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Inhibitory Effect of Red Koji Extracts on Mushroom Tyrosinase

LI-CHEN WU,* YUN-CHEN CHEN, JA-AN ANNIE HO, AND CHUNG-SHI YANG

Department of Applied Chemistry, National Chi-Nan University, Puli, Nantou 545, Taiwan

Red koji has been recognized as a cholesterol-lowering diet supplement because of it contains fungi metabolites, monacolins, which reduce cholesterol synthesis by inhibiting HMG-CoA reductase. In this study, water extracts of red koji were loaded onto a C_{18} cartridge, and the acetonitrile eluate was collected as test fraction. Red koji water extracts and its C_{18} cartridge acetonitrile eluent had total phenols concentrations of 5.57 and 1.89 mg/g of red koji and condensed tannins concentrations of 2.71 and 1.20 mg/g of red koji, respectively. Both exhibited an antioxidant activity and an inhibitory activity to mushroom tyrosinase. The higher antioxidant activity of the red koji acetonitrile eluent was due to the existence of a high percentage of condensed tannins. The results from the kinetic study for inhibition of mushroom tyrosinase by red koji extracts showed that the compounds in the extracts competitively inhibited the oxidation of tyrosine catalyzed by mushroom tyrosinase with an ID_{50} of 5.57 mg/mL.

KEYWORDS: Red yeast rice; red koji; polyphenols; Folin−Ciocalteu; vanillin−HCl assay; antioxidants; free radical scavenging activity; tyrosinase; tyrosinase inhibitor; DL-DOPA

INTRODUCTION

Tyrosinase (EC 1.14.18.1; polyphenol oxidase, PPO) is a multifunctional copper-containing enzyme (mono- and diphenolase activities) that is involved in the synthesis of melanin. It catalyzes the hydroxylation of tyrosine to *o*-diphenol and the oxidation of *o*-diphenol to *o*-quinone. The formation of melanin has been reportedly important not only in insect defensive and developmental processes (*1*) but also in mammalian cells (*2*). In wounded plants, tyrosinase-induced pigmentation is responsible for enzymatic browning, which leads to undesired color quality and loss of nutritional value of plant-derived foods and beverages (*3*, *4*). Many naturally occurring tyrosinase inhibitors have been studied for the prevention of enzymatic browning in the food industry, as well as for the reduction of hyperpigmentation in the cosmetic (*5*) and medical industries (*6*). The modes of the inhibitory effect for those naturally occurring tyrosinase inhibitors were observed as competitive (*7*, *8*), noncompetitive (*9*), or mixed (*10*), mediated through any of the following mechanisms: (i) inhibition of PPO (*11*); (ii) reduction of *o*-quinones to diphenols (*12*) or Cu^{2+} to Cu^{+} (*13*); (iii) interaction with the formation of *o*-quinone products (*14*); or (iv) decrease in the uptake of oxygen for the reaction (*15*). The degree of inhibitory effect is variable because of differences in the sources of tyrosinase and phenolics substrates studied (*16*).

The sources of tyrosinase inhibitors are found mainly in microorganisms and plants (*17*). Kojic acid [5-hydroxy-2-(hydroxymethyl)-γ-pyrone], a fungal secondary metabolite produced by species of *Aspergillus* and *Penicillium* (*18*), has been demonstrated to be a potent inhibitor (*16*, *19*) through the mechanism of reduction of *o*-quinones to diphenols (*10*, *20*) and the chelating of transition metal ions (*7*, *21*). Agaritine [β-*N*-(γ-L-(+)-glutamyl)-4-hydroxymethpyphenylhydrazine], isolated from *Afaricus bisporus*, was evidently capable of inhibiting the monophenolase and diphenolase activities of mushroom polyphenol oxidase (*22*). Phenolics, such as flavonols, isolated from plants, also showed diphenolase inhibitory activity due to their free 3-hydroxyl group (*7*). Because of the broad applications and the importance of tyrosinase inhibitors, there is an urgent need to explore safer and more potent substances as alternatives for those currently used.

Red yeast rice, also known as "red koji" or "angkak," prepared by solid fermentation with the fungal strain *Monascus purpureus* on rice, has been used traditionally in eastern Asia as a food colorant and medicine (*23*). Recent clinical observations showed that red yeast rice significantly reduces total cholesterol, low-density lipoportein (LDL) cholesterol, and total triacylglycerol concentrations (*24*−*31*), partly due to the presence of the secondary metabolites, monacolins, that inhibit cholesterol production by inhibiting HMG-CoA reductase activity (*32*−*34*). The pigments separated from red koji have been proven to inhibit the mutagenicity of activated forms of food pyrolysate mutagens (*35*). Several secondary metabolites of *Monascus*, polyketide pigments [yellow pigments: ankaflavin, monascin, and xanthomonasin A and B; red pigments: rubropunctain, monascorbrin, monascorubramine, rubropunctamine (*35*−*37*)] and polyketide monacolins (monacolins K, J, L, M, and X and dihydromonacolin L) (*38*−*40*), have been isolated and their chemical structures determined to be phenolics (*23*, *37*, *41*−*44*). Phenolic compounds obtained from herbs, plants, and fungi showed a wide range of biological and pharmaceutical

* To whom correspondence should be addressed: Dr. Li-chen Wu, Department of Applied Chemistry, National Chi-Nan University, Puli, Nantou 545 Taiwan. E-mail: lw25@ncnu.edu.tw. Fax: +886-49-2917956.

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benefits, including anticarcinogenic, antioxidative, and hypolipidemic activities (*45*−*48*). However, up to now, there has been little work carried out on the antioxidant effects of red yeast rice and its action against mushroom tyrosinase activity. A new biofunction of red yeast rice may be explored by studying the inhibitory effect of red koji on mushroom tyrosinase.

In the present study, the antioxidant activity and tyrosinase inhibitory effect of red koji water extracts and the red koji acetonitrile eluate from C_{18} cartridge were studied. In addition, the mode of inhibition for red koji acetonitrile eluate was also investigated.

MATERIALS AND METHODS

Materials. Mushroom tyrosinase (EC 1.14.18.1) used for the bioassay was purchased from Sigma Chemical Co. (St. Louis, MO). Red yeast rice powder was a gift, kindly donated from a local food company. All other chemicals and organic solvents were purchased from Sigma Chemical. Whatman no. 1 filter paper was bought from Fisher Scientific (Fair Lawn, NJ).

Sample Preparation. Red yeast rice powder (0.5 g) was dissolved in 10 mL of distilled water, sonicated for 60 min, and centrifuged (5000*g*) for 10 min. Filtered red koji water extracts were then applied at a flow rate of no faster than 1 mL/min to a J. T. Baker C_{18} cartridge (6-mL capacity with 1000 mg of sorbent, Phillipsburg, NJ) that had been preconditioned as per the directions provided by manufacturer. The cartridge was first washed with water, after which the desired fraction of red koji was collected in the test tube with 100% acetonitrile. The collected eluate was vacuum-dried to dryness by a Thermo Savant SpeedVac (Holbrook, NY). The tubes were capped and stored at 4 °C until analysis.

Determination of Phenolic Compounds. *Folin*−*Ciocalteu Method.* The total phenolic content in the extracts was determined according to the Folin−Ciocalteu procedure (*49*). A 100 μL aliquot of the supernatant was added to the test tube combining with 500 μL of Folin−Ciocalteu reagent and 400 μL of sodium carbonate (7.5%). The tubes were votexed for 15 s and then allowed to stand for 30 min at 20 °C. Absorption was measured at 765 nm using the Hewlett-Packard UV−vis spectrophotometer (Palo Alto, CA). The total phenolic content was expressed as gallic acid equivalents in milligrams. Duplicate determinations were performed on each sample; data shown later represent the mean of two measurements.

Vanillin−*HCl Assay.* The amount of condensed tannins was measured by vanillin assay (*50*). One milliliter aliquots of red koji extracts were dispensed into test tubes. Tubes were incubated in the water bath at 30 °C. Five milliliters of the vanillin reagent (1% vanillin in methanol) was added at 1.0 min intervals to one set of samples, and 5.0 mL of the 4% HCl solution was added at 1.0 min intervals to the second set of samples. After 20 min of incubation, the samples were removed and the absorbance at 500 nm was read. The condensed tannins content was expressed as catechin equivalents in milligrams. Duplicate determinations were performed on each sample; data shown later represent the mean of two measurements.

Antioxidant Measurement: α,α-Diphenyl-β-picrylhydrazyl (DPPH) Radical Scavenging Activity. A 0.5 mL aliquot of the methanol extract prepared above was mixed with 0.25 mL of an ethanolic 0.5 mM DPPH solution and 0.5 mL of 100 mM acetate buffer (pH 5.5). The contents of the tubes were mixed for 15 s, and the absorbance of the mixture was measured at 517 nm after 30 min of standing (*51*). The antioxidant activity was expressed as the following formula:

$$\text{activity (\%)} = [(A_{517,\text{extract}} - A_{517,\text{blank}})/A_{517,\text{blank}}] \times 100$$

Bioassay. *Enzyme Activity Assay.* Although it differs somewhat from other sources, this fungal source was used for the present study because it is readily available. The preliminary assays of mushroom PPO system were modified from Chen et al. (*10*). Two milliliters of 5.0 mM tyrosine or DL-DOPA solution was mixed with 0.9 mL of 0.05 M acetate buffer (pH 6.8) and incubated for 10 min at room temperature. Then, 0.1 mL of the aqueous solution of mushroom tyrosinase (0.3 mg/mL, added last) was added to the mixture for immediate measurement of the initial rate of linear increase in optical density at 475 nm (dopachrome formation at 475 nm).

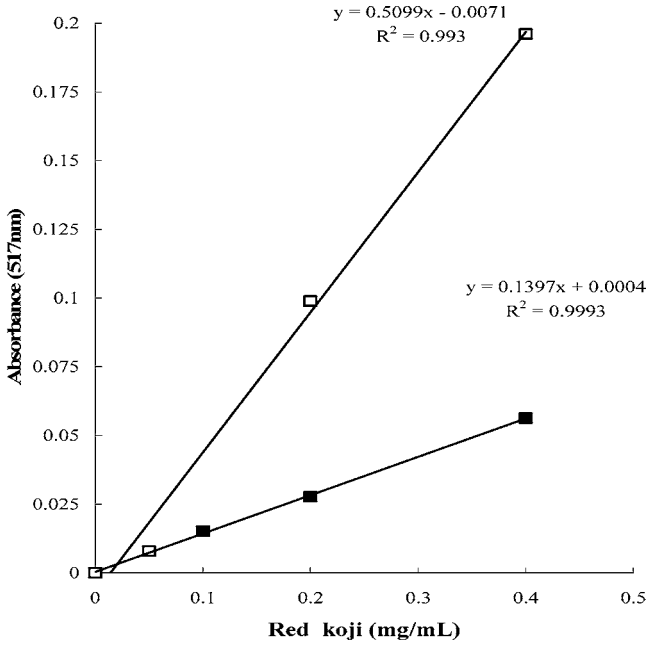

Figure 1. Effect of red koji extracts (water extracts, ■; acetonitrile eluate, □) on the antioxidant capacities. Antioxidant capacity was measured as the DPPH radical scavenging activity of the extracts. Red yeast rice powder (0.5 g) was dissolved in 10 mL of distilled water, sonicated for 60 min, and centrifuged for 10 min followed by filtration. The filtrate was assigned as water extracts for antioxidant capacity determination. Filtered red koji water extracts were further applied at a flow rate of no faster than 1 mL/min to a C_{18} cartridge. The 100% acetonitrile eluate was collected and dried to dryness. The antioxidant capacity of the acetonitrile eluate was measured by dissolving the dried fraction in methanol. Antioxidant activity was expressed by the differences of A_{517} (extract) and A_{517} (blank). Each point represents the mean of two measurements.

Assay for Effect of Inhibition. Substrate and inhibitor structures determine the mode of inhibition; in this study, tyrosine and DL-DOPA were used as the substrates for the determination of monophenolase and diphenolase inhibitory effects, respectively. Because of its insolubility in the aqueous solution, the dry residue from red koji extracts was dissolved in dimethyl sulfoxide (DMSO) as previously described in Masamoto et al. (*52*). The system for determining the inhibitory effect of red koji extracts was similar to that in the enzyme activity assay, except 0.9 mL of acetate buffer (50 mM) was replaced with 0.9 mL of red koji extracts. Inhibition by the addition of tested samples is expressed as a percentage necessary to give a 50% inhibition (ID_{50}).

The preincubation mixture consisted of 1.8 mL of 0.1 M phosphate buffer (pH 6.8), 0.6 mL of water, 0.1 mL of the sample solution, and 0.1 mL of the aqueous solution of mushroom tyrosinase (130 units). The mixture was preincubated at room temperature for 5 min before 0.4 mL of 6.3 mM DL-DOPA solution was added. The reaction mixture was monitored at 475 nm for 2 min.

RESULTS AND DISCUSSION

Determination of Phenolic Content and Antioxidant Capacity. The phenolic metabolites produced by *Monascus* have been isolated and studied (*53*); however, the antioxidation activity and tyrosine inhibitory effect have received less attention. In this study, water was chosen as the extraction solvent system for phenolic metabolites (*37*). Furthermore, acetoniltrile eluate from the C_{18} cartridge of water extracts was also obtained. Total phenols and condensed tannins of the water extracts and the acetonitrile eluate were measured according to

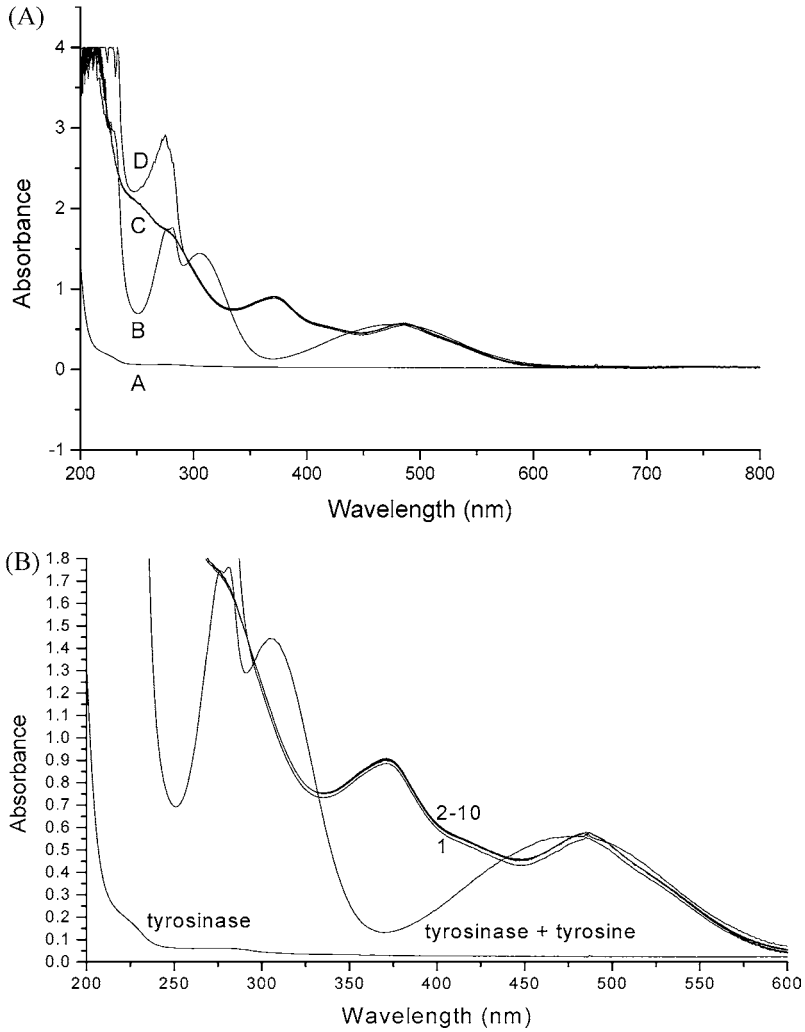

Figure 2. (A) UV–visible spectra of mushroom tyrosinase with tyrosine or red koji water extracts and (B) time course study (lines 2–10, 0, 2, 4, 6, 8, 10, 15, 20, and 30 min; line 1, red koji water extract alone) of the reaction of mushroom tyrosinase and red koji water extracts. Panel A, spectrum A, mushroom tyrosinase (0.3 mg/mL); spectrum B, mushroom tyrosinase (0.3 mg/mL) + tyrosine (2.5 mM); spectrum C, mushroom tyrosinase (0.3 mg/mL) + red koji water extracts (7.7 mg/g); spectrum D, red koji water extracts (7.7 mg/g).

Folin−Ciocalteu method and vanillin−HCl assay, respectively. The Folin−Ciocalteu method is commonly used to measure phenolic content, although it is not completely specific for phenolic compounds, and not all phenolic compounds exhibit the same level of activity in the assay. However, it does give a good general measure of phenolic content. The total phenolic content in acetonitrile eluate was about one-third of those in water extracts (5.57 vs 1.89 mg/g of red koji). That is, about one-third of total phenols of water extracts were more hydrophobic and, therefore, retained by the C_{18} cartridge and eluted by acetoniltrile. The vanillin−HCl method involves reaction of an aromatic aldehyde, vanillin, with the meta-substituted ring of flavanols to yield a red adduct (measured at A_{517}). This method has been widely used to estimate condensed tannins (proanthocyanidin); however, this method also measures simple flavonoids. The total condensed tannins level of water extracts was 2.71 mg/g of red koji, whereas the amount of condensed tannins of acetonitrile was 1.20 mg/g of red koji. It appears that the constituent of the acetonitrile eluent was higher in condensed tannins (63%) than that of water extracts (48%). Many flavonoids and flavonols (condensed tannins) have been reported to exhibit potent antioxidant behavior due to their ability in radical scavenging (*54*, *55*) and in the chelating of metal ions (*56*). Because there were detectable amounts of condensed tannins in red koji, the antioxidant capacity of red koji extracts was determined. The relationship between phenolic content (measured by the Folin−Ciocalteu method) and antioxidant capacity is shown in **Figure 1**. Antioxidant capacity was measured as the DPPH radical scavenging activity of the water extracts and acetonitrile eluate. The increase in phenolic content in the two extracts was linearly correlated with the antioxidant capacity. As the concentration of phenols increased from 0.0125 to 0.05 mg/mL for water extracts and from 0.00625 to 0.05 mg/mL for acetonitrile eluate, the DPPH value increased linearly by about 4-fold ($R^2 = 0.993$) and 25-fold ($R^2 = 0.999$), respectively. It appears that the antioxidant activity of acetonitrile eluate was much stronger than that of water extract. This could be due to the higher content of condensed tannins in acetonitrile eluate, although the concentration of total phenol content was higher in water extract. The chemical structures for some constituents from red koji were established and reported by several groups (*23*, *37*, *38*, *41*, *44*). They are pigments and compounds from the monacolin family; some of them are structurally similar to flavonoids. In addition, Aniya et al. (*57*) also reported that the extract of *Monascus anka* showed radical scavenging action. The antioxidant activity of acetonitrile eluate at a concentration of 0.05 mg/mL was comparable to that of kojic acid at 0.4 mM (23.99 vs 24.08%). Kojic acid is also a well-known strong natural antioxidant, which scavenges reactive oxygen species (*58*) and chelates metal ions.

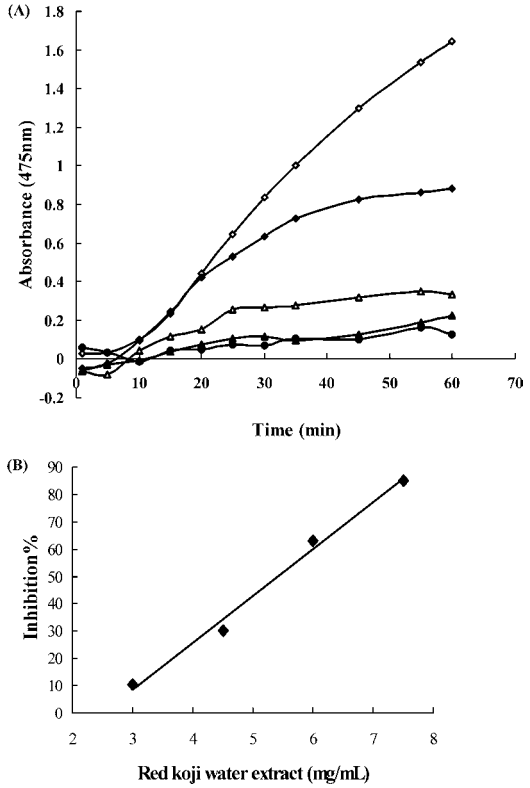

Figure 3. Effects of red koji water extracts on (A) the hydroxylation of monohydroxyphenol and (B) the dose-dependent inhibitory effects by mushroom PPO for the catalysis of tyrosine. Red koji extracts (0.9 mL) of different concentrations (◆ 4.5; △ 6.0; ▲ 7.5; and ● 9.0 mg/mL) were incubated with 2.0 mL of 5.0 mM tyrosine for 10 min at ambient temperature followed by the addition of 0.1 mL of mushroom PPO (0.3 mg/mL). The reaction was monitored at 475 nm (25 °C) for 60 min. Control (◇) was run similarly except that red koji extracts were replaced by an equivalent volume of acetate buffer. Each point represents the mean of two measurements.

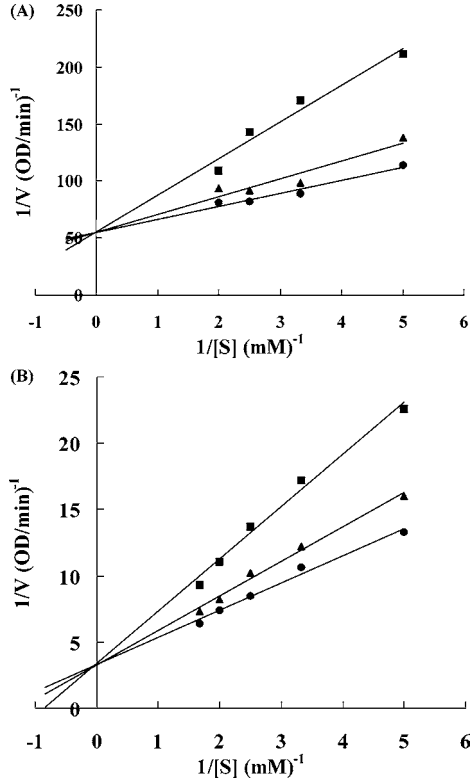

Figure 4. Lineweaver–Burk plots for the inhibition of red koji acetonitrile eluate on mushroom tyrosinase for the catalysis of (A) tyrosine and (B) DOPA at 25 °C, pH 6.8. Concentrations of red koji acetonitrile eluate for curves were 0 mg/g (●), 0.09 mg/g (▲), and 0.18 mg/g (■) respectively; the enzyme concentration was 0.3 mg/mL. 1/V: 1/△ 475 nm/min.

Usually phenolic antioxidants can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals, and also by acting as oxygen scavengers. Therefore, the antioxidant activity of red koji extracts might be due to the existence of a high percentage of condensed tannins (flavonoids). Because kojic acid is a depigmenting agent used as a cosmetic material with a skin-whitening effect and as a medical agent for hyperpigmentation disorders (*59*), it functions as a tyrosinase inhibitor. Hence, red koji extracts might also have demonstrated an inhibitory effect on tyrosinase activity. However, the assumption should be made that the red koji extract was not the substrate of the enzyme tyrosinase.

Inhibitory Effects of Red Koji Extracts on Mushroom Tyrosinase. As shown in **Figure 2**, mushroom tyrosinase incubated with its substrate, tyrosine, for 10 min peaked noticeably at 475 nm due to the formation of dopachrome. Red koji extracts (1.02 mg/mL), on the other hand, incubated with the enzyme for 0, 2, 4, 6, 8, 10, 15, 20, and 30 min showed nearly no significant shift with the increase of time. It was similar to that of red koji extract alone, except for the significant drop at ~275 nm. This drop could be attributed to the new chemistry that occurred between the enzyme and red koji extracts, which broke the symmetry of its original structure. Further confirmation study by NMR is ongoing. Tyrosinase catalyzes two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity), both using molecular oxygen incorporated into the products. Because phenolics in red koji extracts did not show absorbance increase at 475 nm with the presence of tyrosinase, we concluded that the red koji extracts was not the substrate of the enzyme.

Further study was conducted to investigate whether the red koji extracts was able to inhibit tyrosinase activity. Enzyme activity assay with red koji water extracts and red koji acetonitrile eluate was therefore carried out to explore the kinetics and the mode of the inhibitory effect. As shown in **Figure 3**, a dose-dependent inhibitory effect of red koji on tyrosine oxidation by mushroom tyrosinase was observed. The lag time, which is the time period known for the oxidation of monophenolic substrates to diphenols, was extended by the addition of the inhibitors, red koji extracts. It has been reported by the Kubo group (*7*) that some of the potent tyrosinase inhibitors, such as quercetin, activate the monophenolase activity as a cofactor but inhibit *o*-diphenolase activity; kaempherol and galangin, however, did not activate the monophenolase but inhibited *o*-diphenolase activity.

The ID_{50} value of red koji water extracts was estabilished as 5.57 mg/mL for tyrosine. The ID_{50} value for red koji acetonitrile eluate was hampered due to the severe interference of its genuine red color. Although the ID_{50} value of red koji extracts was much lower than those of koji acid (5.00×10^{-3} mg/mL) and ascorbic acid (5.00×10^{-2} mg/mL), the concentration of the pure effective compounds in the extracts might be lower than the concentration of the above two potent inhibitors used in the study.

The inhibition kinetics for the red koji acetonitrile eluate was analyzed by a Lineweaver−Burk plot as shown as **Figure 4**. The three lines, obtained from the uninhibited enzyme and from the two different concentrations of red koji acetonitrile eluate,

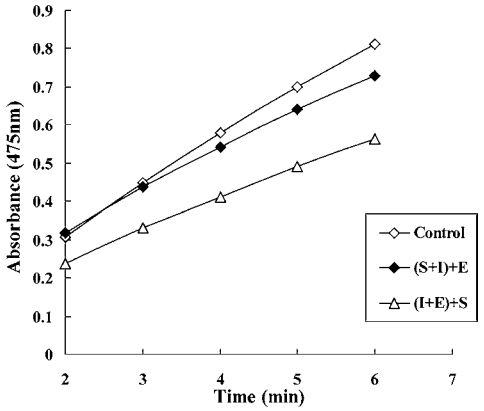

Figure 5. Effect of preincubation of red koji extracts and mushroom tyrosinase on inhibitory activity. The preincubation mixture consisted of 1.8 mL of 0.1 M phosphate buffer (pH 6.8), 0.6 mL of water, 0.1 mL of the sample solution, and 0.1 mL of the aqueous solution of mushroom tyrosinase (130 units). [◇, the mixture was preincubated at room temperature for 5 min before 0.4 mL of 6.3 mM DL-DOPA solution was added; ◆, DL-DOPA (S) preincubated with red koji acetonitrile eluate (I), followed by the addition of tyrosinase (E)]. The reaction mixture was monitored at 475 nm for 2 min.

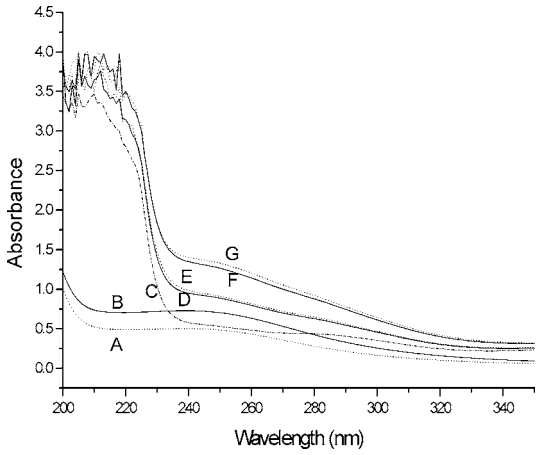

Figure 6. UV–visible spectrum of red koji extracts without and with $CuSO_4$ and mushroom tyrosinase: (A) 0.5 mM $CuSO_4$; (B) 1.0 mM $CuSO_4$; (C) red koji acetonitrile eluate (0.05 mg/g); (D) 0.5 mM $CuSO_4$ + red koji acetonitrile eluate (0.05 mg/g); (E) 0.5 mM $CuSO_4$ + red koji acetonitrile eluate (0.05 mg/g) + mushroom tyrosinase (0.3 mg/mL); (F) 1.0 mM $CuSO_4$ + red koji acetonitrile eluate (0.05 mg/g); (G) 1.0 mM $CuSO_4$ + red koji acetonitrile eluate (0.05 mg/g) + mushroom tyrosinase (0.3 mg/mL).

intersected in the horizontal axis. This result indicates that red koji extracts exhibited a competitive inhibition for tyrosine and DL-DOPA by mushroom tyrosinase. It also means the red koji extracts inhibited effectively the activities of diphenolase and monophenolase. Furthermore, a preincubation experiment for the enzyme with 0.1 mg/mL red koji acetonitrile eluate and in the absence of the substrate resulted in an increase of inhibitory activity from 6.4 to 30% (**Figure 5**). There are three forms of active center in tyrosinase, namely, *oxy*-, *deoxy*-, and *met*-tyrosinase, classified by the geometric and electronic structure of the active site. The enzyme preincubated was mostly *met*-tyrosinase (85%), the resting form of the enzyme (*3*). The binuclear copper active center of *met*-tyrosinase is bicupric and unable to bind oxygen. Supposedly, red koji extracts will not bind with *met*-tyrosinase as a monophenol. The inhibitory effect might come from the chelation of the copper in the active site; the Kubo group (*7*) asserted that flavonols chelated copper because of their free 3-hydroxyl group. However, some flavonoids still show their inhibitory functionality without this characteristic (*60*). As shown in **Figure 6**, red koji acetonitrile eluate showed a noticeable shift in the spectrum and rise at 250 nm. This might be contributed to by the compounds in the eluate interacting with copper. The result also explains why the red koji extracts were not oxidized by the enzyme as substrate.

Finding tyrosinase inhibitory activity with red koji extracts led us to evaluate and compare it with other well-known tyrosinase inhibitors such as ascorbic acid and kojic acid. Because the most effectively inhibitory compound has not yet been isolated from red koji extracts, crude red koji extracts therefore did not exhibit superior activity compared to ascorbic acid and kojic acid in terms of their ID_{50} values. However, from safety and nutritional points of view, red koji might be a useful alternative as a tyrosinase inhibitor, especially for use in food products. Many studies have demonstrated that red koji has significant effects on lowering cholesterol and is antimutagenic. On the basis of our data, the inhibitory action of crude red koji extracts against tyrosinase activity may also be applied in cosmetics products as a potential anti-hyperpigmentation or skin-whitening agent.

Conclusions. Red koji extracts showed a dose-dependent inhibitory effect on tyrosine oxidation by tyrosinase from mushroom. They also showed an inhibitory effect with an ID_{50} value of 5.57 mg/mL on mushroom tyrosinase activity. A study of the kinetics for inhibition of mushroom tyrosinase exhibited the red koji extracts as a competitive inhibitor of the enzyme with tyrosine and DL-DOPA as the substrates. Kojic acid has been extensively used as a cosmetic material with excellent skin-whitening effects and as a medical agent for the treatment of hyperpigmentation disorders. It is also a good chelator of copper and has been shown to inhibit tyrosinase from various sources including fungi, plants, and animal tissues; its depigmenting action is attributed to an ability to chelate copper at the active site of tyrosinase. Red koji extracts, containing a high percentage of condensed tannins, inhibited tyrosine oxidation by mushroom tyrosinase in a manner similar to that of kojic acid. Like kojic acid, red koji extracts also showed an antioxidant activity. Therefore, a similar property of skin-whitening effects would also be expected in red koji extracts. Ongoing investigations are focusing on the isolation of the effective compound from red koji and its inhibitory effect on melanin synthesis of melanocytes in vitro.

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