# Effect of Kojic Acid on the Oxidation of *N*-Acetyldopamine by Mushroom Tyrosinase

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Kojic acid inhibited mushroom tyrosinase, as judged by the inhibition it exerted on the rate of oxygen uptaked during *N*-acetyldopamine (NADA) oxidation. Preincubation of mushroom tyrosinase with kojic acid did not result in the inactivation of the enzyme (irreversible loss of activity). Kojic acid had a pronounced effect on the spectrum of product(s) obtained when NADA was oxidized by mushroom tyrosinase. In its absence, NADA quinone ($\lambda_{max} = 390 \pm 10$ nm) was formed; in its presence, a stable final yellow product(s), characterized by a peak at $340 \pm 20$ nm, was obtained. A maximum level of the final yellow product(s) was formed when the ratio between NADA quinone and kojic acid was 1:1. Spectral data suggest that NADA quinone oxidizes kojic acid to the yellow product(s).

**Keywords:** *N-Acetyldopamine (NADA); mushroom tyrosinase; kojic acid inhibition*

## INTRODUCTION

Tyrosinase (monophenol, dihydroxyphenylalanine; oxygen oxidoreductase EC 1.14.18.1) (also known as polyphenol oxidase [PPO], phenolase, catecholase, and cresolase) is a copper-containing enzyme that is widespread in nature.

In insects, tyrosinase plays a key role in cuticular tanning and sclerotization as well as in defense against pathogens (Andersen and Roepstorff, 1982; Andersen et al., 1992; Barrett, 1991; Brunet, 1967; Czapla et al., 1989; Kramer et al., 1991; Peter, 1989; Sugumaran, 1987, 1988; Sugumaran and Lipke, 1983). *N*-Acetylcatecholamines are the major precursors participating in the sclerotization and tanning of insect cuticles (Czapla et al., 1989; Peter, 1989). Cuticular tyrosinase oxidizes *N*-acetylcatecholamines to the corresponding *o*-quinones, which, due to the action of an isomerase, are converted to the corresponding *p*-quinone methides (Saul and Sugumaran, 1989a,b). These quinonid derivatives are highly reactive, and during sclerotization they form adduct(s) or cross-links via covalent bonds between the ring or the side-chain carbon of the *N*-acetylated catecholamine to nucleophillic groups of cuticular proteins (Andersen et al., 1992; Czapla et al., 1989).

The topic of molecular mechanisms for cuticular sclerotization and tanning in insects has been comprehensively reviewed in the literature over the years (Andersen and Roepstoff, 1982; Barrett, 1991; Brunet, 1967; Sugumaran 1987, 1988; Sugumaran and Lipke, 1983); we have referred above only to references pertinent to the present study.

Kojic acid (5-hydroxy-2-(hydroxymethyl)-4*H*-pyran-4-one), a mycotoxin isolated from *Aspergillus flavus* and other species of *Aspergillus*, has insecticidal properties (Beard and Walton, 1969; Wilson, 1971). For example, it inhibits the growth of *Trogoderma* larvae and induces sterility in males and females of this genus (Sehgal, 1976) and inhibits the development of *Musca domestica* (Beard and Walton, 1969), as well as that of *Drosophila melanogaster* (banana fly) (Dobias et al., 1977). Dowd (1988) showed that both kojic acid and fusaric acid synergized the toxicity of naturally occurring mycotoxins as well as allelochemicals and insecticides.

Kojic acid is a good chelator of transition metal ions (Wiley et al., 1942) and a good scavenger of free radicals (Mishima et al., 1988; Niwa and Akamatsu, 1991). It inhibits the tyrosinases of various *Aspergillus* species (Tanaka et al., 1989; Saruno et al., 1979), *Neurospora crassa* (Hider and Lerch, 1989), and mushrooms (Chen et al., 1991a), as well as those of some plants and crustaceans (Chen et al., 1991b). Recently, hemolymph serum tyrosinase from larvae of the noctuid moth *Spodoptera littoralis* was shown to be effectively inhibited by kojic acid when L-DOPA was used as the substrate (Lee and Anstee, 1995).

We have shown recently that kojic acid inhibits effectively the rate of formation of pigmented product(s) and of oxygen uptake when catecholamines such as DL-DOPA, norepinephrine, and dopamine were oxidized by mushroom tyrosinase (Kahn, 1995). We further found that the spectrum of product(s) formed when these catecholamines were oxidized by tyrosinase in the presence of kojic acid was different than that formed in its absence and suggested that a chemical interaction occurs between the *o*-quinones of these catecholamines and kojic acid, probably due to the ability of these *o*-quinones to oxidize kojic acid to a yellow product(s) (Kahn, 1995).

In view of the above, it occurred to us that kojic acid might have the property of an insecticide due to its inhibitory effect on tyrosinase as well as to its ability to interact with *o*-quinones of catecholamines, thus preventing the sclerotization process. It was, therefore, decided to study the effect of kojic acid on the oxidation of *N*-acetyldopamine (NADA) by tyrosinase and to examine the possible interaction of NADA quinone with kojic acid since, as pointed out above, NADA is one of the major catecholamine derivatives participating in insect sclerotization. The results of this study are presented below.

We have used for the present study mushroom tyrosinase rather than tyrosinase isolated from insects since the latter is known to (a) be highly unstable, (b) stick

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to glassware, (c) undergo self-polymerization, and (d) lose its activity (Sugumaran et al., 1995).

## MATERIALS AND METHODS

**Materials.** Mushroom tyrosinase, kojic acid, and NADA were obtained from Sigma. All other chemicals were reagent grade.

**Methods.** The effect of kojic acid on the oxidation of NADA by mushroom tyrosinase was assayed in a total volume of 1.5−3.0 mL, which included NADA, sodium phosphate buffer (pH 6.5), and mushroom tyrosinase in the absence or presence of kojic acid, as specified in the legends to the figures. The experiments were conducted in replicates.

The spectrophotometric assays were conducted using a Varian DMS 90 spectrophotometer equipped with a recorder. The spectra were scanned at the rate of 100 nm/min. The cuvettes were mixed before each scanning.

The polarographic measurements were assayed in a 3 mL reaction mixture, previously saturated with air, in a Yellow Springs biological oxygen monitor (Model 53) equipped with a recorder. Changes in the initial oxygen concentration (percentage) were recorded as a function of time.

## RESULTS

Kojic acid in aqueous solution is colorless and is characterized by a relatively high peak at 217 nm and a relatively low peak at 270 nm.

Control experiments showed that incubation of kojic acid, in the presence of sodium phosphate buffer (pH 6.5) for a period of at least 20 h, did not yield any pigmented product(s). Moreover, the potential ability of kojic acid to inactivate tyrosinase was tested by incubating 200 $\mu$g of tyrosinase in a total volume of 3 mL containing 47 mM sodium phosphate buffer (pH 6.5), in the absence (control) or presence (experimental) of 3.3, 6.6, or 13.3 mM kojic acid for 60 min, dialyzing the samples, and then assaying the remaining *o*-dihydroxyphenolase activity using DL-DOPA as the substrate. Identical activities were found in the control and the experimental samples, indicating that kojic acid does not inactivate tyrosinase, i.e., that preincubation of tyrosinase with kojic acid does not cause an irreversible loss of activity, in agreement with the findings of Chen et al. (1991a).

**Effect of Kojic Acid on the Changes with Time in the Spectrum of Product(s) Obtained When NADA Is Oxidized by Tyrosinase.** According to Saul and Sugumaran (1989a,b) and Sugumaran et al. (1989), NADA is oxidized by mushroom tyrosinase to NADA quinone ($\lambda_{max}$ = 392−400 nm), which can be converted nonenzymatically to NADA quinone methide. The latter is very unstable and undergoes a rapid nonenzymatic hydration to yield *N*-acetylnorepinephrine ($\lambda_{max}$ = 278 nm).

The data in Figure 1 demonstrate that kojic acid has a pronounced effect on the changes with time in the spectrum of product(s) obtained when NADA is acted upon by tyrosinase. In the absence of kojic acid, the NADA quinone formed when NADA is oxidized by tyrosinase is rather stable with time, since its intensity increased for a period of 13 min. However, after 34 min, the spectrum of the product(s) formed became undefined, indicating that NADA quinone was converted to other oxidation product(s) (Figure 1, part A). Under the same conditions but in the presence of kojic acid, a yellow product(s), with a symmetrical peak at 325 nm, was formed within 4 min. The peak at 325 nm then shifted to 340−360 nm, and its intensity increased as a function of time for a period of approximately 14 min. The peak at 340−360 nm remained stable during further incubation for at least 27−30 min (Figure 1, parts B and C). The intensity of the peak at 340−360 nm when the peak became stable was higher in the presence of 0.16 than in the presence of 0.1 mM kojic acid (Figure 1, parts B and C, respectively).

**Effect of Kojic Acid on the Rate of NADA Oxidation by Tyrosinase to Pigmented Product(s) Absorbing at 392 and 340 nm.** In view of the above, we examined the effect of kojic acid on the rate of NADA oxidation by tyrosinase to pigmented product(s) absorbing at both 392 and 340 nm. The data in Figure 2A illustrate the effect of various concentrations of kojic acid on the rate of NADA oxidation to pigmented product(s) absorbing at 392 nm. The rate of NADA oxidation in the absence of kojic acid was rapid and linear initially and then decreased rapidly. The decrease probably represents the conversion of NADA quinone to *N*-acetylnorepinephrine (Sugumaran, 1989a). Under identical conditions but in the presence of kojic acid (0.033−0.26 mM), the rate of formation of pigmented product(s) absorbing at 392 nm was not linear but was characterized by a lag period; the higher the kojic acid concentration, the more pronounced was the lag period and the slower was the maximum absorbance value reached (Figure 2A).

The effect of kojic acid on the rate of NADA oxidation to the yellow product(s) absorbing at 340 nm was also examined. Controls showed that relatively little material absorbing at 340 nm was detected when NADA was oxidized by tyrosinase in the absence of kojic acid, due to the formation of NADA quinone, which has a peak at 392 nm and, therefore, contributes some absorbance at 340 nm (Figure 2B). In the presence of kojic acid, the rate of formation of yellow product(s) absorbing at 340 nm had an initial lag period, followed by a rapid rate of reaction which afterward plateaued (Figure 2B). The higher the kojic acid concentration in the range tested (0.033−0.13 mM), the shorter was the lag period, the faster was the rate of formation of the yellow product(s) after the lag period, and the higher was the absorbance value at 340 nm, at which the plateau occurred (Figure 2B).

The lag period in the formation of the yellow product(s) ($\lambda_{max}$ = 340 nm) (Figure 2B) is probably the time in which some NADA quinone is formed, and the relatively fast rate of the reaction thereafter is probably the time in which kojic acid interacts with NADA quinone to yield the yellow product(s). As soon as the kojic acid in the reaction is depleted, a leveling-off occurs in the rate of formation of the yellow product(s). The interaction between NADA quinone and kojic acid is probably a reaction in which kojic acid is oxidized by the *o*-quinone to yellow product(s) absorbing at 340 nm (see further below).

A look at the data presented in parts A and B of Figure 2 indicates that the inhibition exerted by kojic acid on the rate of formation of NADA quinone ($\lambda_{max}$ = 392 nm) is equivalent to the period in which the formation of the yellow product ($\lambda_{max}$ = 340 nm) occurred. Namely, the inhibition exerted by kojic acid on the rate of NADA oxidation by tyrosinase to the corresponding *o*-quinone ($\lambda_{max}$ = 392 nm) as depicted in Figure 2A is probably due, in part, to the ability of kojic acid to interact with the *o*-quinones formed in the course of the reaction and to yield yellow product(s) ($\lambda_{max}$ = 340 nm).

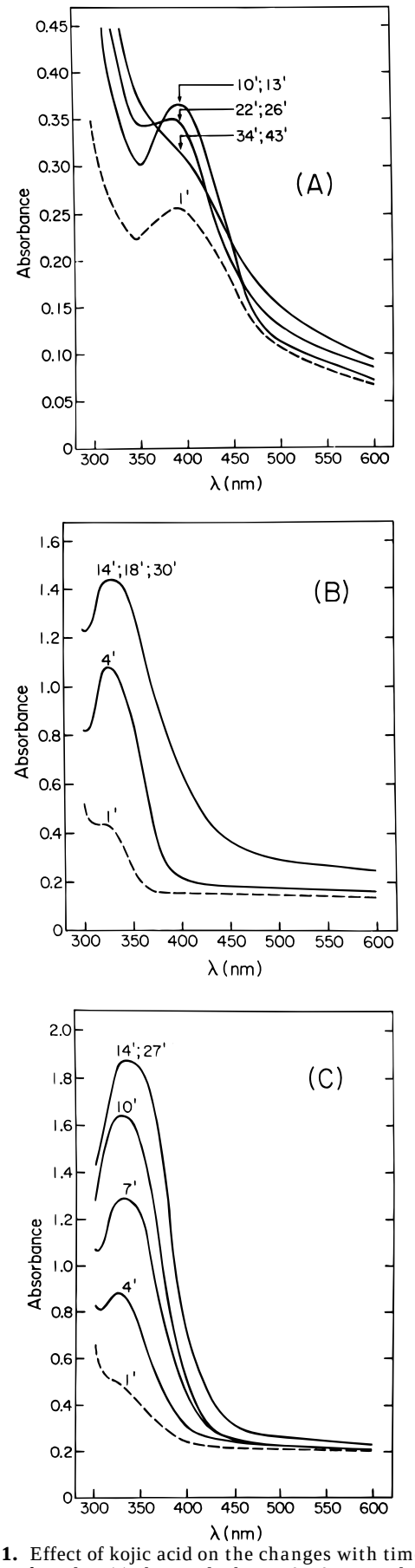


**Figure 1.** Effect of kojic acid on the changes with time in the spectrum of product(s) obtained when NADA is acted upon by tyrosinase. The reaction mixture included, in a total volume of 1.5 mL, 2.6 mM NADA, 26 mM sodium phosphate buffer (pH 6.5), 50 $\mu$g of tyrosinase (added last), and kojic acid as follows: A, none; B, 0.1 mM; and C, 0.16 mM.

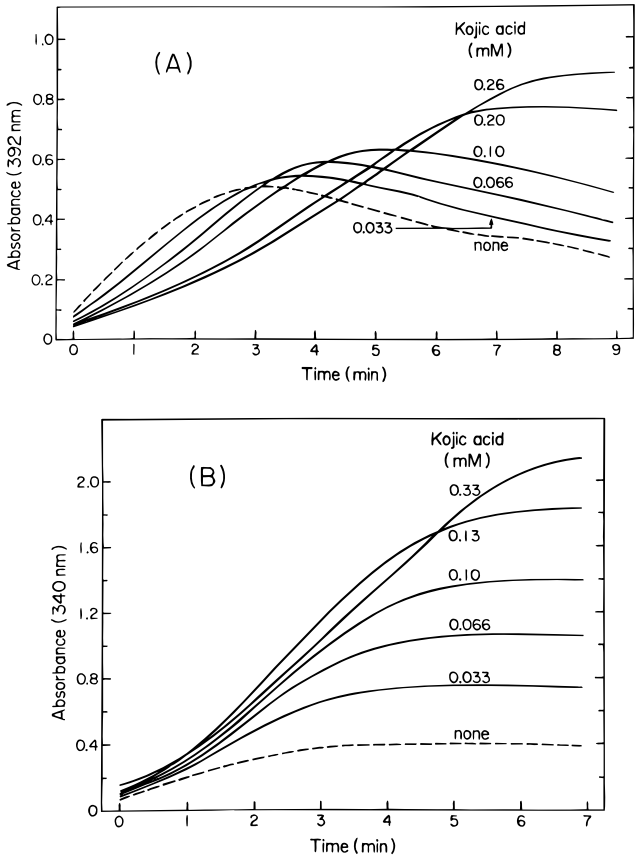


**Figure 2.** Effect of various concentrations of kojic acid on the rate of formation of pigmented product(s) absorbing at 392 and 340 nm when NADA is acted upon by tyrosinase. The reaction mixture included, in a total volume of 3 mL, 3.3 mM NADA, 47 mM sodium phosphate buffer (pH 6.5), kojic acid as indicated, and 20 $\mu$g of tyrosinase (added last). The kinetics of the reaction was followed on identical reaction mixtures at 392 and 340 nm in parts A and B, respectively.

**Effect of Kojic Acid on the Rate of Oxygen Uptake When NADA Is Oxidized by Tyrosinase.** As shown in Figure 3, kojic acid inhibited the rate of oxygen uptake when NADA was acted upon by tyrosinase. In the range tested (0.66−3.3 mM), the higher the kojic acid concentration, the more effective was the inhibition. From a plot of the rate of oxygen uptake vs kojic acid concentration (Figure 3B), it was estimated that 50% inhibition of the reaction occurred at 0.54 mM kojic acid. The finding that kojic acid inhibits the rate of oxygen uptake during NADA oxidation by tyrosinase (Figure 3) can be taken as proof that kojic acid inhibits tyrosinase, probably by binding to Cu(II) at the active site of the enzyme, since kojic acid is a good chelator of Cu(II) (Wiley et al., 1942).

**Effect of Kojic Acid on the Final Pigmented Product(s).** The pigmented product(s) obtained after 20 h incubation of NADA with tyrosinase is referred to as the final pigmented product(s). The effect of various concentrations of kojic acid on the spectrum of the final pigmented product(s) obtained when a fixed concentration of NADA was acted upon by a fixed concentration of tyrosinase was examined in an attempt to determine the ratio of kojic acid:NADA that yields the maximum yellow product(s) ($\lambda_{max} = 340$ nm). The data presented in Figure 4A show that a final pigmented product(s) absorbing at 340 ± 20 nm was formed when kojic acid was included in the reaction mixture but not in its absence. A plot of the maximum absorbance ($\lambda_{max} = 340$ nm) of the final pigmented product(s) obtained from the

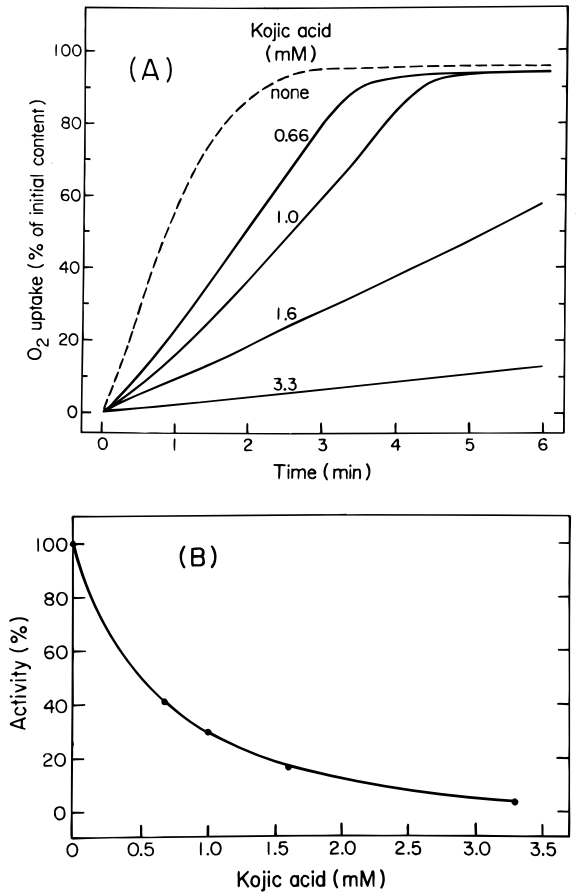


**Figure 3.** Effect of various concentrations of kojic acid on the rate of oxygen uptake when NADA is acted upon by tyrosinase. (A) The reaction mixture included, in a total volume of 3 mL, 2.6 mM NADA, 47 mM sodium phosphate buffer (pH 6.5), 20 $\mu$g of tyrosinase (added last), and kojic acid as indicated. (B) Activities, estimated from the initial linear portions of the kinetic data shown in part A, are plotted as a function of kojic acid concentration. Activity in the absence of kojic acid was considered to be 100%.

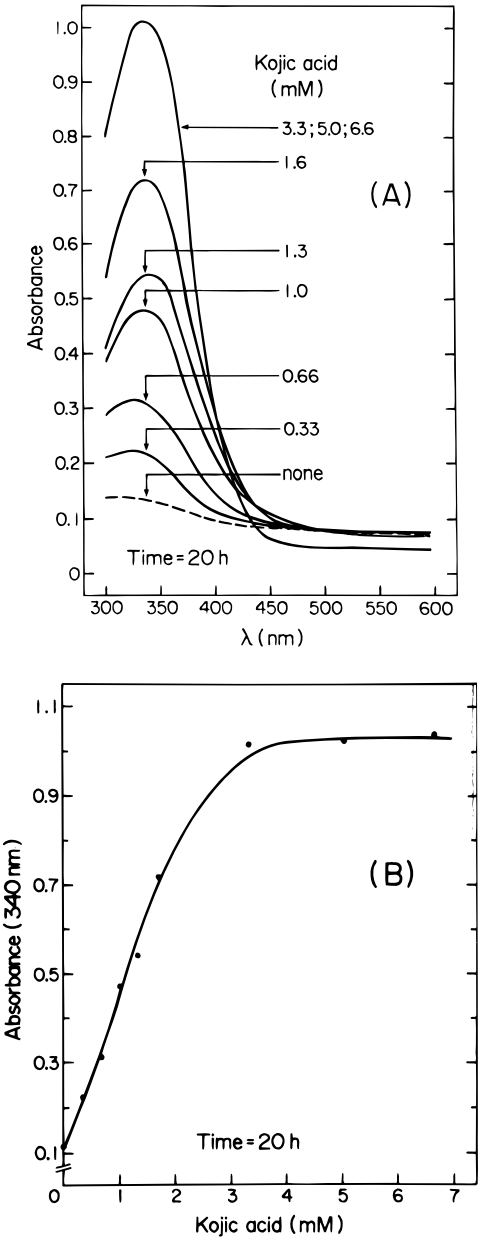


**Figure 4.** Effect of various concentrations of kojic acid on the spectrum of the final pigmented product(s) formed when NADA is acted upon by tyrosinase. The reaction mixture included, in a total volume of 3 mL, 3.3 mM NADA, 47 mM sodium phosphate buffer (pH 6.5), 20 mg of tyrosinase (added last), and kojic acid as indicated. The sample was incubated for 20 h and diluted 1:18 with water before scanning. The maximum absorbance values at 340 nm obtained from the spectral data shown in part A are plotted in part B as a function of various concentrations of kojic acid.

spectral data presented in Figure 4A is presented in part B as a function of kojic acid concentration. As shown in Figure 4B, a linear relationship exists between maximum absorbance value at 340 nm and kojic acid concentration up to 3.3 mM. Kojic acid at concentrations of 3.3 mM or above yielded the same absorbance value at 340 nm.

The reaction mixture used in the experiment presented in Figure 4 had 3.3 mM NADA; therefore, the finding that a plateau in absorbance at 340 nm of the final pigmented product(s) occurred at 3.3 mM kojic acid indicates that a maximum level of the yellow product(s) is formed when the ratio between NADA quinone and kojic acid is 1:1.

The final pigmented product(s) obtained when NADA was oxidized by tyrosinase in the presence of kojic acid, but not in its absence, was fluorescent, as shown in Table 1.

## DISCUSSION

The finding that kojic acid inhibits the rate of oxygen uptake when NADA is oxidized by tyrosinase (Figure 3) indicates that kojic acid inhibits the enzyme *per se*, possibly by chelating copper at the active site of the enzyme, since kojic acid is a good chelator of Cu(II) (Wiley et al., 1942).

The observation that a yellow product(s) ($\lambda_{max}$ = 340 nm) was formed when NADA was oxidized by tyrosinase in the presence of kojic acid, but not in its absence (Figures 1, 2, and 4), can be taken as proof that a chemical interaction occurs between NADA quinone and kojic acid and that it yields the yellow product(s).

We have shown recently that a yellow product(s) is formed when kojic acid interacts with the *o*-quinones of catechol, protocatechuic acid, and 3,4-dihydroxyphenylpropionic acid (Kahn et al., 1995a), the *o*-quinones of DL-DOPA, norepinephrine, and dopamine (Kahn, 1995), as well as the *o*-quinones of 4-methyl gallate and *n*-propyl gallate (Kahn and Zakin, 1995). Moreover, we found that a yellow product(s) is formed when kojic acid

**Table 1. Fluorescence Properties of the Final Pigmented Product(s) Formed When NADA Is Acted upon by Tyrosinase in the Absence and Presence of Kojic Acid[a]**

| | λ (nm) | | |
|---|---|---|---|
| kojic acid concn (mM) | em | exc | $F_{max}$ |
| 0 | 430 | 365 | 6 |
| 1.3 | 410 | 350 | 973 |
| 5.3 | 510 | 350 | 200 |

[a] The reaction mixture included, in a total volume of 3 mL, 2.6 mM NADA, 26 mM sodium phosphate buffer (pH 6.5), 80 μg of tyrosinase, and kojic acid as indicated. The sample was incubated for 48 h prior to determining the fluorescence spectrum.

is acted upon by horseradish peroxidase in the presence of $H_2O_2$ (Kahn et al., 1995b), by hemoglobin in the presence of $H_2O_2$ (Kahn et al., 1995c), and by $NaIO_4$, $Ag_2O$, and $KMnO_4$ (unpublished data). In view of the observation that a yellow product(s) was formed in all the above-mentioned reactions, we proposed that the yellow product(s) was formed as the result of kojic acid oxidation by a certain *o*-quinone, by the HRP/$H_2O_2$ or the Hb/$H_2O_2$ system, and by chemical oxidants (Kahn et al., 1995a,b; Kahn and Zakin, 1995).

By analogy with the above, it is likely that the yellow product(s) formed when NADA is oxidized by tyrosinase in the presence of kojic acid, but not in its absence, is due to the oxidation of kojic acid by NADA quinone. That is, it is probable that the oxidation−reduction potential of kojic acid vs NADA quinone is such that NADA quinone oxidized kojic acid to a yellow product(s). The oxidation of kojic acid to the yellow product(s) probably results in the reduction of NADA quinone to NADA, in agreement with the finding of Chen et al. (1991a), who reported that kojic acid reduces dopaquinone to DOPA.

The results presented in Figure 4B indicate that the interaction between NADA quinone and kojic acid is most effective when the ratio between them is 1:1. We know that the yellow product(s) is fluorescent (Table 1), but we do not have any information regarding the structure of the yellow product(s) formed in the above reactions.

As pointed out in the Introduction, tyrosinase plays a key role in cuticular tanning and sclerotization in insects (Andersen and Roepstorff, 1982; Brunet, 1967; Czapla et al., 1989; Kramer et al., 1991; Peter, 1989; Sugumaran, 1987, 1988), and NADA is one of the major catecholamines participating in the process (Czapla et al., 1989; Peter, 1989; Saul and Sugumaran, 1989). Moreover, kojic acid was reported to inhibit effectively tyrosinase activity from fungi (Chen et al., 1991a; Hider and Lerch, 1989; Saruno et al., 1979; Takana et al., 1989), plants, and crustaceans (Chen et al., 1991a,b), as well as from larvae of the noctuid moth *Spodoptera littoralis* (Lee and Anstee, 1995).

The data presented in this paper show that kojic acid is an effective inhibitor of mushroom tyrosinase when NADA is the substrate and that kojic acid interacts chemically with NADA quinone. Assuming that tyrosinase isolated from various insects would respond to kojic acid as mushroom tyrosinase does, it may be concluded that kojic acid might have an important effect on the endogenous pool of NADA quinone needed for sclerotization in insects by inhibiting tyrosinase activity *per se* and by interacting nonenzymatically with NADA quinone formed enzymatically *in vitro*. Since kojic acid can potentially have an important effect on the availability of NADA quinone for the sclerotization reaction, it is tempting to speculated that it acts as an insecticide, due to its above properties.

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