria Politécnica, Universidad de Castilla–La Mancha, E-02006 Albacete, Spain, and Departamento de Bioquímica, Universidad de Murcia, Espinardo, E-30001 Murcia, Spain

A kinetic study of inhibition of the catecholase activity of grape polyphenol oxidase by tropolone has been made. The results obtained showed that tropolone inhibits grape polyphenol oxidase in a nonclassical manner. A decrease in initial velocity to a steady-state inhibited velocity can be observed on a time scale of minutes. The time dependence, which is unaltered by prior incubation of the enzyme with the inhibitor, is consistent with a first-order transition. Kinetic data obtained correspond to those for a postulated mechanism that involves rapid formation of an enzyme–inhibitor complex that subsequently undergoes a relatively slow reversible reaction, since inhibition was reversed by the addition of $CuSO_4$ to the reaction medium. Likewise, kinetic parameters characterizing this type of inhibition have been evaluated by means of nonlinear regression of product accumulation curves.

INTRODUCTION

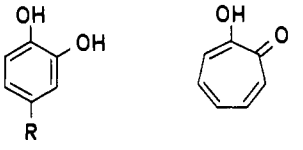
Polyphenol oxidase (monophenol, dihydroxy-L-phenylalanine:oxygen oxidoreductase E.C. 1.10.3.1) is a widely distributed copper-containing protein, responsible for browning in fruits and vegetables. Three different forms of binuclear copper in the active site involved in the reaction mechanism are known: met, oxy, and deoxy (Lerch, 1981; Robb, 1981). The enzyme catalyzes two different reactions, both using molecular oxygen: hydroxylation of monophenols to *o*-diphenols (cresolase activity) and oxidation of *o*-diphenols to *o*-quinones (catecholase activity). The quinones thus formed lead by polymerization to the creation of brown pigments, which generally cause a decrease in the quality of processed food.

Because of the deleterious effect of enzymatic browning on fruits and vegetables, a considerable number of inhibitors of polyphenol oxidase are known. An important group is constituted by compounds structurally analogous to phenolic substrate, which generally show competitive inhibition with respect to this substrate, although it can vary depending on enzyme source and substrate used (Lerner et al., 1971; Walker, 1975; Mayer and Harel, 1979; Vámos-Vigyázó, 1981).

Tropolone (2-hydroxy-2,4,6-cycloheptatrien-1-one) is the progenitor of a compound group called tropolones, some of them being found in several plants and molds. It is structurally analogous to orthodiphenolic substrates of polyphenol oxidase, as well as an effective copper chelator (Bryant et al., 1953). The structure of tropolone is compared with the normal orthodiphenolic substrate in Chart I. It was reported to be a potent inhibitor of dopamine β-hydroxylase and mushroom polyphenol oxidase (Kahn and Andrawis, 1985a), both being copper-containing enzymes, as well as a substrate of horseradish peroxidase in the presence of hydrogen peroxide (Kahn and Andrawis, 1985b). It is, therefore, an agent that allows differentiation between polyphenol oxidase and peroxidase (Kahn, 1985).

The aims of the present paper are the kinetic study of the inhibition of catecholase activity of grape polyphenol oxidase by tropolone and an evaluation of kinetic parameters and constants characterizing the system.

Chart I. Structures of a Normal Orthodiphenolic Substrate of Polyphenol Oxidase (Catechols) (I) and Tropolone (II)



(I) (II)

MATERIALS AND METHODS

The grapes (*Vitis vinifera* L. cv. Airen) used in this study were harvested at maturation stage at Villarrobledo (Albacete, Spain) and stored at −25 °C until used.

4-Methylcatechol and tropolone were purchased from Sigma Chemical Co. All other chemicals were of analytical grade.

Extraction of grape polyphenol oxidase and measurement of catecholase activity by following at 400 nm the appearance of the 4-methyl-*o*-benzoquinone product of the reaction were performed as previously described (Valero et al., 1988). Unless otherwise stated, the reaction media at 25 °C contained 10 mM 4-methylcatechol, 10 mM sodium acetate buffer (pH 4.75), tropolone at the indicated concentration, and 0.15 unit of enzymatic activity. One unit of enzymatic activity was defined as the amount of enzyme that produces 1 μmol of 4-methyl-*o*-benzoquinone/min. Protein concentration was determined by the method of Bradford (1976).

Kinetic parameters were evaluated by fitting the experimental progress curves to the equation

$$Y = c_1 t + c_2(1 - e^{-c_3 t}) + c_4$$

by means of *n*-parameter nonlinear regression (Marquardt, 1963), where the parameter c_4 was included to correct any possible deviation from the initial absorption value.

RESULTS AND DISCUSSION

When grape polyphenol oxidase was assayed at pH 4.75 and 25 °C in the presence of tropolone with 4-methylcatechol as substrate, a biphasic response was observed with a progressive decrease in initial activity followed by a constant rate (Figure 1); both the initial and constant rate decreased as the inhibitor concentration increased. Because the inhibition produced by tropolone was expressed slowly, it may be a slow-binding inhibitor (Morrison, 1982) that is not necessarily tight binding or stoichiometric since tropolone concentration in the progress

* Author to whom correspondence should be addressed.
† Universidad de Castilla–La Mancha.
‡ Universidad de Murcia.

0021-8561/91/1439-1043$02.50/0

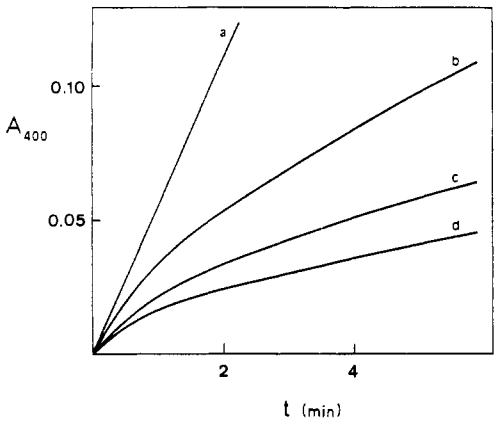
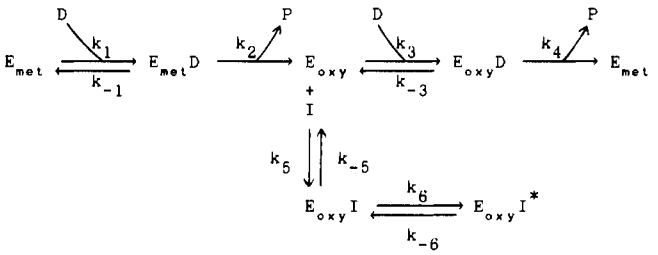


Figure 1. Progress curves for the inhibition of grape polyphenol oxidase by tropolone. Tropolone concentrations used were (a) 0, (b) 10, (c) 20, and (d) 30 μM. The reaction was started by the addition of the enzyme.

Scheme I. Mechanism Postulated by Cabanes et al. (1987a) for the Slow Inhibition of Frog Epidermis Polyphenol Oxidase by L-Mimosine[a]



[a] E_{met}, met form of polyphenol oxidase; E_{oxy}, oxy form of polyphenol oxidase; D, orthodiphenolic substrate; $E_{oxy}I^*$, enzyme (oxy form)–inhibitor complex formed by slow isomerization of $E_{oxy}I$ complex.

curve experiments was always much higher than the enzyme concentration (Williams and Morrison, 1979). This time-dependent inhibition of polyphenol oxidase has also been found by other authors (Cabanes et al., 1984, 1987a) using compounds structurally analogous to phenolic substrate, such as *m*-coumaric acid and L-mimosine, and using frog epidermis and mushroom as sources of enzyme.

The curves shown in Figure 1 can be described by the general equation (Frieden, 1970) for a first-order process

$$P = v_s t + (v_0 - v_s)(1 - e^{-k_{app}t})/k_{app} \quad (1)$$

where v_0, v_s, and k_{app} represent, respectively, the initial rate, the steady-state rate, and the apparent first-order rate constant, the meaning of the last depending on the mechanism under study. Data analysis of these product accumulation curves can be performed by making an overall fit of the experimental data to eq 1 by nonlinear regression, as has been described by Morrison (1982).

To study in depth this effect of tropolone on polyphenol oxidase, experiments were performed in which the enzyme was preincubated with different tropolone 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 1 were obtained (data not shown). This result indicates that tropolone 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 polyphenol oxidase (Scheme I) (Galindo et al., 1983; Lerch, 1983; Cabanes et al., 1987b), revealing that tropolone must bind to the oxy form of the enzyme. This form is an obligatory intermediate in the catalytic turnover, and so

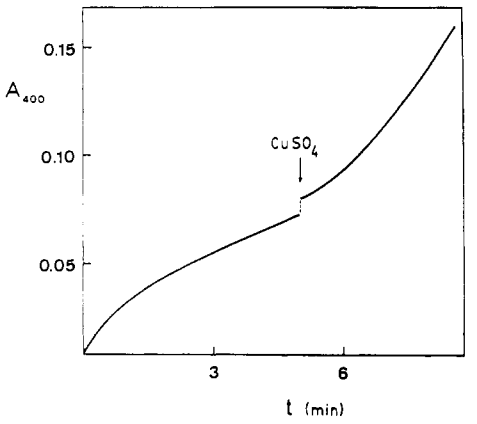


Figure 2. Effect of addition of $CuSO_4$ to the reaction medium. [Tropolone] = 20 μM; once the steady-state was reached, 100 μL of 500 μM $CuSO_4$ was added to the reaction medium.

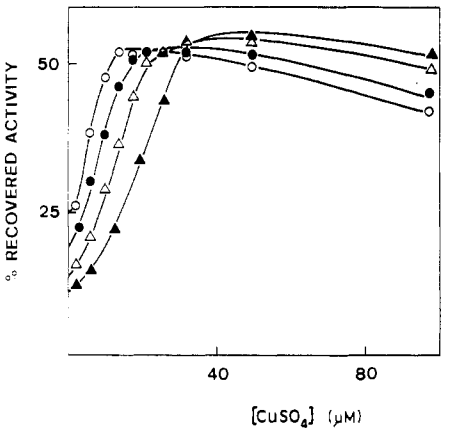


Figure 3. Effect of addition of $CuSO_4$ to the reaction medium as shown in Figure 2, at different tropolone concentrations: (○) 10, (●) 15, (△) 20, and (▲) 30 μM. Percentage of recovered activity is referred to the activity of a standard assay.

the presence of the substrate and, therefore, of the catalytic activity is necessary to observe the slow binding of tropolone to the enzyme.

As has been pointed out above, tropolone is known to be a copper chelator. Thus, to check the reversibility of binding of tropolone to the enzyme, we carried out several experiments in which $CuSO_4$ was added to the reaction medium when the constant rate had been reached. We found that activity recovered (Figure 2) after a lag period. This result indicates that tropolone inhibits polyphenol oxidase reversibly, perhaps by binding to the copper at the active site of the enzyme. Figure 3 shows the results obtained at different tropolone concentrations. In all cases the regained activity was less when higher concentrations of $CuSO_4$ were added to the reaction medium, which may be due to the fact that copper sulfate alone inhibits catecholase activity of polyphenol oxidase, as has been reported by Kahn and Andrawis (1985a).

Parts A and B of Figure 4 show the effect of tropolone on the initial and constant rates, respectively, at five different concentrations of tropolone. A simple competitive inhibition was shown, in contrast to the mixed-type inhibition reported in the literature for polyphenol oxidase from other sources (Kahn and Andrawis, 1985a; Moore and Flurkey, 1990). Furthermore, the fact that v_0 decreases with tropolone concentration shows, according to the behavior of slow-binding inhibitors reported by Morrison and Walsh (1989), that an enzyme–inhibitor complex (EI) is rapidly formed and subsequently undergoes a slow reversible isomerization reaction to a second complex (EI*).

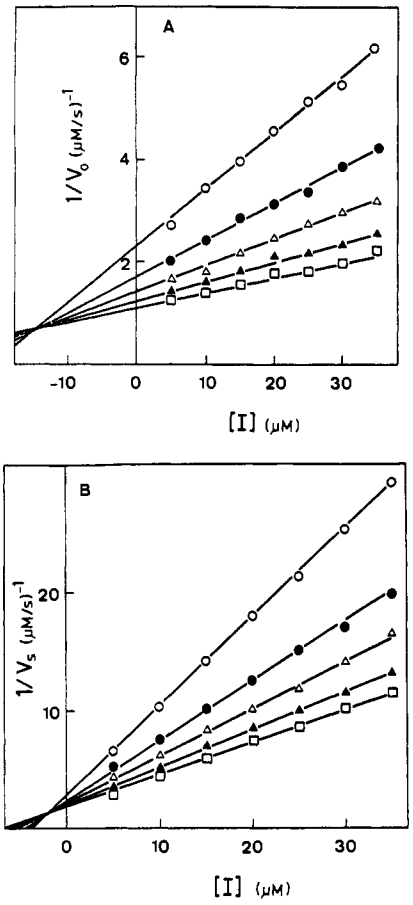


Figure 4. Dixon plots of the effect of tropolone on initial (A) and steady-state (B) rates of the catecholase activity of grape polyphenol oxidase. 4-Methylcatechol concentrations used were (○) 5, (●) 7.5, (△) 10, (▲) 12.5, and (□) 15 mM.

Table I. Variation of v_0, v_s, k_{app}, and k_{-6} with Tropolone Concentration

[I], μM	v_s, μM/s	v_0, μM/s	k_{app}, $s^{-1} \times 10^2$	k_{-6}, $s^{-1} \times 10^3$
5	0.23	0.60	1.2	4.5
10	0.16	0.55	1.6	4.7
15	0.12	0.46	1.9	4.9
20	0.10	0.41	2.2	5.3
25	0.08	0.36	2.4	5.5
30	0.07	0.34	2.7	5.6
35	0.06	0.32	3.1	5.8

In this way, the apparent constant for EI complex dissociation, K_I = 16 μM, and the apparent constant for EI* dissociation, K_I' = 1.5 μM, were then evaluated from the intersection points in parts A and B of Figure 4, respectively.

All these effects of tropolone on grape polyphenol oxidase are very similar to those obtained by Cabanes et al. (1987a) for the slow inhibition of mushroom polyphenol oxidase by L-mimosine, the pyridone analogue of Dopa, which has a structure similar to that of tropolone. These authors proposed the mechanism shown in Scheme I to explain this type of inhibition, which is in agreement with the results obtained here. According to the equations obtained when the kinetic analysis of this mechanism is carried out, the constant of slow transition k_{-6} can be evaluated, as is shown in Table I. Once k_{-6} and k_{app} are known, k_6 can be evaluated according to eq 2 by means of

$$k_{app} = k_{-6} + \frac{[I]k_6}{K_I(1 + ([D]/K_M)) + [I]} \quad (2)$$

a double-reciprocal plot of $k_{app} - k_{-6}$ vs [I] (Figure 5). A

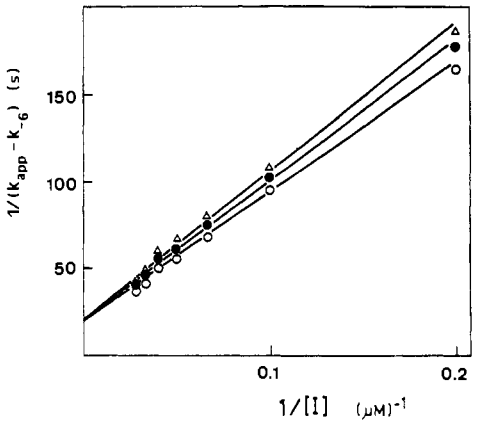


Figure 5. Graphical calculation of k_6 for the inhibition of grape polyphenol oxidase by tropolone. Experimental conditions were as in Figure 4. 4-Methylcatechol concentrations were (○) 5, (●) 10, and (△) 15 mM.

series of straight lines intersecting at a point on the ordinate axis equal to $1/k_6$ was obtained, from which a value of 0.0505 s^{-1} was calculated for k_6.

CONCLUSION

The results obtained in this paper show clearly that tropolone can be classified as a slow-binding competitive inhibitor of grape polyphenol oxidase when 4-methylcatechol is used as substrate. This is a complex response of polyphenol oxidase to several compounds structurally analogous to phenolic substrate 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 for studies of the inhibition of polyphenol oxidase by compounds structurally analogous to phenolic substrate since expressed activity is not constant in time in the presence of the inhibitor. Thus, mistaken values of rate are obtained if the product increase is only followed in a determined time and so different patterns of velocity vs substrate are obtained as a function of the level of inhibitor used.

ACKNOWLEDGMENT

This work was partially supported by a grant from the Comisión Interministerial de Ciencia y Tecnología (Spain), Proyecto No. AGR-89-0296, and by the Consejería de Cultura, Educación y Turismo de la Comunidad Autónoma de la Región de Murcia, Proyecto No. PCT-89/04.

LITERATURE CITED

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

Bryant, B. E.; Fernelius, W. C.; Douglas, E. Formation constants of metal complexes of tropolone and its derivatives. 1. Tropolone. *J. Am. Chem. Soc.* **1953**, *75*, 3784–3790.

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

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* **1987b**, *923*, 187–195.

Frieden, C. Kinetic aspects of regulation of metabolic processes. *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.

Kahn, V. Tropolone—A compound that can aid in differentiating between tyrosinase and peroxidase. *Phytochemistry* **1985**, *24*, 915–920.

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

Kahn, V.; Andrawis, A. Tropolone as a substrate for horseradish peroxidase. *Phytochemistry* **1985b**, *24*, 909–914.

Lerch, K. Copper monooxygenases: tyrosinase and dopamine-β-monooxygenase. In *Metal Ions in Biological Systems*; Sigel, H., Ed.; Zürich, 1981.

Lerch, K. *Neurospora* tyrosinase: structural, spectroscopic and catalytic properties. *Mol. Cell. Biochem.* **1983**, *52*, 125–138.

Lerner, H. R.; Harel, E.; Lehman, E.; Mayer, A. M. Phenylhydrazine, a specific irreversible inhibitor of catechol oxidase. *Phytochemistry* **1971**, *10*, 2637–2640.

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.

Moore, B. M.; Flurkey, W. H. Sodium dodecyl sulfate activation of a plant polyphenol oxidase. *J. Biol. Chem.* **1990**, *265*, 4982–4988.

Morrison, J. E. The slow-binding and slow tight-binding inhibition of enzyme-catalysed reactions. *Trends Biochem. Sci.* **1982**, *7*, 102–105.

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

Robb, D. A. Molecular properties of plant tyrosinases. In *Biochemistry of Fruits and Vegetables*; Friend, J., Rhodes, M., Eds.; Academic Press: London, 1981.

Valero, E.; Varón, R.; García-Carmona, F. Characterization of polyphenol oxidase from Airen grapes. *J. Food Sci.* **1988**, *53*, 1482–1485.

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

Walker, J. R. L. Enzymic browning in foods: a review. *Enzyme Technol. Dig.* **1975**, *4*, 89–100.

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

Received for review August 14, 1990. Accepted January 4, 1991.

Registry No. II, 533-75-5; Cu, 7440-50-8; polyphenol oxidase, 9002-10-2.