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Kojic Acid, a Cosmetic Skin Whitening Agent, is a Slow-binding Inhibitor of Catecholase Activity of Tyrosinase

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Abstract—It was found that kojic acid, which is used in cosmetics for its excellent whitening effect, inhibits catecholase activity of tyrosinase in a non-classical manner. A decrease in the initial velocity to a steady-state inhibited velocity can be observed over a few minutes. This time-dependence, which is unaltered by prior incubation of the enzyme with the inhibitor, is consistent with a first-order transition. The kinetic data obtained correspond to those for a postulated mechanism that involves the rapid formation of an enzyme inhibitor complex that subsequently undergoes a relatively slow reversible reaction. Kinetic parameters characterizing this type of inhibition were evaluated by means of nonlinear regression of product accumulation curves.

Kojic acid (5-hydroxy-2-hydroxymethyl-4H-pyran-4-one) is an antibiotic produced by many species of *Aspergillus* and *Penicillum* in an aerobic process from a wide range of carbon sources (Lee et al 1986; Kwak & Rhee 1992). Kojic acid has been extensively used as a cosmetic agent with an excellent whitening effect (Obara et al 1985; Ohyama 1990) because it inhibits tyrosinase (Saruno et al 1979; Chen et al 1991a, b).

Tyrosinase, or polyphenoloxidase (EC. 1.14.18.1), is a bifunctional copper protein complex widely distributed on the phylogenetic scale and responsible for melanization in animals and browning in plants. Three different forms of binuclear copper in the active site are involved in the reactions (Lerch 1981; Robb 1981). The enzyme catalyses two different reactions: cresolase activity, or hydroxylation of monophenols to *o*-diphenols and catecholase activity, or oxidation of *o*-diphenols to *o*-quinone.

The expression of the cresolase activity of the enzyme in the presence of its substrate (monophenol) shows a lag period (Pomerantz 1966; Garcia-Carmona et al 1979) that has recently been explained by taking into account the chemical steps involved in the tyrosinase reaction (Cabanes et al 1987b; Garcia-Carmona et al 1982, 1987, 1988), while catecholase activity shows no slow-transition phenomena.

Both the lag period of the cresolase activity and the steady-state rate are affected by kojic acid acting as a competitive and mixed-type inhibitor, depending on the phenolic substrates as has been described by Chen et al (1991a). Those authors include kojic acid in an important group of classical inhibitors formed of compounds structurally analogous to phenolic substrates, towards which they generally show competitive inhibition, although this inhibition varies depending on the enzyme source and substrate used (Walker 1975; Mayer & Harel 1979; Vámos-Vigyázó 1981; Khan & Andrawis 1985). L-Mimosine and tropolone have been described by us as competitive, slow-binding inhibitors (Cabanes et al 1987a; Valero et al 1991), according to the classification of reversible enzyme inhibitors established by Morrison (1982). These inhibitors have an oxo group ortho to the hydroxyl group, thus presenting structures which are intermediate between the diphenolic substrate of the enzyme and the enzymatic product (Fig. 1). They result in an inhibition of catecholase activity that is characterized by a long transition phase.

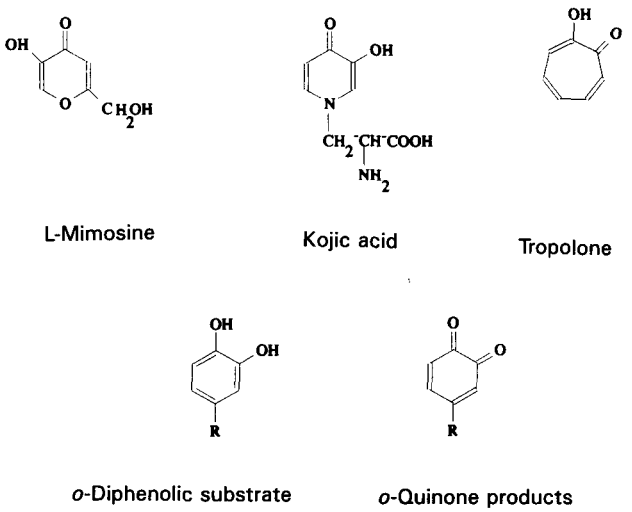

FIG. 1. Structures of the inhibitors, a normal orthodiphenolic substrate and orthoquinonic products of tyrosinase.

In view of the above, we thought it would be interesting to test whether kojic acid is also an effective inhibitor of tyrosinase according to the above classification.

Materials and Methods

L-Dopa was purchased from Sigma Chemical Co. (St Louis, MO). All other chemicals used were of analytical grade.

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Frogs (*Rana esculenta ridibunda*) were obtained from local suppliers from November to March. Epidermis was separated from dermis after incubation with a 2 M NaBr for 24 h at 0–4°C. After washing several times with bidistilled water, a negative bromide reaction took place and the epidermis was lyophilized and kept at 0–4°C until used. Frog epidermis protyrosinase was extracted, partly purified and activated by the procedure of Lozano et al (1975).

Catecholase activity was determined spectrophotometrically at 475 nm by the appearance of dopachrome ($\epsilon = 3700\,M^{-1}\,cm^{-1}$) using L-dopa (5·6 mM) as substrate in sodium phosphate buffer (pH 6·5). One unit of enzyme was taken as the amount of enzyme that produced 0·5 μmol dopachrome min^{-1}, since this involves the production of 1 μmol dopaquinone (Garcia-Cánovas et al 1982). Protein concentration was determined by the method of Lowry et al (1951).

The progress curves were fitted by nonlinear regression to the following equation, using Marquardt's algorithm (Marquardt 1963):

$$P = C_2t + (C_1 - C_2)(1 - e^{-C_3t})/C_3 + C_4 \qquad (1)$$

Equation 1 is equivalent to equation 2 (see below), C_4 representing the experimental uncertainty at the zero-time absorbance, caused by addition of enzyme to start the reaction.

Results and Discussion

When frog epidermis tyrosinase was assayed with L-dopa as substrate, a steady-state rate was immediately attained; the absorbance changes were linear up to at least 0·3 absorbance units (Fig. 2, curve A). When the reaction was started by addition of the enzyme in the presence of kojic acid, a biphasic response was observed with a progressive decrease in initial activity followed by a constant rate (Fig. 2, curves B–D); both the initial and the constant rate decreased as the inhibitor concentration increased.

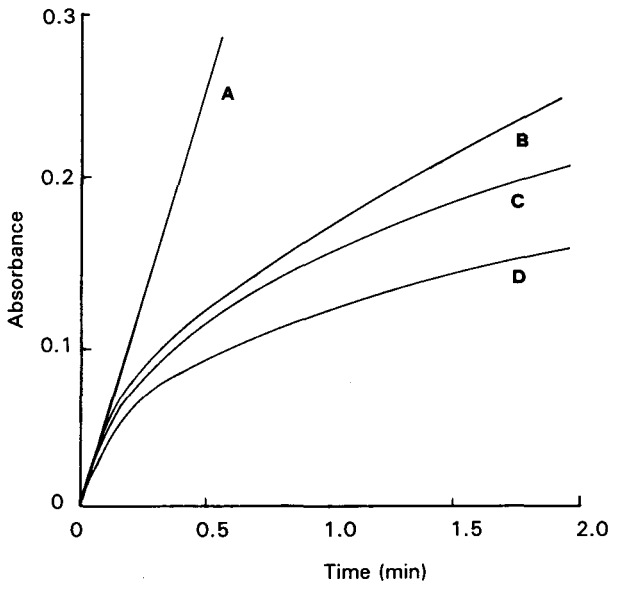
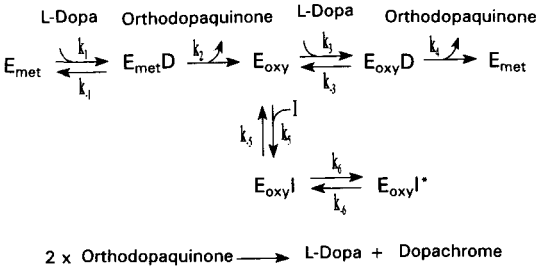


FIG. 2. Progress curves for the inhibition of frog epidermis tyrosinase by kojic acid. Kojic acid concentrations were A 0, B 0·32, C 0·94, D 1·57 μM. The reaction was started by the addition of the enzyme.



SCHEME 1. Mechanism postulated by Cabanes et al (1987b) for the slow inhibition of frog epidermis tyrosinase by L-mimosine. E_{met}, met form of tyrosinase; E_{oxy}, oxy form of tyrosinase, $E_{oxy}I^*$, enzyme-inhibitor complex formed by slow isomerization of $E_{oxy}I$ complex.

This result indicates that the inhibition produced by kojic acid is expressed slowly, thus behaving as a slow-binding inhibitor, according to the definition given by Morrison (1982). The inhibition expressed is not necessarily tightly bound or stoichiometric since kojic acid concentration in the progress curve experiments was always much higher than the enzyme concentration (Williams & Morrison 1979).

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

$$[P] = V_st + (V_s - V_0)(1 - e^{-k_{app}t})/k_{app} \qquad (2)$$

were V_0, V_s and k_{app} represent, respectively, the initial and the steady-state rates and the apparent first-order rate constant (the meaning of which depends on the mechanism). Data analysis of these product-accumulation curves can be performed by making an overall fit of the experimental data to equation 2 by nonlinear regression, as has been described by Morrison (1982).

To study this effect of kojic acid on frog tyrosinase, experiments were performed in which the enzyme was preincubated with different kojic acid concentrations at various times, at the end of which the reaction was started by the addition of the substrate; progress curves similar to those in Fig. 2 were obtained (data not shown). This result indicates that kojic acid 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 (Scheme 1) (Galindo et al 1983; Lerch 1983; Cabanes et al 1987b). This scheme postulates the successive binding of two diphenolic substrates to complete the catalytic cycle with the appearance of an enzymatic oxy form, which, in comparison with the met form, has a greater affinity for the substrate (Galindo et al 1983). Thus, the inhibitor competes with the binding of the second molecule of L-dopa, and so kojic acid must bind to the oxy form of the enzyme. This 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 kojic acid to the enzyme to be observed. Thus, kojic acid is different from other inhibitors previously

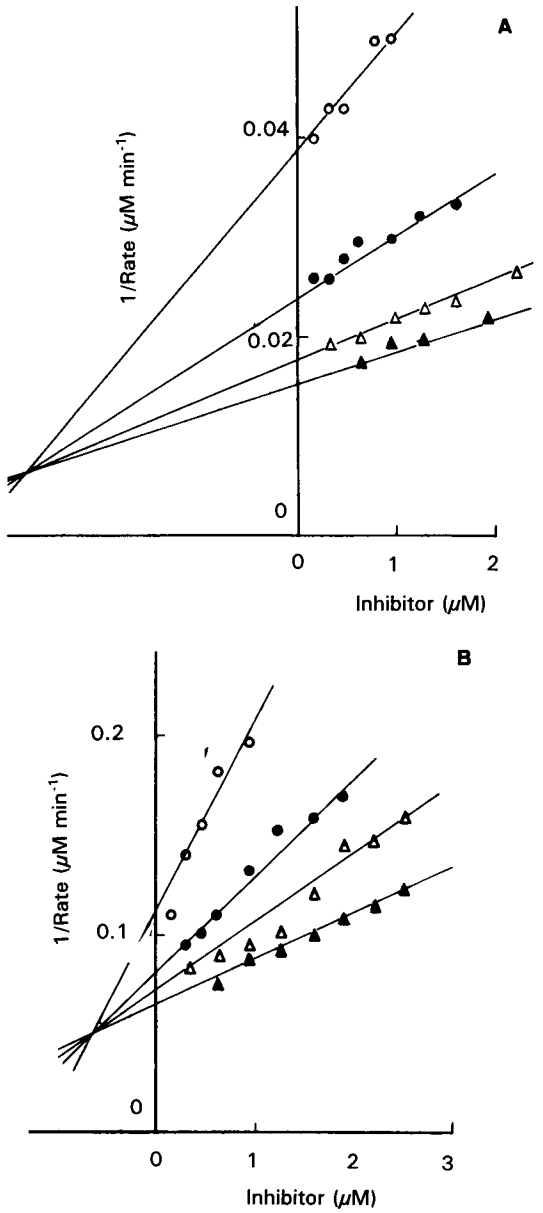


FIG. 3. Dixon plots of the effect of kojic acid on initial (A) and steady-state (B) rates of the catecholase activity of frog epidermis tyrosinase. ○ 1·49, ● 2·78, △ 4·47, and ▲ 5·96 μM, L-dopa.

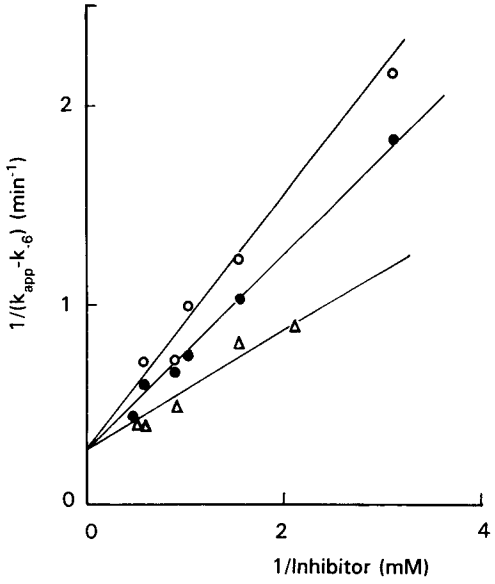


FIG. 4. Graphical calculation of k_6 for the inhibition of epidermis tyrosinase by kojic acid. △ 2·78, ● 4·47, ○ 5·96 mM, L-dopa.

described as slow-binding inhibitors to other enzymes (Belda et al 1983), since kojic acid requires an enzymatic turnover to exhibit its inhibitory effect. It cannot, therefore, be considered as a classical competitive inhibitor (Khan & Andrawis 1985).

The initial velocities (V_0) decrease with inhibitor concentration (Fig. 3) indicating, according to the behaviour of slow-binding inhibitors reported by Morrison & Walsh (1989), that an enzyme-inhibitor complex is rapidly formed with a dissociation constant (K_d) of 2·75 μM. When the steady-state rates are plotted according to the Dixon equation at four different substrate concentrations (Fig. 3), the value of the overall dissociation constant (K_d') can be calculated, giving a value of 0·62 μM. Thus, the rapidly formed complex subsequently undergoes a slow reversible isomerization to a second complex. The more enzyme drawn into the second complex, the more pronounced the inhibition becomes.

Thus all these effects of kojic acid on frog tyrosinase can be explained by the mechanism proposed by us previously for the slow inhibition of mushroom tyrosinase by L-mimosine, the pyridone analogue of L-dopa (Cabanes et al 1987a). According to the equations obtained when the kinetic analysis of this mechanism (Scheme 1) is carried out, the slow transition constant k_{-6} can be evaluated:

$$V_s = \frac{k_{-6}}{k_{app}} V_0 \qquad (3)$$

since V_s, k_{app}, and V_0, can be obtained experimentally.

Once k_{-6} and k_{app} are known, k_6 can be evaluated according to equation 3 by means of a double reciprocal plot of $(k_{app} - k_{-6})$ vs concentration on the inhibitor (Fig. 4):

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

A series of straight lines intersecting at a point on the ordinate axis equal to $1/k_6$ was obtained, from which a value of 0·06 s^{-1} was calculated for k_6.

The results obtained in this paper show clearly that kojic acid can be classified as a slow-binding competitive inhibitor of frog epidermis tyrosinase when L-dopa is used as substrate. This is a complex response of tyrosinase 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 concerning the inhibition of tyrosinase by compounds structurally analogous to phenolic substrate, since expressed activity does not remain uniform in the

presence of the inhibitor. Thus, incorrect rate values are obtained if the product increase is followed over a limited time and so different patterns of velocity vs substrate are obtained depending on the level of inhibitor used.

In agreement with the kinetic mechanism for tyrosinase (Cabanes et al 1987b), only the oxy form, of the three different forms of the enzyme, acts on L-tyrosine, hydroxylating it to L-dopa. On the other hand, both oxy and met forms act on L-dopa to give dopaquinone, which evolves to give L-dopa and dopachrome. Thus, the inhibition produced by kojic acid affects both catecholase and cresolase activity through the oxy form. As mentioned previously, cresolase activity shows a complex kinetic response (Cabanes et al 1987b), with a slow transition phase, which basically expresses the accumulation of the L-dopa necessary to maintain this activity. Control of the level of L-dopa produced in these conditions has recently been established as an important mechanism for the regulation of the monophenolase activity of tyrosinase (Garcia-Carmona et al 1987, 1988; Riley 1993).

The presence of a slow transition phase such as the lag period in the cresolase activity of tyrosinase makes it impossible to quantify the kinetic constants of the interaction of kojic acid and the enzyme. However, since the enzymatic intermediaries, oxy and met, are the same in both cresolase and catecholase activity, it might reasonably be assumed that K_d, k_6, and k_{-6} are the same for both activities, since these constants exclusively reflect the presence of the oxy form and kojic acid (Scheme 1).

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