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Inhibition Mechanism of Kojic Acid on Polyphenol Oxidase†

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The inhibition mode of kojic acid on mushroom, potato, apple, white shrimp, and spiny lobster polyphenol oxidase (PPO) was investigated. Using a polarographic method, kojic acid was shown to inhibit melanosis by interfering with the uptake of O_2 required for enzymatic browning. Spectrophotometric and chromatographic methods demonstrated that kojic acid was capable of reducing *o*-quinones to diphenols to prevent the final pigment (melanin) forming. The preincubation temperature did not significantly affect ($P > 0.05$) PPO inhibition by kojic acid.

INTRODUCTION

The undesirable melanosis (blackening) of agricultural products caused by polyphenol oxidase (PPO, EC 1.14.18.1) has been of great concern to food technologists and processors (Joslyn and Ponting, 1951). The darkening of these products, although in most cases innocuous to consumers, renders the products less acceptable.

Enzymatic browning of plant products and crustaceans due to PPO activity has been extensively studied (Bailey et al., 1960a,b; Ferrer et al., 1989a; Flurkey and Jen, 1978; Macrae and Duggleby, 1968; Ogawa et al., 1984; Walker, 1962, 1964). PPO is a copper-containing enzyme capable of hydroxylating monophenols to diphenols or oxidizing diphenols to benzoquinones. However, the capability of PPO in hydroxylating monophenols to diphenols varies with enzyme sources. Oxygen, PPO, and phenolic substrates are involved in the enzymatic browning reaction. Winkler et al. (1981) proposed the reaction by PPO was through the following sequences: met-PPO [Cu(II)Cu(II)] is first reduced by reductants, such as ascorbic acid, to deoxy-PPO [Cu(I)Cu(I)], which then interacts with oxygen, forming oxy-PPO [Cu(II)Cu(II)O_2] capable of catalyzing mono- or diphenols. Many compounds have been reported for the inhibition of melanosis through the following mechanisms: by inhibiting PPO (Kahn and Andrawis, 1986; Sayavedra-Soto and Montgomery, 1986); by reducing *o*-quinones to diphenols (Golan-Goldhirsh et al., 1984) or Cu^{2+} to Cu^{+} (Hsu et al., 1988); by interacting with the formation of *o*-quinone products (Embs and Markakis, 1965; Ferrer et al., 1989b); or by decreasing the uptake of O_2 for the reaction (Embs and Markakis, 1965).

Kojic acid [5-hydroxy-2-(hydroxymethyl)-γ-pyrone], a fungal metabolite, produced by many species of *Aspergillus* and *Penicillium* (Kinosita and Shikata, 1964) has been reported for its inhibitory effect on mushroom PPO (Saruno et al., 1979). Recently, Chen et al. (1991) showed the inhibitory effect of kojic acid on mushroom, plant (potato and apple), and crustacean (white shrimp, grass prawn, and Florida spiny lobster) PPO. These workers observed competitive inhibition of potato and apple PPO and mixed-type inhibition of crustacean PPOs by kojic acid. A competitive and mixed-type inhibition occurred for mushroom PPO depending on the phenolic substrates studied. Since information concerning the mode of action of kojic acid on PPO was still limited, this study was undertaken to elucidate its action on some of these (mushroom, potato, apple, Florida spiny lobster, and white shrimp) PPOs.

MATERIALS AND METHODS

To maintain uniformity and clarity, the enzyme used in this study is abbreviated PPO instead of tyrosinase, phenolase, monophenol oxidase, or cresolase. Mushroom tyrosinase (PPO) with activity of 2200 units/mg of solid was purchased from Sigma Chemical Co. (St. Louis, MO). The other four PPO enzymes (Russet potato tuber, Red Delicious apple, white shrimp, and Florida spiny lobster) were extracted and purified according to the procedures of Chen et al. (1991). Potato PPO activity (10 900 units/mg of protein) was determined at 25 °C for 5 min by adding 0.1 mL of enzyme to 2.9 mL of 0.97 mM chlorogenic acid in 1 mM potassium phosphate buffer (pH 7.0). Maximal initial velocity for quinone formation was monitored at 395 nm using a DU-7 spectrophotometer (Beckman Instruments Inc., Irvine, CA). One

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unit of PPO activity was defined as an increase in absorbance of 0.001/min at 395 nm and 25 °C.

Apple PPO activity (97 400 units/mg of protein) was measured at 30 °C by adding 0.2 mL of enzyme to 1.8 mL of 0.05 M 4-methylcatechol in 0.1 M sodium phosphate buffer (pH 6.0). Maximal initial velocity for quinone formation was determined at 395 nm. One unit of PPO activity was defined as an increase in absorbance of 0.001/min at 395 nm and 30 °C.

The activities of spiny lobster PPO (7000 units/mg of protein) and white shrimp PPO (5400 unit/mg of protein) were measured by adding 0.1 mL of enzyme to 1.4 mL of 10 mM DL-Dopa in 0.05 M sodium phosphate buffer (pH 6.5), and the reaction was monitored for 5 min at 25 and 40 °C, respectively. One unit of PPO activity was defined as an increase in absorbance of 0.001/min at 475 nm at 25 or 40 °C.

Effect of Preincubation Temperature on PPO Inhibition by Kojic Acid. Reaction mixture containing 0.95 mL of 0.13 mM kojic acid in 0.05 M sodium phosphate buffer (pH 6.5) and 50 μL of mushroom PPO (0.5 mg/mL and 2200 units/mg) or lobster PPO (7000 units/mg of protein) was incubated for 15 min in a cuvette at 0, 25, or 37 °C. After the mixture was equilibrated to ambient temperature, 0.5 mL of 10 mM DL-Dopa in 0.05 M sodium phosphate buffer (pH 6.5) was added and the reaction monitored at 475 nm (25 °C) for 5 min. Controls were run consecutively with kojic acid being replaced by phosphate buffer. Percentage inhibition (I) was expressed as $[(A - A^*)/A] \times 100$, where A and A^* were enzyme activities in the absence and presence of the inhibitor (kojic acid), respectively (Chen et al., 1991).

Similarly, 50 μL of potato PPO (10 900 units/mg of protein) was added to a cuvette containing 0.5 mL of 1 mM sodium phosphate buffer (pH 7.0) and 0.45 mL of 0.56 mM kojic acid. After preincubation at 0, 25, or 37 °C for 15 min and equilibration back to ambient temperature, 0.5 mL of 20 mM catechol in 1 mM phosphate buffer (pH 7.0) was added. The reaction was monitored at 395 nm (25 °C) for 5 min. Percentage inhibition was determined as described above. For apple PPO, the preincubation conditions were performed identically to that of potato PPO except the reaction was carried out at 30 °C by adding 0.5 mL of 20 mM catechol in 0.1 M sodium phosphate buffer (pH 6.0) to the cuvette containing 50 μL of PPO (97 400 units/mg of protein), 0.5 mL of 0.1 M sodium phosphate buffer (pH 6.0), and 0.45 mL of 0.56 mM kojic acid.

Effect of Kojic Acid on the Hydroxylation Capability of PPO. A 0.1-mL kojic acid solution (0.35–5.63 mM) in 0.05 M sodium phosphate buffer (pH 6.5) was incubated with 60 μL of mushroom PPO (0.5 mg/mL) in water at ambient temperature for 15 min. Following incubation, 0.84 mL of 1 mM L-tyrosine in the same buffer was added, and the reaction was monitored at 475 nm (25 °C) for 90 min. Controls were run simultaneously with kojic acid being replaced by buffer.

Effect of Kojic Acid on O_2 Uptake by PPO Reaction. The effect of kojic acid on the inhibition of PPO was also conducted using a polarographic method. Apple PPO (97 400 units/mg of protein) (0.1 mL) was added with 0.1 mL of kojic acid solution (0.28, 0.56, or 1.06 mM) in 0.1 M sodium phosphate buffer (pH 6.0) into the sample chamber of a biological oxygen monitor (YSI Model 53, Yellow Springs Instrument Co., Yellow Springs, OH). Following preincubation at ambient temperature for 30 min, 2.9 mL of 0.1 M 4-methylcatechol or chlorogenic acid in 0.1 M sodium phosphate buffer (pH 6.0) was added. The reaction was allowed to proceed at ambient temperature for 10 min, and the consumption rate of O_2 was monitored using a Brinkmann Servogor 210 recorder at a chart speed of 1 cm/min. The rate of O_2 consumption was determined amperometrically against time. Control had phosphate buffer substituted for kojic acid. Background O_2 consumption determinations for enzyme, kojic acid, or substrate alone and the combination of kojic acid plus substrate or kojic acid plus PPO were also carried out.

A similar study was conducted for lobster PPO in which 2.9 mL of 10 mM DL-Dopa or catechol in 0.05 M sodium phosphate buffer (pH 6.5) was used as substrate. Percentage of inhibition (I) on the rate of O_2 consumption was defined as $[(U - U^*)/U] \times 100$, where U and U^* were the rates of O_2 consumption in the absence or presence of kojic acid, respectively.

Effect of Kojic Acid on the Reduction of Cu^{2+}. The method of Andrawis and Kahn (1990) was adopted to study whether kojic acid possessed any reducing capability from the reducing of cupric copper to cuprous copper. After a 10-min incubation at ambient temperature of the mixture containing 1 mL of kojic acid in 0.05 M sodiun phosphate buffer (pH 7.0) and 0.5 mL of 0.4 mM cupric sulfate (Fisher) in the same buffer, a 0.5-mL aliquot of 4 mM aqueous bathocuproinedisulfonic acid was added. Since bathocuproinedisulfonic acid could interact with Cu^+ to form a red-color complex having an optimal absorption at 483 nm (Blair and Diehl, 1961), the reducing ability of kojic acid can be determined from the measurement of the absorbance at this wavelength using a DU-40 spectrophotometer (Beckman) of the reaction following incubation at ambient temperature for 20 min. The final concentration of kojic acid in the mixture was 0.02–1.40 mM. Controls had phosphate buffer (1 mL) substituted for kojic acid.

Effect of Kojic Acid on Quinone Products. A reaction mixture containing 1.2 mL of 10 mM DL-Dopa and 0.8 mL of mushroom PPO (0.125 mg/mL) in 0.05 M sodium phosphate buffer (pH 6.5) was incubated at ambient temperature for 30 min. Following red color development due to dopaquinone formation, the mixture was scanned from 220 to 700 nm using a spectrophotometer. The effect of kojic acid on dopaquinone was also studied by adding 0.6 mL of 5.63 mM kojic acid in 0.05 M sodium phosphate buffer (pH 6.5) to a mixture containing 0.2 mL of mushroom PPO (0.5 mg/mL) and 1.2 mL of 10 mM DL-Dopa. Following incubation at ambient temperature for 30 min, the solution was scanned as previously described. An identical study was also conducted on spiny lobster PPO.

The effect of kojic acid on dopaquinone formation was further investigated using thin-layer chromatography. One milliliter of mushroom PPO (0.5 mg/mL) was mixed with 1.0 mL of 5 mM DL-Dopa in 0.05 M sodium phosphate buffer (pH 6.5), and the reaction was allowed to proceed at ambient temperature for 2 h. Following the reaction, 0.5 mL of kojic acid (1 mg/mL) in 0.05 M sodium phosphate buffer (pH 6.5) or the same phosphate buffer was respectively added to a 0.5-mL aliquot of reaction mixture, and the incubation was held at ambient temperature. After 2 h, 80-μL aliquots were spotted on TLC plates (20 × 20 cm Redi/plt Sil, Gel G, Fisher) and the plates developed using a butanol–acetic acid–water (4:1:5 v/v) solvent system. An equivalent volume of 10 mM DL-Dopa in 0.05 M sodium phosphate buffer (pH 6.5) was used as the TLC standard. The chromatographic pattern was examined, and the R_f value for each compound was determined following the spraying of the plate with a ninhydrin reagent (Sigma).

Enzymatic Activities of Kojic Acid Treated PPO. Two milliliters of mushroom (2200 unit/mg of solid), white shrimp (5400 units/mg of protein), and lobster PPO (7000 units/mg of protein) were individually incubated with 0.5 mL of kojic acid (0.56 or 1.12 mM) in 0.05 M sodium phosphate buffer (pH 6.5) in a Spectra/Por membrane (Spectrum Medical Industries Inc., Los Angeles, CA) with molecular weight cutoff of 12 000–14 000 at ambient temperature for 30 min. The chamber containing the enzyme–kojic acid mixture was then dialyzed overnight (4 °C) against three changes of 2 L of phosphate buffer. For the control sample, kojic acid was replaced with an equivalent volume of buffer. Studies on potato and apple PPO were similarly conducted except kojic acid at 1.12 or 5.60 mM was used, respectively. The enzyme activity of mushroom, white shrimp, and lobster PPO was respectively determined by adding 60 μL of PPO to the cuvette containing 840 μL of 10 mM DL-Dopa in 0.05 M sodium phosphate buffer (pH 6.5). The reaction was monitored for 5 min at 475 nm and 25 °C for mushroom and lobster PPO and at 40 °C for white shrimp PPO. For potato and apple PPO, the activity was determined by adding 60 μL of enzyme to the cuvette containing 840 μL of 10 mM catechol in 1 mM sodium phosphate buffer (pH 7.0) or 0.1 M sodium phosphate buffer (pH 6.0), respectively. The reaction was monitored for 5 min at 395 nm and 25 °C for potato PPO and 30 °C for apple PPO.

Kojic acid in the enzyme preparations was quantified using the method of Bentley (1957) with slight modification. Five hundred microliter of enzyme preparation was incubated with 1.5 mL of $FeCl_3$ solution at ambient temperature for 40 min, and

Table I. Effect of Different Preincubation Temperatures on the Inhibition of Various Polyphenol Oxidases by Kojic Acid

	% inhibition		
enzyme source[a]	0 °C	25 °C	37 °C
mushroom	78.5[b]	77.3	75.1
potato	26.2	25.5	25.4
apple	45.6	41.8	41.1
lobster	78.1	77.0	76.8

[a] In the absence of kojic acid, the ΔA_{475nm}/min for mushroom PPO and spiny lobster PPO was 0.137 and 0.027, respectively; the ΔA_{395nm}/min for potato and apple PPO was 0.146 and 0.059, respectively. Final concentration of kojic acid in mushroom PPO and lobster PPO assay systems was 84 μM, while that in potato and apple PPO systems was 168 mM. [b] Values within the same row are not significantly different ($P > 0.05$) from each other.

the absorbance of the reaction mixture was measured at 505 nm. Kojic acid at various concentrations (10–250 μg/mL) was used as standard and distilled, deionized water as the blank.

Statistical Analysis. Statistical analysis was carried out using a PC SAS package (SAS, 1985). Duncan's multiple range test ($P = 0.05$) was performed to determine any significant difference among various treatments. Unless otherwise specified, all the experiments conducted in this study were carried out two times in triplicate.

RESULTS AND DISCUSSION

Effect of Preincubation Temperature on PPO Inhibition by Kojic Acid. Kojic acid was shown to inhibit crustacean PPO activities (Chen et al., 1991). It can be used as a potential substitute for bisulfite compounds for shrimp dipping at refrigerated temperature to inhibit enzymatic browning of the product.

Different preincubation temperatures did not affect the inhibitory effect of kojic acid on the activity of various PPOs (Table I). More than 75% inhibition was observed with mushroom PPO and lobster PPO when 0.28 mM kojic acid was introduced into the assay mixture containing DL-Dopa and enzyme. However, only 25–26% and 41–46% of the potato and apple PPO activities were inhibited, respectively, when 0.56 mM kojic acid was added. Although preincubation at 0 °C usually gave a higher percentage of inhibition than at higher temperatures, the difference was insignificant ($P > 0.05$). Preincubation at 37 °C for 5, 10, 15, or 30 min did not significantly affect the enzyme activity (data not shown). Thus, kojic acid can be potentially used as an enzymatic browning inhibitor at either refrigeration or ambient temperature.

Effect of Kojic Acid on the Hydroxylation Capability of PPO. The lag period for hydroxylation of monohydroxyphenol by PPO increased with increasing concentrations of kojic acid (Figure 1). Kojic acid at higher concentrations was also shown to have a profound inhibitory effect on the oxidation of DL-Dopa by PPO (Chen et al., 1991). Thus, kojic acid did not behave as reducing agents, such as ascorbate, hydroquinone, H_2O_2, and NH_2OH, that were reported to be capable of reducing the lag period of hydroxylation of monohydroxyphenol when added at small concentrations (Kahn, 1983; Kahn and Andrawis, 1986; Sato, 1969; Vaughan and Butt, 1970).

Effect of Kojic Acid on O_2 Uptake by PPO Reaction. O_2 consumption did not take place with the presence of kojic acid, the substrates (4-methylcatechol and chlorogenic acid for apple PPO and DL-Dopa and catechol for lobster PPO), or the kojic acid–substrate mixtures (data not shown). When PPO was added to the mixture containing substrate and buffer, O_2 consumption occurred immediately. Although O_2 uptake by the PPO–substrate mixture still took place when kojic acid was added, the

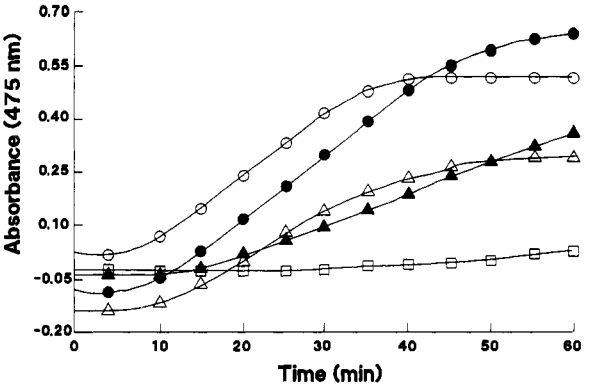

Figure 1. Effect of kojic acid on the hydroxylation of monohydroxyphenol by mushroom PPO. Kojic acid (0.1 mL) of different concentrations (●, 0.35; △, 1.41; ▲, 2.81; and □, 5.63 mM) was incubated with 60 μL of mushroom PPO (0.5 mg/mL) at ambient temperature for 15 min followed by the addition of 0.84 mL of 1 mM L-tyrosine. The reaction was monitored at 475 nm (25 °C) for 90 min. Control (○) was run similarly except that kojic acid was replaced by an equivalent volume of phosphate buffer.

Table II. Inhibitory Effect of Kojic Acid on the Consumption of O_2 by Polyphenol Oxidase

	% inhibition			
	lobster PPO		apple PPO	
kojic acid, mM	DL-Dopa	catechol	4-methyl-catechol	chlorogenic acid
0	0	0	0	0
0.28	50.2	47.4	ND[a]	ND
0.56	60.3	65.0	36.7	24.8
1.06	80.3	77.5	50.9	41.9
1.41	ND	ND	55.4	52.1

[a] ND, not determined.

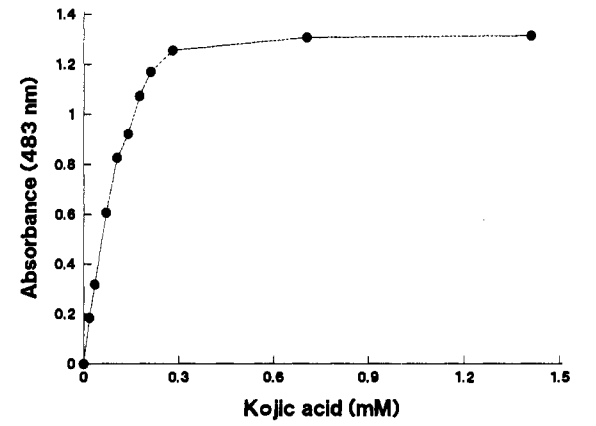

Figure 2. Capability of kojic acid on the reduction of Cu^{2+} to Cu^{+} in a model reaction mixture containing 1 mL of kojic acid (0.04–2.81 mM), 0.5 mL of 0.4 mM $CuSO_4$, and 0.5 mL of 4 mM bathocuproinedisulfonic acid. The mixture was incubated at ambient temperature for 20 min followed by absorbance measurement at 483 nm.

percentage of O_2 consumption in these mixtures decreased with increasing concentration of kojic acid (Table II). For example, O_2 consumption for the oxidation of DL-Dopa by lobster PPO in the presence of 0.56 and 1.06 mM kojic acid was inhibited by 60.3 and 80.3%, respectively.

Effect of Kojic Acid on the Reduction of Cu^{2+}. The absorbance of the reaction mixture increases with increasing concentrations of kojic acid and then reaches a plateau when kojic acid exceeds 0.28 mM (Figure 2). On the basis of molar extinction coefficient for the Cu^{+}–bathocuproinedisulfonate complex (Blair and Diehl, 1961), all of the Cu^{2+} present in the reaction mixture of this model

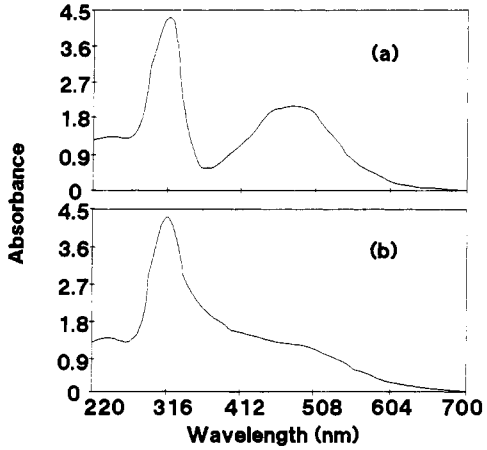

Figure 3. Change of absorption spectra of dopaquinone mixture due to the addition of kojic acid: (a) dopaquinone mixture produced from the action of mushroom PPO on DL-Dopa only; (b) dopaquinone mixture plus kojic acid.

system was reduced to Cu^+ when kojic acid was added at greater than 0.28 mM. Data obtained from this study demonstrate that kojic acid possesses reducing capability.

Effect of Kojic Acid on Quinone Products. Spectrophotometric scanning of the product generated from the reaction of DL-Dopa and mushroom PPO revealed two distinct absorption peaks at 316 and 480 nm (Figure 3a). The addition of kojic acid to this solution caused the change of the color from red-brown to violet and the disappearance of the 480-nm peak which represents dopaquinone (Figure 3b) (Fling et al., 1963). Similar phenomena occured when kojic acid was added to the reaction mixture containing lobster PPO and DL-Dopa (data not shown). Thus, the formation of dopaquinone from DL-Dopa through the action of PPO was affected in the presence of kojic acid.

This finding was further verified from the TLC analyses of the DL-Dopa standard and the reaction mixture containing kojic acid. A reddish purple spot with a R_f value of 0.76 was detected for these two samples on the TLC plate following spraying with a ninhydrin reagent. However, no such reddish purple spot was detected on the plate for the control sample that contained no kojic acid.

The kojic acid effect on the quinone products formed by the action of PPO on DL-Dopa is attributed to the reduction of dopaquinones to diphenols (DL-Dopa). Many reagents including cysteine, bisulfite, and ascorbic acid are known to retard enzymatic browning through this mechanism (Eskin et al., 1971; Synge, 1975; Whitaker, 1972).

Enzymatic Activities of Kojic Acid Treated PPO. The dialysate of the control sample and kojic acid treated PPO preparations showed an equivalent volume. Data listed on Table III show that there was no significant difference ($P > 0.05$) in the enzyme activity between the control sample and kojic acid treated PPO following dialysis against phosphate buffer. The restoration of enzymatic activity of kojic acid treated PPO was attributed to the removal of kojic acid from enzyme solution; the failure to detect kojic acid residue in kojic acid treated enzyme preparations following dialysis verified this assumption. A similar approach employing gel filtration (Sephadex G-100, Pharmacia) chromatography also demonstrated that the enzyme activity was restored after the removal of kojic acid from the PPO. These results thus indirectly suggested that kojic acid did not bind irreversibly to the enzyme and its inhibition on PPO was reversible. Such an inhibitory characteristic is in contrast to that of sulfite and its derivatives (Sayavedra-Soto and Montgomery, 1986; Ferrer et al., 1989b).

Table III. Enzymatic Activity of Kojic Acid Treated Polyphenol Oxidase[a]

		kojic acid, mM		
enzyme	control	0.56	1.12	5.60
mushroom[b]	0.80[d]	0.80	0.79	ND[e]
lobster[b]	0.124	0.118	0.120	ND
white shrimp[b]	0.032	0.031	0.032	ND
potato[c]	0.059	ND	0.060	0.061
apple[c]	0.183	ND	0.188	0.187

[a] Enzyme was incubated with kojic acid for 30 min followed dialysis against phosphate buffer. [b] DL-Dopa was used as substrate. The activity was determined as ΔA_{475nm}/min. [c] Catechol was used as substrate. The activity was determined as ΔA_{395nm}/min. [d] Result was an average of three observations. Values within the same row were not significantly different ($P > 0.05$) from each other. [e] ND, not determined.

CONCLUSION

The inhibitory mode of kojic acid on the various polyphenol oxidases is thus through the following actions: (1) by interfering with the uptake of O_2 required for the enzymatic reaction as demonstrated from the polarographic study; (2) by reducing quinone compounds to diphenols to prevent melanin formation via polymerization as evidenced by analyses using spectrophotometric and chromatographic methods; and/or (3) by combination of the above two actions.

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