Inhibitory Effect of Kojic Acid on Some Plant and Crustacean Polyphenol Oxidases†

Jon S. Chen, Cheng-i Wei,* Rosa S. Rolle, W. Steven Otwell, Murat O. Balaban, and Marty R. Marshall*

Food Science and Human Nutrition Department, University of Florida, Gainesville, Florida 32611-0163

Kojic acid exhibited a competitive inhibition for the oxidation of chlorogenic acid and catechol by potato polyphenol oxidase (PPO) and of 4-methylcatechol and chlorogenic acid by apple PPO. This compound showed a mixed-type inhibition for white shrimp, grass prawn, and lobster PPO when DL-β-3,4-dihydroxyphenylalanine (DL-Dopa) and catechol were used as substrates but a mixed-type and a competitive inhibition for mushroom PPO when DL-Dopa and L-tyrosine were used, respectively. Potato and apple PPOs were shown to have much higher affinities for Dopa than for chlorogenic acid, 4-methylcatechol, and catechol. These two PPOs also had a higher affinity for Dopa than the other four PPOs. Kojic acid is thus an effective inhibitor of PPO on the oxidation of Dopa.

INTRODUCTION

Unfavorable darkening of many food products resulting from enzymatic oxidation of phenols to *o*-quinones by polyphenol oxidase (PPO, E.C. 1.14.18.1) has been of great concern to food technologists and processors (Joslyn and Ponting, 1951). The darkening of food products, although innocuous to consumers, causes a decrease in market value and economic loss because it connotes spoilage.

Enzymatic browning of plant products and crustaceans due to PPO has been widely studied (Bailey et al., 1960a,b; Ferrer et al., 1989a; Flurkey and Jen, 1978; Harel et al., 1966; Ogawa et al., 1984; Walker, 1962, 1964). Compounds capable of inhibiting melanosis in these products through the interference of PPO-mediated reactions or through the reduction of *o*-quinones to diphenols have been identified (Bailey and Fieger, 1954; Ferrer et al., 1989b; Golan-Goldhirsh and Whitaker, 1984; Harel et al., 1967; Madero and Finne, 1982; Palmer and Roberts, 1967; Robb et al., 1966; Sayavedra-Soto and Montgomery, 1986; Wagner and Finne, 1984; Walker, 1975, 1976). However, the number of chemicals that can actually be used in food systems to inhibit melanosis is limited due to off-flavors, off-odors, toxicity, and economic feasibility (Eskin et al., 1971).

Sulfiting agents have been widely used to prevent melanosis in agricultural and seafood products. Due to health concerns, the use of sulfiting agents as food additives is being re-evaluated by the FDA and, in some products, banned for use (Lecos, 1986). It has been necessary to search for alternatives that show effective inhibitory effect on melanosis but are devoid of health concerns to consumers.

Kojic acid [5-hydroxy-2-(hydroxymethyl)-γ-pyrone], a fungal metabolite produced by many species of *Aspergillus* and *Penicillium* (Kinosita and Shikata, 1964; Parrish et al., 1966), was shown to inhibit mushroom tyrosinase activity and melanosis in pink shrimp (Saruno et al., 1979; Applewhite et al., 1990). Kojic acid is mixed with ascorbic acid and citric acid to constitute a Japanese product for inhibiting tyrosinase in foods. Since only limited information was available on the inhibitory effect of kojic acid on PPO, this study was undertaken to investigate the inhibitory activity of this compound on various plant (mushroom, potato, and apple) and crustacean (white shrimp, Florida spiny lobster, and grass prawn) PPO and to elaborate on mechanisms involved.

* Correspondence should be addressed to Dr. Wei or Dr. Marshall.

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MATERIALS AND METHODS

Mushroom tyrosinase with an activity of 2200 units/mg of solid was purchased from Sigma Chemical Co. (St. Louis, MO). Russet potatoes and Red Delicious apples were purchased from a local supermarket. Fresh Florida spiny lobster (*Panulirus argus*) tails were obtained from Dr. R. A. Gleeson of the Whitney Marine Laboratory, Marineland, FL; frozen grass prawn (*Penaeus monodon*) was from Dr. J. S. Yang of the Food Industry Research and Development Institute, Hsinchu, Taiwan, Republic of China. Fresh, nonsulfited white shrimp (*Penaeus setiferus*) was obtained from a local seafood store.

Extraction and Purification of Potato PPO. The method of Patil and Zucker (1965) with modifications was used. After ammonium sulfate precipitation and dialysis, crude PPO preparation was subjected to chromatography with a DEAE-cellulose (0.95 mequiv/g, Sigma) column (40 cm length × 26 mm i.d., K 26/40, Pharmacia Fine Chemicals) pre-equilibrated with 1.0 mM potassium phosphate buffer (pH 7.0). Following removal of the unbound phenolic compounds and proteins with 250 mL of 1 mM phosphate buffer (pH 7.0), PPO was eluted by using a linear gradient (0–1.0 M) of NaCl in 1.0 mM potassium phosphate buffer at 24 mL/h. Four-milliliter fractions were collected, and protein was monitored by spectrophotometry at 280 nm. Fractions showing PPO activity were pooled and concentrated via ultrafiltration using an Amicon stirred cell (Model 8050, Amicon Co., Danvers, MA) fitted with an Amicon YM 10 filter.

The partially purified enzyme preparation was loaded onto a Sephadex G-100 (Pharmacia) gel filtration column (Pharmacia K 26/40) pre-equilibrated with 1.0 mM potassium phosphate buffer (pH 7.0) and then eluted at 4 °C with 400 mL of the same buffer at 24 mL/h. The 4-mL fractions showing PPO activity were pooled and concentrated via ultrafiltration. Concentrated samples were dialyzed at 4 °C overnight against three changes of 2 L of elution buffer.

Extraction and Purification of Apple PPO. The method of Stelzig et al. (1972) with modifications was followed. Crude apple PPO after partial purification and dialysis against H_2O was loaded onto an HT hydroxylapatite (Bio-Rad) column (K 26/40). The enzyme was desorbed from the gel by using 250 mL of 0.005–0.3 M (linear gradient) sodium phosphate buffer (pH 7.6) containing 5% ammonium sulfate at 24 mL/h. Four-mil-

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liter fractions were collected, and fractions showing PPO activity were pooled and dialyzed overnight (4 °C) against two changes of 4 L of H_2O. The dialysate was concentrated via ultrafiltration.

Extraction and Purification of White Shrimp and Grass Prawn PPO. The methods of Chen et al. (1991) and Rolle et al. (1991) with slight modification were followed to purify white shrimp and grass prawn PPO, respectively. The cephalothorax powder was suspended in 3 volumes (w/v) of 0.05 M sodium phosphate buffer (pH 7.2) containing 1 M NaCl (extraction buffer) and 0.2% (v/v) Brij 35 (Fisher Scientific Co., Orlando, FL) and stirred at 4 °C for 3 h. Following centrifugation at 23000*g* (4 °C) for 30 min, the supernatant was fractionated with ammonium sulfate between 0 and 40% saturation; protein precipitate was collected by centrifugation at 23500*g* at 4 °C for 30 min.

For white shrimp, the precipitate was dissolved in 0.05 M phosphate buffer (pH 7.2) and dialyzed at 4 °C overnight against three changes of 4 L of the same buffer (pH 7.2). The dialyzed PPO was loaded onto a DEAE-cellulose (0.95 mequiv/g) column (K 26/40) pre-equilibrated with 0.05 M phosphate buffer (pH 7.2). Following desorbing of the unbound phenolic compounds and proteins with 60 mL of the same buffer at 0.2 mL/min, PPO was eluted by using 300 mL 0.05 M sodium phosphate buffer (pH 7.2) containing a linear gradient (0–1.0 M) of NaCl. Those 3-mL fractions possessing PPO activity were pooled and concentrated via ultrafiltration.

The concentrated PPO was loaded onto a Sephadex G-100 gel column (K 26/40) pre-equilibrated with 0.05 M sodium phosphate buffer (pH 7.2); PPO was eluted with 300 mL of the same buffer at 0.15 mL/min. Again, the 3-mL fractions showing PPO activity were pooled and concentrated via ultrafiltration. Concentrated PPO was then dialyzed at 4 °C overnight against three changes of 2 L of H_2O.

For grass prawn, the precipitate was resuspended in extraction buffer containing 40% ammonium sulfate. After homogenization using a Dounce manual tissue grinder (Wheaton, Millville, NJ), the sample was centrifuged at 23500*g* (4 °C) for 20 min. The precipitate was homogenized in extraction buffer and centrifuged as previously described. The resulting precipitate was homogenized in extraction buffer and then subjected to high-performance hydrophobic interaction chromatography at 4 °C using a preparative Phenyl-Sepharose CL-4B (Sigma) column (K 16/40) pre-equilibrated with extraction buffer.

PPO was eluted with a stepwise gradient of elution buffer [100% extraction buffer (9 mL), 50% extraction buffer in water (24 mL), and 10% extraction buffer in water (24 mL)], 50% ethylene glycol (12 mL), and then distilled water (150 mL) at a flow of 0.2 mL/min. Four-milliliter fractions were collected, and fractions exhibiting PPO activity were pooled and concentrated via ultrafiltration.

Extraction and Purification of Lobster PPO. The modified procedures of Simpson et al. (1987) were followed. Crude lobster PPO (1 mL), after dialysis at 4 °C against three changes of 4 L of 0.05 M sodium phosphate buffer (pH 6.5), was applied to nondenaturing preparative polyacrylamide gel electrophoresis (PAGE) tubes (12 cm length × 14 mm i.d.) containing 5% acrylamide gel and run at a constant current of 10 mA/tube. PPO was visualized by using a specific enzyme–substrate staining method (Constantinides and Bedford, 1967) using 10 mM DL-β-3,4-dihydroxyphenylalanine (DL-Dopa) as substrate. Gels containing PPO were sectioned and homogenized in 0.05 M sodium phosphate buffer (pH 6.5) utilizing a Dounce tissue grinder. Following filtration through a Whatman No. 4 filter paper, the filtrate was concentrated via ultrafiltration. The PPO was further purified by subjecting the concentrated filtrate to electrophoresis using 7.5% acrylamide gel.

Protein Quantitation and Enzyme Purity Determination. Protein content of all preparations was quantitated by using the Bio-Rad protein assay kit with bovine serum albumin (Sigma) as standard. Enzyme purity was examined by using a minigel system (Mini-Protean II dual slab cell, Bio-Rad, 1985). PPO at 20 μg of protein/well was loaded; electrophoresis was carried out at 200 V for 35 min. The purity of enzyme preparations was determined by comparing gels stained with 10 mM DL-Dopa in 0.05 M sodium phosphate buffer (pH 6.5) (Constantinides and Bedford, 1967) and then with a Coomassie brilliant blue R-250 (Eastman Kodak Co., Rochester, NY) solution.

Enzyme Activity Assay. Potato PPO activity was determined at 25 °C for 5 min by mixing 2.9 mL of 0.97 mM chlorogenic acid in 1 mM potassium phosphate buffer (pH 7.0) with 0.1 mL of enzyme. Maximal initial velocity for quinone formation was monitored at 395 nm by using a DU-7 spectrophotometer (Beckman Instruments Inc., Irvine, CA). One unit of PPO activity was defined as an increase in absorbance of 0.001/min at 395 nm and 25 °C. Apple PPO activity was measured at 30 °C for 5 min by mixing 0.2 mL of PPO preparation with 1.8 mL of 0.05 M 4-methylcatechol in 0.1 M sodium phosphate buffer. Maximal initial velocity for quinone formation was determined at 395 nm, and 1 unit of PPO activity was defined as an increase in absorbance of 0.001/min at 30 °C.

White shrimp PPO activity was carried out at 40 °C for 5 min by adding 80 μL of PPO to 1.12 mL of 10 mM L-Dopa in 0.05 M sodium phosphate buffer (pH 6.5). Maximal initial velocity for dopachrome formation was determined at 475 nm. One unit of PPO activity was defined as an increase in absorbance of 0.001/min at 40 °C. Grass prawn and spiny lobster PPO activities 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). The reaction was monitored at 25 °C for 5 min. Maximal initial velocity was determined as (ΔA_{475nm}/min), and 1 unit of PPO activity was defined as an increase in absorbance of 0.001/min at 25 °C.

Effect of Kojic Acid on Enzyme Activity. The method of Saruno et al. (1979) was adopted. PPO preparation was incubated with sodium acetate, potassium or sodium phosphate buffer, and kojic acid (Sigma) solutions in distilled water at 37 °C for 15 min. Following equilibration to ambient temperature, specific substrate for each system in buffer solution was added to the mixture and the change in absorbance of the reaction product, benzoquinone, was spectrophotometrically monitored for 5 min. For control sample, an equivalent volume of H_2O was used to replace kojic acid solution. Percentage inhibition (I) was expressed as $[(T - T^*)/T] \times 100$, where T^* and T were enzyme activities in the presence and absence of kojic acid, respectively (Saruno et al., 1979).

The mushroom PPO system was composed of 0.9 mL of kojic acid (20–200 μg/mL), 0.1 mL of enzyme (1 mg/mL) in distilled H_2O, and 2.0 mL of 0.83 mM L-tyrosine or 5 mM DL-Dopa in 0.05 M acetate buffer (pH 6.8). The reaction was monitored at 475 nm and 25 °C. The potato PPO system contained 0.9 mL of kojic acid (20–800 μg/mL), 0.1 mL of enzyme, and 2.0 mL of 1.4 mM chlorogenic acid or 5 mM catechol in 1.0 mM phosphate buffer (pH 7.0). The reaction was monitored at 395 nm and 25 °C. The apple PPO system contained 1.15 mL of kojic acid (0.02–2.0 mg/mL), 0.1 mL of enzyme, and 2.0 mL of 0.05 M 4-methylcatechol or chlorogenic acid in 0.1 M phosphate buffer (pH 6.0). The reaction was monitored at 395 nm and 30 °C.

White shrimp PPO system contained 0.45 mL of kojic acid (20–200 μg/mL), 50 μL of enzyme, and 1.0 mL of 5 mM L-Dopa or catechol in 0.05 M phosphate buffer (pH 6.5). The reaction was monitored at 40 °C and at 395 and 475 nm, respectively, for catechol and L-Dopa oxidation. Grass prawn and lobster PPO systems contained 0.9 mL of kojic acid (20–150 μg/mL), 0.1 mL of enzyme, and 2.0 mL of 5 mM DL-Dopa or catechol in 0.05 M phosphate buffer (pH 6.5). The reaction was monitored at 25 °C and 395 and 475 nm, respectively.

Enzyme Kinetics Study. Michaelis constants, K_m, for the various plant and crustacean PPOs were determined by using the Lineweaver–Burk equation (Lineweaver and Burk, 1934). The substrates used for mushroom PPO, potato PPO (10 900 units/mg of protein), and apple PPO (97 400 units/mg of protein) were DL-Dopa (0.30–3.33 mM) or L-tyrosine (13.8–153 μM) in 0.05 M acetate buffer (pH 6.8), chlorogenic acid (0.60–6.67 mM) or catechol (0.90–10.0 mM) in 1 mM phosphate buffer (pH 7.0), and 4-methylcatechol (1.0–9.5 mM) or chlorogenic acid (1.0–7.0 mM) in 0.1 M sodium phosphate buffer (pH 6.0), respectively. Enzyme activity for each plant PPO was monitored as previously described.

L-Dopa and catechol at 1.5–7.0 mM and DL-Dopa and catechol at 1.67–9.92 mM in 0.05 M sodium phosphate buffer (pH 6.5) were respectively used as substrates for white shrimp PPO (5400

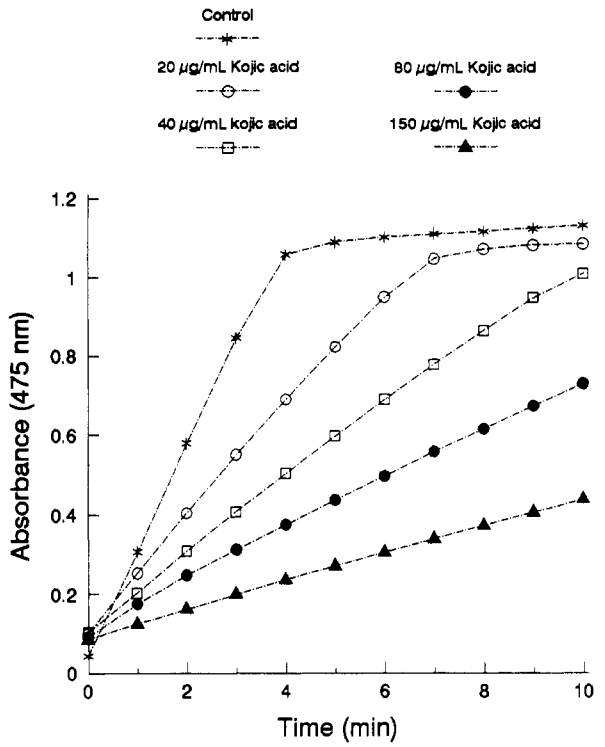

Figure 1. Effect of concentration-related inhibitory effect of kojic acid on mushroom tyrosinase activity on DL-Dopa. A 0.9-mL aliquot of kojic acid solution at 20–150 μg/mL was added to the assay system.

units/mg of protein) and grass prawn (900 units/mg of protein) and lobster PPO (7000 units/mg of protein). The assay for white shrimp PPO was conducted for 5 min at 40 °C and at 25 °C for grass prawn and lobster PPO. Enzyme activity on L-Dopa or DL-Dopa and catechol was monitored at 475 and 395 nm, respectively.

The inhibitory mechanism of kojic acid on enzyme activities was also investigated. Except for the substitution of 0.5 mL of kojic acid solutions for buffer, substrate concentrations used in the previous study were employed. Kojic acid solutions at 0.28, 0.56, and 1.06 mM in distilled water were used in the mushroom, white shrimp, grass prawn, and lobster PPO assay systems, while those at 0.56, 1.06, and 1.41 mM were used in the potato and apple PPO systems. Prior to the addition of the substrate, the enzyme–inhibitor mixture was incubated at 37 °C for 15 min. The assays for mushroom, potato, grass prawn, and lobser PPO were carried out as previously described. Kinetic parameters of K_{mapp} and K_i for enzyme activities were also determined according to the equations of Lineweaver and Burk (1934) and Dixon (1953).

The kinetic constants (K_m, apparent K_m, and K_i) of potato, apple, and white shrimp PPO with DL-Dopa as the substrate were also determined. Sixty microliters of PPO was added to 940 μL of DL-Dopa in 0.05 M sodium phosphate buffer (pH 6.5). The final concentration of DL-Dopa varied from 1.4 to 8.9 mM. The reaction was monitored at 475 nm and 25 °C for 10 min. The inhibitory effect of kojic acid on the enzyme activity in oxidizing DL-Dopa in these systems was determined by adding 50 μL of kojic acid at 0.56 or 1.12 mM to the cuvette containing PPO and sodium phosphate buffer (pH 6.5). The mixture was incubated at 37 °C for 15 min. Following the equilibration to ambient temperature, DL-Dopa was added and the reaction was monitored at 475 nm and 25 °C for 10 min. All the assays to determine enzyme activity, the inhibitory effect of kojic acid on the various enzymes, and the enzyme kinetics were conducted at least three times with three different sample preparations.

RESULTS AND DISCUSSION

Effect of Kojic Acid on Plant PPO Activity. Kojic acid showed a concentration-dependent inhibitory effect on mushroom tyrosinase oxidation of DL-Dopa (Figure 1). The addition of kojic acid (20 μg/mL) to the assay system

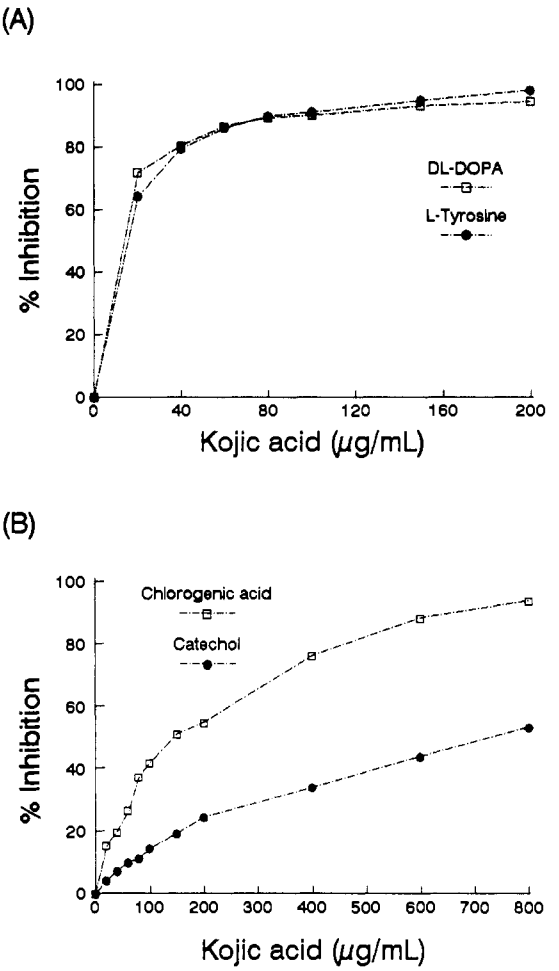

Figure 2. Concentration-related inhibitory effect of kojic acid on the oxidation of DL-Dopa (□) and L-tyrosine (●) by mushroom tyrosinase (A) and of chlorogenic acid (□) and catechol (●) by potato polyphenol oxidase (B). A 0.9-mL aliquot of kojic acid at 20–200 or 20–800 μg/mL was added to the assay mixture in (A) or (B), respectively.

containing DL-Dopa and L-tyrosine caused the inhibition of tyrosinase by 72 and 64%, respectively (Figure 2A). The inhibition was elevated to 90% when kojic acid at 80 μg/mL was added. Saruno et al. (1979) reported a 20–30% inhibition of tyrosinase activity when kojic acid at 20 μg/mL was applied. The difference in enzyme preparations could have affected the inhibitory effectiveness of kojic acid.

Inhibition of potato PPO (Figure 2B) and apple PPO (data not shown) by kojic acid was not as effective as mushroom tyrosinase. Kojic acid when added at 200 μg/mL only inhibited 55 and 25% of potato PPO on the oxidation of chlorogenic acid and catechol, respectively (Figure 2B). The inhibition was increased to about 90 and 53%, respectively, when kojic acid at 800 μg/mL was used.

Effect of Kojic Acid on Crustacean PPO Activity. Kojic acid inhibition on white shrimp (Figure 3A) and grass prawn PPO (Figure 3B) was less effective than on spiny lobster PPO (Figure 3C). The former two enzymes were only inhibited by about 20%, while the oxidation of DL-Dopa and catechol by lobster PPO was inhibited to 80 and 70%, respectively, when kojic acid at 20 μg/mL was used. Kojic acid appeared to be more effective in inhibiting lobster PPO than white shrimp and grass prawn PPO and plant PPO.

Simpson et al. (1988) found that *p*-aminobenzoic acid (PABA) or NaN_3 was more effective than EDTA and cys-

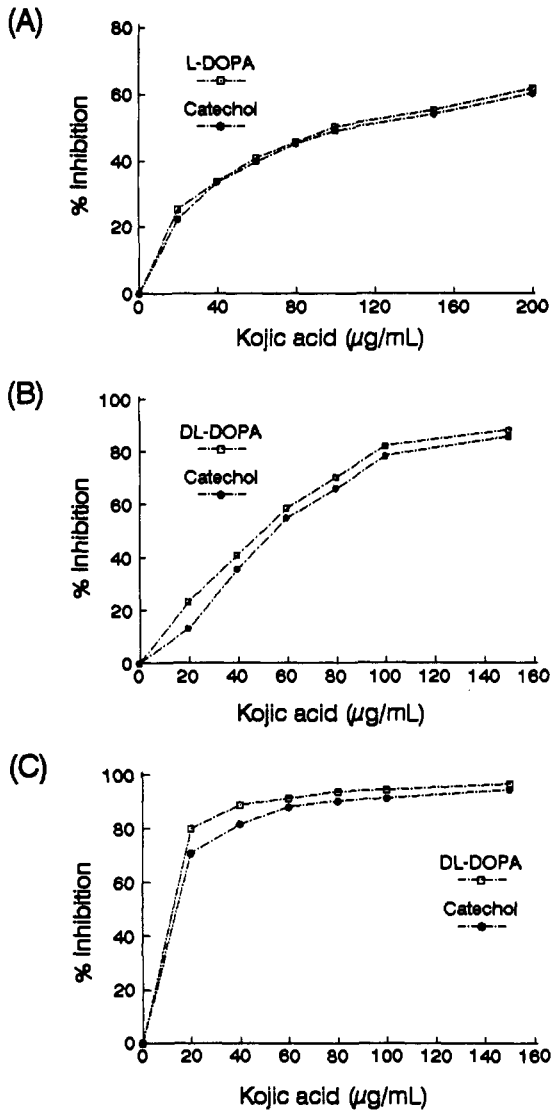

Figure 3. Concentration-related inhibitory effect of kojic acid on the oxidation of L-Dopa (□) and catechol (●) by white shrimp polyphenol oxidase (A), DL-Dopa (□), and catechol (●) by grass prawn polyphenol oxidase (B) and of DL-Dopa (□) and catechol (●) by spiny lobster polyphenol oxidase (C). A 0.25-mL aliquot of kojic acid (20–200 μg/mL) was added to the assay mixture in (A), and a 0.9-mL aliquot (20–150 μg/mL) was added to the assay mixtures in (B) and (C).

teine in inhibiting pink and white shrimp PPO. On the basis of the molar ratio between crustacean PPO and inhibitor used, kojic acid was found to be less effective in inhibiting white shrimp, grass prawn, and lobster PPO than PABA and NaN_3 on pink and white shrimp PPO.

Enzyme Kinetics. The kinetic parameters (K_m, K_{mapp}, and K_i) for various enzymes utilizing phenolic substrates in the absence or presence of kojic acid and the type of inhibition as verified by the Dixon plot are listed in Table I. Kojic acid was a competitive inhibitor for both potato and apple PPO but a mixed-type inhibitor for white shrimp, grass prawn, and Florida piny lobster PPO. For mushroom PPO, kojic acid was a competitive inhibitor for L-tyrosine while a mixed-type inhibitor for DL-Dopa.

The Michaelis constants for the oxidation of DL-Dopa and L-tyrosine by mushroom tyrosinase were 0.29 and 0.69 mM, respectively. The K_m value for DL-Dopa was close to that for the oxidation of catechol (0.22 mM) and chlorogenic acid (0.22 mM) by Sisler and Evans (1958) but was lower than the values reported by Smith and Krueger (1962) on catechol (2.5–4.0 mM). Similarly, the Michaelis constant for L-tyrosine was lower than that for the oxidation of *p*-cresol (1.5–10 mM) observed by Sisler and Evans (1958). Mayer et al. (1966) have attributed this discrepancy to different enzyme preparations and assay methods used. On the basis of the K_m values for these two substrates, it was noted that mushroom tyrosinase had a higher affinity for DL-Dopa than for L-tyrosine. Bouchilloux et al. (1963) isolated four different forms of tyrosinase from mushroom, all of which were capable of oxidizing Dopa more actively than tyrosine. A similar observation was also reported by Harrison et al. (1967) using a fluorescence spectrophotometric technique.

The apparent K_m values for the oxidation of L-tyrosine and DL-Dopa by mushroom tyrosinase in the presence of kojic acid were determined to be 2.02 and 0.66 mM, respectively. The inhibitor constant (K_i) was determined to be 0.03 mM for the former (competitive inhibition) and 0.02 mM for the latter (mixed-type inhibition). Since kojic acid was a competitive inhibitor, it would compete with L-tyrosine for the active site (Segel, 1976). The results also showed that the inhibitory properties of kojic acid on mushroom tyrosinase depend upon whether *o*-diphenol (DL-Dopa) or monophenol (L-tyrosine) is used as a substrate. The differences in inhibition mechanism and inhibitor constant found with DL-Dopa and L-tyrosine indicate the existence of more than one active site in mushroom tyrosinase (Macrae and Duggleby, 1968). In this study, a mixed-type inhibition implies that kojic acid affected the affinity of the enzyme for DL-Dopa but did not bind at the active site (Webb, 1963).

The K_m value for the oxidation of chlorogenic acid by potato PPO was 5.20 mM, while it was 7.89 mM for the oxidation of catechol. These values were lower than those reported by Abukharma and Woolhouse (1966), Alberghina (1964), and Macrae and Duggleby (1968). Variations in enzyme preparation and assay method could have contributed to these differences in K_m values (Macrae and Duggleby, 1968).

In the presence of kojic acid, the apparent Michaelis constant for the oxidation of chlorogenic acid became 7.23 mM, while it was 10.5 mM for catechol. The inhibitor constant for the oxidation of chlorogenic acid was 0.60 and 0.71 mM for catechol oxidation. Thus, kojic acid was more competitive with chlorogenic acid than with catechol for the active site. The K_i values for kojic acid with chlorogenic acid and catechol as substrate were lower than those reported for *p*-nitrophenol, ferulic acid, *p*-coumaric acid, 2,3-dihydroxynaphthalene, and cinnamic acid (Macrae and Duggleby, 1968).

The Michaelis constants for the oxidation of 4-methylcatechol and chlorogenic acid by apple PPO were determined to be 3.85 and 8.20 mM, respectively. The K_m value for 4-methylcatechol was close to that reported by Harel et al. (1965) and Mayer et al. (1964), whereas the K_m for chlorogenic acid was higher than reported by Walker (1964).

When kojic acid was added, the apparent Michaelis constants for 4-methylcatechol and chlorogenic acid were changed to 4.28 and 10.3 mM, respectively. The inhibitor constants of kojic acid for 4-methylcatechol and chlorogenic acid oxidation were 0.06 and 0.13 mM, respectively. The former was similar to the K_i of 2,3-naphthalenediol (Mayer et al., 1964). However, both K_i values were lower than that of cinnamic acid (1.4 and 0.14 mM, respectively) when cinnamic acid was used as an inhibitor in a solubilized PPO system (Walker and Wilson, 1975). The Michaelis constants for the oxidation of L-Dopa and catechol by white shrimp PPO were determined to be 3.48

Table I. Inhibitory Mechanism of Kojic Acid on Polyphenol Oxidase Obtained from Various Sources

enzyme source	substrate	Michaelis constant (K_m), mM	type of inhibition	apparent K_m, mM	inhibitor constant (K_i), mM
mushroom	L-tyrosine	0.69	competitive	2.02	0.03
	DL-Dopa	0.24	mixed	0.66	0.02
potato	chlorogenic acid	5.20	competitive	7.23	0.60
	catechol	7.89	competitive	10.5	0.71
	DL-Dopa	0.06	competitive	0.08	0.06
apple	4-methylcatechol	3.85	competitive	4.28	0.13
	chlorogenic acid	8.20	competitive	10.3	0.06
	DL-Dopa	0.04	competitive	0.07	0.03
white shrimp	L-Dopa	3.48	mixed	4.29	0.15
	catechol	4.27	mixed	5.18	0.18
	DL-Dopa	3.20	mixed	4.20	0.09
spiny lobster	DL-Dopa	3.27	mixed	4.37	0.07
	catechol	4.98	mixed	7.31	0.10
grass prawn	DL-Dopa	3.64	mixed	7.77	0.05
	catechol	5.29	mixed	7.78	0.07

and 4.27 mM, respectively. The former (L-Dopa) K_m value was slightly higher than that reported for DL-Dopa (2.8 mM) by Simpson et al. (1988). Regarding lobster PPO, the K_m values for oxidation of DL-Dopa and catechol were 3.27 and 4.98 mM, respectively. These values were lower than those reported earlier (Chen et al., 1991). Lobster PPO used in this study was further purified by 7.5% acrylamide gel and thus possessed a higher specific activity. For grass prawn PPO, the K_m values for DL-Dopa and catechol were 3.64 and 5.29 mM, respectively. The former value was close to that reported by Rolle et al. (1991). In comparison to pink shrimp (Simpson et al., 1988), white shrimp, grass prawn, and lobster PPO showed comparatively higher K_m values when either L-Dopa or DL-Dopa was used as substrate.

When kojic acid was added as an inhibitor, a mixed-type inhibition was observed for the oxidation of both substrates by these three crustacean PPOs. Antony and Nair (1975) studied the inhibitory effect of several chemicals on prawn phenolase activities and found L-tyrosine (K_i = 0.38 mM) was a competitive inhibitor to the oxidation of Dopa. In contrast, L-cysteine and sodium diethyl dithiocarbamate were found to behave as mixed-type inhibitors. Madero (1982) reported that bisulfite was a competitive inhibitor to brown shrimp PPO. The observation of lower K_i values for grass prawn and lobster PPO than white shrimp PPO further demonstrates that kojic acid exhibited a greater inhibitory effect on the former two enzymes than the latter one (Table I). Also, when the same amount of kojic acid was applied to the assay mixtures, a higher inhibitory effect was observed for DL-Dopa than for catechol (Figure 3B,C).

It was noted that the K_m value of white shrimp PPO for DL-Dopa (3.20 mM) was close to that of enzyme for L-Dopa (3.48 mM). For potato and apple PPO, both enzymes showed a much higher affinity for DL-Dopa than for chlorogenic acid, 4-methylcatechol, and catechol (Table I). Also, these two enzymes showded higher affinity for DL-Dopa than the rest of the PPO enzymes. When kojic acid existed as an inhibitor, it exhibited the same inhibitory mechanisms on the oxidation of DL-Dopa as well as on other diphenolic substrates by white shrimp, potato, and apple PPO (Table I). As K_i shown in Table I indicates, kojic acid exerts more effective inhibition on DL-Dopa oxidation by potato and apple PPO than other diphenolic substrates previously described.

The results thus indicated that kojic acid could be potentially applied to prevent melanosis in plant and seafood products. Significant inhibitory effects with different types of inhibition mechanism were observed with these PPOs when Dopa was used as substrate.

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Registry No. DL-Dopa, 63-84-3; PPO, 9002-10-2; kojic acid, 501-30-4; L-tyrosine, 60-18-4; chlorogenic acid, 327-97-9; catechol, 120-80-9; 4-methylcatechol, 452-86-8.