JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Isolation and Identification of Flavonoids in Licorice and a Study of Their Inhibitory Effects on Tyrosinase

BOQIANG FU,[†] HUAN LI,[†] XIAORU WANG,[†,‡,§] FRANK S. C. LEE,*[,‡,§] AND SHUFEN CUI[†,||]

Department of Chemistry and the Key Laboratory of Analytical Sciences of the Ministry of Education, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen, China, Material Transport &Transformation in Environment & Life Processes Laboratory, First Institute of Oceanography, QingDao, China, QingDao key Lab of Analytical Science and Standardization of Chinese Medicines, QingDao, China, and Department of Biological and Applied Engineering, Shenzhen Polytechnic, Shenzhen, China

Five different flavonoids were isolated from licorice after multistep chromatographic fractionation. The aim was to identify and characterize active components in licorice responsible for antibrowning activities and to seek new tyrosinase inhibitors for applications as antibrowning and depigmenting agents in the food and cosmetic industries. The isolated flavonoids were identified as liquiritin, licuraside, isoliquiritin, liquiritigenin (from *Glycyrrhiza uralensis* Fisch.), and licochalcone A (from *Glycyrrhiza inflate* Bat.) by UV, MS, ^{1}H NMR, and ^{13}C NMR analyses. The inhibitory potencies and capacities of these flavonoids toward monophenolase activity of mushroom tyrosinase were investigated. The IC_{50} values of licuraside, isoliquiritin, and licochalcone A for monophenolase activity were 0.072, 0.038, and 0.0258 mM, respectively. A study of the mechanisms of monophenolase inhibition by these flavonoids indicated that they are all competitive inhibitors. Different from the above flavonoids, no inhibitory activity was observed for liquiritin, whereas liquiritigenin activated the monophenolase activity as a cofactor. The inhibitory effect of licuraside, isoliquiritin, and licochalcone A on diphenolase activity with L-DOPA as the substrate was much lower than those with l-tyrosine. Results suggest that licuraside, isoliquiritin, and licochalcone A have the high potential to be further developed into effective antibrowning and depigmenting agents.

KEYWORDS: Licorice; flavonoids; isolation; identification; tyrosinase; inhibition

INTRODUCTION

Tyrosinase (monophenol or *o*-diphenol, oxygen oxidoreductase, EC 1.14.18.1), also known as polyphenol oxidase (PPO) (*1*), is a copper-containing monooxygenase. The enzyme catalyzes two distinct reactions involving molecular oxygen: (i) the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and (ii) the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity). Quinones are highly reactive compounds that can polymerize spontaneously to form high molecular weight brown pigments (melanins) or react with amino acids and proteins to enhance the brown color of the pigment produced (*2*).

Tyrosinase is widely distributed in nature and is responsible for not only browning in plants but also melanization in animals. The tyrosinase-induced unfavorable browning of wounded raw fruits, vegetables, and beverages is a major problem in the food industry. It is one of the main causes of loss in appearance and nutritional value during the postharvest handling processes, resulting in shorter shelf life and reduced market value. In the food and beverage industries today, the most widely used antibrowning agents are reducing reagents (sulfites, ascorbic acid, etc.). However, these compounds could have adverse health effects (especially sulfites) or react with other components in food to produce unwanted side products (*3*). Melanin is the pigment responsible for the color of human skin and hair. However, the accumulation of an excessive level of melanin is of concern because such dermatological disorder may cause melasama, age spots, and sites of actinic damage (*4*). Biosynthesis of melanin can be inhibited by tyrosinase. Previously, 1,4-dihydroquinone was one of the most widely prescribed (*5*) skin-lightening agents. Recently, however, the compound has been reported to induce mutations, because it acts by affecting both DNA and RNA synthesis, and is a potent melanocyte cytotoxic agent. Thus, in the diverse areas of food processing, the medical field, and cosmetic industries, there is a common

* Corresponding author. E-mail: fsclee@yahoo.com.
[†] Xiamen University.
[‡] First Institute of Oceanography.
[§] QingDao key Lab of Analytical Science and Standardization of Chinese Medicines.
[||] Shenzhen Polytechnic.

10.1021/jf051258h CCC: $30.25
Published on Web 08/27/2005

need to explore safer and more potent substitutes for the tyrosinase inhibitors currently in use.

Microorganisms and plants are the main sources of natural tyrosinase inhibitors (*6*, *7*). Flavonoids are the most abundant and best-studied plant polyphenols with such activities. Some flavonoids inhibit tyrosinase activity by active site chelation, while others acted as cofactor and/or substrate of tyrosinase (*7*, *8*). Licorice (gancao in Chinese) is the roots and rhizomes of certain *Glycyrrhiza* species. Besides being a popular food additive used worldwide as additives in tobaccos, chewing gums, and candies, etc, licorice is also one of the most widely used medicinal herbs in China, with antitumorigenic, antimicrobial, and antiulcer, antioxidant activities. Glycyrrhizic acid and flavonoids are the two major bioactive components in licorice. In recent studies, some flavonoids in licorice have been demonstrated to exhibit inhibitory activities on tyrosinase. Yokota et al. (*9*) showed that UV-B-induced pigmentation and erythema were inhibited by topical application of 0.5% glabridin. Nerya et al. (*10*) indicated that glabrene and isoliquiritigenin (2′,4′,4-trihydroxychalcone) in the licorice extract can inhibit both mono- and diphenolase tyrosinase activities and also inhibit melanin formation in melanocytes. The reported IC_{50} values for glabrene and isoliquiritigenin are 3.5 and 8.1 μM, respectively. In Japan, *Glycyrrhiza glabra* extract containing favonoids such as glabridin and glabrene has been used as a depigmentation agent in cosmetics. The whitening cosmetic products of some famous cosmetic brands, such as Lancome and Avon, all contain the licorice extract.

The aim of the present experiment is to isolate, purify, and identify some flavonoids from *Glycyrrhiza uralensis* Fisch. and *Glycyrrhiza inflate* Bat., to evaluate their inhibitory capacity on the monophenolase activity of mushroom tyrosinase and to study the kinetics and mechanisms of the inhibition processes.

MATERIAL AND METHODS

Chemicals and Reagents. Tyrosinase from mushroom (EC 1.14.18.1, Sigma-Aldrich Product, with an activity of 2440 units/mg), L-tyrosine, and DPPH (2,2-diphenyl-1-picrylhydrazyl) were all purchased from Fluka Chemie. L-DOPA (3,4-dihydroxy-L-phenylalanine) and DMSO (dimethyl sulfoxide) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO). Sodium dihydrogen phosphate, potassium phosphate monobasic, and copper sulfate pentahydrate obtained from Shanghai Chemical Co. (Shanghai, P. R. China) were of analytical-grade. Freshly deionized water (18 MΩ) prepared by a Millipore Milli-Q50 device (Millipore Corp., Waltham, MA) was used in all experiments. Liquiritin, licuraside, and isoliquiritin were isolated from the aqueous ethanol (30:70 v/v) extract of the roots of *G. uralensis* Fisch. (from Elion Resources Group Co., Inner Mongolia, China). The authenticity of the licorice plant species was established by Prof. Shouquan Lin of Institute of Medicinal Plants, Chinese Academy Medical Sciences. Liquiritigenin was the hydrolysate of liquiritin. Licochalcone A was the generous gift of Dr. Qiaoer Wang from our group and was isolated and purified by high-speed counter-current chromatography (HSCCC) from *G. inflate* Bat. (*11*). All compounds subject to testings were prepared in DMSO solutions, and the concentrations of liquiritin, licuraside, isoliquiritin, liquiritigenin, and licochalcone A in the solutions were 5.203, 4.636, 2.974, 3.43, and 2.92 mM, respectively.

Extraction, isolation, and Identification of Flavonoids. A 250-g sample of licorice (*G. uralensis* Fisch.) was minced and ground into an 80-mesh powder by a muller (Hangzhou Chunjiang Pharmacy Machine Co., Ltd.). The sample powder was extracted twice, each time with 2500 mL of a water−ethanol mixture (30:70 v/v) by sonication in an ultrasonic bath (Model SK3200LH, Shanghai KUDOS Ultrasonic Instrument Co., Ltd.) at room temperature for 30 min. The two extracts were combined and centrifuged at 6000 rpm for 10 min using a refrigerated centrifuge (MIKRO 22R, Hettich Zentrifugen GmbH Co. KG). After centrifugation, the clear supernatant was evaporated to one tenth of its original volume in a rotary evaporator heated at 40 °C. The concentrate (60 g) was fractionated on a column packed with macroporous resin XDA-1 (Grancent Exchange & Adsorbent Material Ltd.), and eluted by a solvent sequence with increasing ratio of ethanol to water. A total of 120 fractions, each of 10 mL volume, were collected. These fractions arranged in the order of elution time were spot checked by HPLC, and those with similar HPLC profiles were combined. Such grouping resulted in four major fractions with distinct compositional features. Fraction 2 was further separated on a Sephadex LH-20 (Pharmacia Fine Chemical Co., Ltd) column with water−methanol (60:40 v/v) solvent elution for the isolation of liquiritin (3.3 g). Fraction 3 was fractionated on a polyamide column eluted with 60% methanol in water, and a total of 55 fractions based on elution order (10 mL each) were collected. After HPLC screening of selected fractions, fractions 21−48 (total dry weight 1.8 g) were found to contain licuraside and isoliquiritin as the two major components. These fractions were combined and further purified over a Sephadex LH-20 column using methanol−H_2O (70:30) as the eluent to yield 1.1 g of licuraside and 0.39 g of isoliquiritin. A solution of liquiritin (100 mg) in methanol with 5% H_2SO_4 was refluxed at 80 °C for 1.5 h. The hydrolyzed products were evaporated to dryness by rotary evaporation. The residue (64 mg) was washed with water to pH 6.5, dissolved in 8 mL of methanol, and then purified over Sephadex LH-20 with methanol−H_2O (40:60, v/v) elution. Colorless, needlelike crystals of liquiritigenin (25 mg) were obtained as the final product after the concentrate was left at room temperature (20 °C) overnight.

Table 1. NMR Data of Liquiritin and Liquiritigenin

position	δ_H (ppm) liquiritin	δ_H (ppm) liquiritigenin	δ_C (ppm): liquiritin
2	5.48 (dd, J = 2.7,12.8 Hz)	5.45 (dd, J = 2.0,12.7 Hz)	79.3
3	2.75 (dd, J = 3,17 Hz, cis)	2.63 (dd, J = 2.4,16.7 Hz, cis)	43.6
	3.05 (dd, J = 13,17 Hz, trans)	3.13 (dd, J = 13,16.6 Hz, cis)	
4			191.8
5	7.75 (d, J = 8.7 Hz)	7.65 (d, J = 8.6 Hz)	128.5
6	6.53 (dd, J = 2.2,8.7 Hz)	6.51 (dd, J = 8.6,2.1 Hz)	110.4
7-OH	10.63 (s)	10.59 (s)	165.4
8	6.38 (d, J = 2.2 Hz)	6.34 (d, J = 2.1 Hz)	102.4
9			164.0
10			113.6
1′			133.0
2′, 6′	7.46 (d, J = 8.6 Hz)	7.33 (d, J = 8.2 Hz)	127.4
3′, 5′	7.16 (d, J = 8.6 Hz)	6.80 (d, J = 8.3 Hz)	116.4
4′-OH		9.59 (s)	157.8
1″	4.96 (d, J = 6.2 Hz)		100.8
2″	3.33–3.50 (μ)		73.5
3″			76.6
4″			69.9
5″			76.8
6″	3.73 (dd, J = 5.5,12.0 Hz)		61.1
	3.91 (dd, J = 1.8,12.0 Hz)		

The purified products as described above were each characterized by UV/vis, LC−MS, and NMR analyses. UV spectra were recorded on an Agilent 8453 UV/vis spectrophotometer. LC/MS analysis was performed on an Agilent 1100 LC/MS analyzer (Agilent Technologies). The mobile phase used in HPLC was acetonitrile−water (50:50, v/v) under isocratic conditions at a flow rate of 0.05 mL·min^{-1}. MS data with PI-ESI detection (positive ion detection with electrospray ionization) was acquired in the m/z range of 200−800, with a step size of 0.1 unit. The collision-induced dissociation (CID) voltage was 100 V and the electrospray voltage (ESV) was set to 4000 V.

The ^{1}H and ^{13}C spectra were recorded on a Bruker Ultra shield 500 MHz nuclear magnetic resonance spectrometer (Bruker, Fällanden, Switzerland). NMR spectra of liquiritin and isoliquiritin were acquired in deuterated methanol solution, while those of licuraside and liquiritigenin were recorded in deuterated DMSO. The NMR data are summarized in **Tables 1** and **2**.

The UV(MeOH), MS, and ^{1}H and ^{13}C NMR spectra of the four isolated licorice flavonoids, liquiritin, licuraside, isoliquiritin, and liquiritigenin, are all consistent with their respective spectral data reported in the literature (*16*, *17*). Other than the NMR data, the other

Table 2. NMR Data of Licuraside and Isoliquiritin

	δ_H (ppm)		δ_C (ppm)	
position	licuraside	isoliquiritin	licuraside	isoliquiritin
b	7.79 (s)	7.83 (d, J = 15.4 Hz)	143.9	143.3
a		7.71 (d, J = 15.2 Hz)	119.6	118.7
C=O			191.9	191.9
1′			113.4	113.2
2′			165.6	165.4
3′	6.29 (d, J = 2.4 Hz)	6.30 (d, J = 2.2 Hz)	103.0	102.4
4′			166.3	166.2
5′	6.40 (dd, J = 2.4,8.6 Hz)	6.44 (dd, J = 2.2,8.8 Hz)	108.6	107.9
6′	8.20 (d, J = 8.9 Hz)	8.01 (d, J = 8.9 Hz)	133.5	132.1
1			128.8	129.1
2, 6	7.86 (d, J = 8.6 Hz)	7.75 (d, J = 8.5 Hz)	131.2	130.0
3, 5	7.07 (d, J = 8.5 Hz)	7.18 (d, J = 8.7 Hz)	116.7	116.6
4			159.7	159.6
1″	5.06(d, J = 7.5 Hz)	5.0 (d, J = 7.3 Hz)	98.8	100.4
2″	3.35–3.65(μ)	3.33–3.52(μ)	77.4	73.4
3″			76.2	76.9
4″			70.3	69.9
5″			76.5	76.5
6″	3.73 (dd, J = 5.4,12.0 Hz)	3.74 (dd, J = 5.5,12.0 Hz)	61.0	61.1
	3.94 (dd, J = 2.2,12.0 Hz)	3.92 (dd, J = 2.2,12.0 Hz)		
1‴	5.3 (d, J = 1.6 Hz)		109.1	
2‴	3.95 (d, J = 1.6 Hz)		77.4	
3‴			79.7	
4‴	3.52 (s)		74.4	
5‴	3.80, 4.66 (J = 7.5 Hz)		64.7	

spectral information can be summarized as follows. (1) Liquiritin: UV (MeOH) spectrum λ_{max} 276, 313 nm; major ESI-MS peaks m/z 419 $[M + H]^+$, 441 $[M + Na]^+$, and 257 $[M - 162 + H]^+$. (2) Licuraside: UV (MeOH) spectrum λ_{max} 228 sh, 259 sh, 336 sh, and 361 nm; major ESI-MS peaks m/z 551 $[M + H]^+$, 573 $[M + Na]^+$, 419 $[M - 132 + H]^+$, and 257 $[M - 132 - 162 + H]^+$. (3) Isoliquiritin: UV (MeOH) spectrum λ_{max} 228 sh, 260 sh, 336 sh, and 361 nm; major ESI-MS peaks m/z 419 $[M + H]^+$, 441 $[M + Na]^+$, and 257 $[M - 162 + H]^+$. (4) Liquiritigenin: UV (MeOH) spectrum λ_{max} 276 and 313 nm; major ESI-MS peaks m/z 257 $[M + H]^+$, 279 $[M + Na]^+$, and 535 $[M + M + Na]^+$. The molecular structures of the above four licorice flavonoids along with the previously isolated licochalcone A are shown respectively in **Figure 1**.

Tyrosinase Assay. The assay was performed according to the methods described previously (*10*, *12*) with some modifications. First, 10−30 μL of 5.0 mM L-tyrosine or 10.0 mM L-DOPA aqueous solution mixed with 50 μL of 0.25 M phosphate buffer (pH 6.8) was added into 96-well plates and incubated at 30 °C for 5 min. Then, 1−6 μL of the sample solution in DMSO and 30 μL of the aqueous solution of the mushroom tyrosinase (217 units/mL) were added to the mixture. The final concentration of DMSO in the test solution was 3%, and the final volume of the reaction system was 200 μL. The reaction was carried out under a constant temperature of 30 °C. The linear increase in optical density of the solution at 475 nm was continuously monitored for 25 min at 5-min intervals using a SPECTRA max M_2 Microplate spectrophotometer (Molecular Devices Co.). All experiments were performed at least three times in order to ensure the reproducibility of the results, and the mean values are reported here. The monophenolase activity of tyrosinase was expressed by the rate of dopachrome formation, which was derived from the slope of the plot of optical density versus reaction time. The extent of inhibition by the testing samples was expressed as the concentration of sample needed to inhibit 50% of the enzymatic activity (IC_{50}).

To study the chelation of licorice flavonoids by Cu^{2+}, the UV/vis (220−600 nm) spectra of licorice flavonoids and their Cu-complexed counterparts were measured and compared. The mixture consisting of 1.0 mL of 100 μM sample solution, 1.0 mL of methanol, and 1.0 mL of the aqueous solution of $CuSO_4$ (200 μM) or water (control) was incubated at 25 °C for 10 min before UV/vis spectral analysis.

Determination of Antioxidant Activity by the DPPH• Radical-Scavenging Method. The antioxidant activity of licorice flavonoids was measured according to a modified method of Gadow et al. (*13*) in terms of hydrogen-donating or radical-scavenging ability toward stable radical DPPH (*14*). Special care was taken to minimize the loss of free radical activity of DPPH• in stock solutions during experimentation as recommended by Blois (15). Solutions of DPPH• in methanol were freshly prepared every day and stored at 4 °C in a volumetric flask shielded from light. 50 μL of the flavonoids solution (in DMSO) was placed in a cuvette, and 2 mL of a 6×10^{-5} M methanolic solution of DPPH•, with 0.5 mol of antioxidant per mole of DPPH•, was added into it. The solution was allowed to stand at room temperature in the dark. The decrease in optical density of the resulting solution at 516 nm was monitored continuously at 1-min intervals for about 30 min on an Agilent 8453 UV/vis spectrophotometer until the observed optical density was stabilized. Methanol was used to zero the spectrophotometer, and a sample of 50 μL of DMSO was used as the control. All determinations were performed in duplicate. The percentage of remaining DPPH• radical was calculated according to the formula as follows:

$$\text{remaining percentage (\%)} = (OD_{A(30)})/OD_{C(0)}) \times 100$$

where $OD_{C(0)}$ is the optical density of the control at $t = 0$ min and $OD_{A(30)}$ is the optical density of the antioxidant at $t = 30$ min.

RESULTS AND DISCUSSION

Effect of Isolated Licorice Flavonoids on Monophenolase Activity of Tyrosinase. The inhibitory potency and capacity of the five licorice flavonoids toward the monophenolase activity of tyrosinase were investigated. The results indicated that licuraside, isoliquiritin, and licochalcone A were the most potent inhibitors to monophenolase activity, with IC_{50} values of 0.072, 0.038, and 0.0258 mM, respectively. These IC_{50} values were derived from the curves shown in **Figure 2** after multinomial equation fit. The IC_{50} values for licuraside, isoliquiritin, licochalcone A were much lower than those of other known tyrosinase inhibitors, such as kojic acid, for which 0.14 mM was reported to inhibit 64% of monophenolase activity (*18*). Liquiritin did not inhibit monophenolase activity of tyrosinase, whereas liquiritigenin, on the other hand, activated such activity as a cofactor. The inhibitory effect of chalcones licuraside,

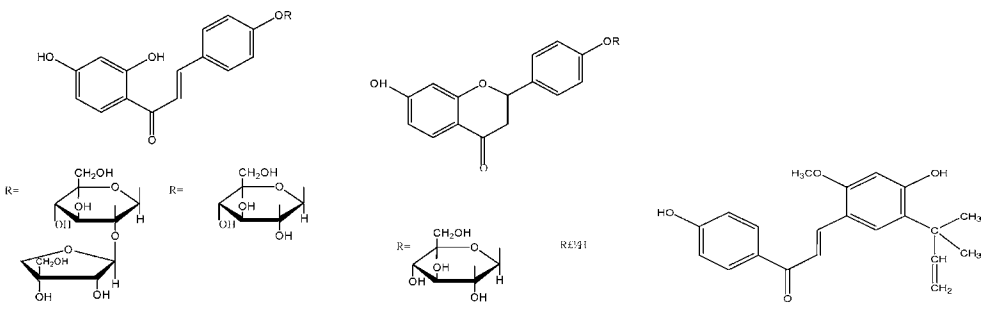

Figure 1. Molecular structures of the five licorice flavonoids investigated in this study.

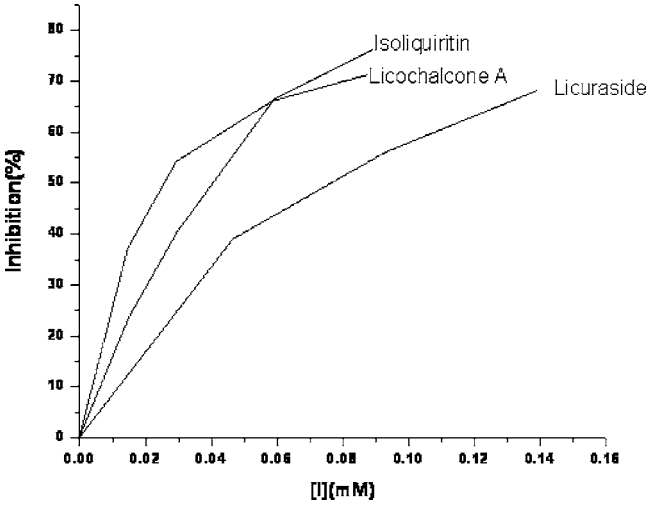

Figure 2. The percentage of inhibition of monophenolase activity as a function of the concentrations [I] of the three inhibitors, including licuraside, isoliquiritin, and licochalcone A.

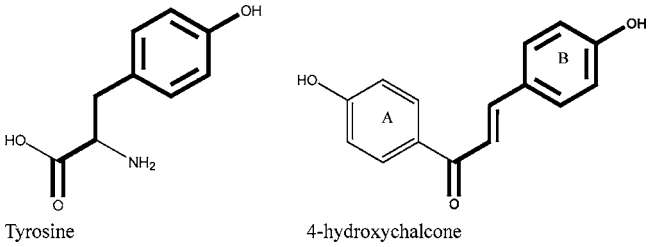

Figure 3. The structural similarity of tyrosine and 4-hydroxychalcone.

isoliquiritin, and licochalcone A on diphenolase activity with L-DOPA as the substrate was much lower than that with L-tyrosine. Even at high concentrations of inhibitors, e.g., 0.1 mM, the diphenolase activity of tyrosinase and the L-DOPA oxidation reactions were not inhibited.

The different inhibitory effects observed between the monophenolase and diphenolase activities could be due to the involvement of different substrate molecules in the two reactions. Most competitive inhibitors have molecular structures that closely resemble those of the substrate. Nerya Ohad et al. reported that the OH group at position 4 (ring B) of the chalcone molecules is the major factor affecting inhibitory potency, because it results in a molecular skeleton closely similar to that of tyrosine. On the other hand, an increase of the number of OH groups on chalcone aromatic rings does not necessarily lead to enhanced inhibitory potency (*19*). When there is an OH in position 4 (ring B), even with the formation of 4-*O*-glycoside, the structures of chalcones such as licuraside, isoliquiritin, and licochalcone A are somewhat similar to that of L-tyrosine (shown in the bold lines in **Figure 3**), the natural ligand of tyrosinase. These chalcones thus could act as a competitive substrate to L-tyrosine.

The IC_{50} value of licochalcone A is higher than the other two chalcones, licuraside and isoliquiritin, probably because the free 4-OH molecular configuration of licochalcone A closely resembles that of tyrosine, and with less steric hindrance than the other two chalcones. Furthermore, the hydrophobic 3,3-dimethyl propylenyl group in position 5 (ring B) of licochalcone A could upset the balance of the weak nonbonding interactions that help maintain its native conformation. This disrupts the quaternary structure of tyrosinase, resulting in the inhibition of enzyme activity. In the case of licuraside and isoliquiritin, the bulky sugar moiety attached to the 4′-hydroxyl group in the

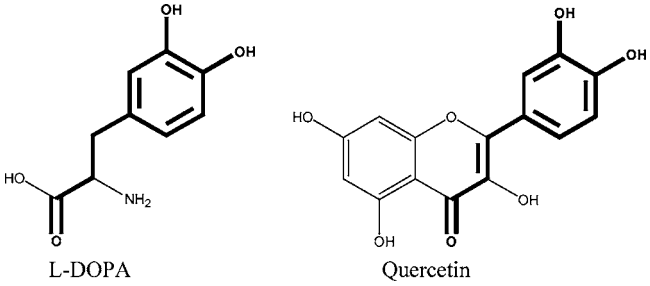

Figure 4. The structural similarity of L-DOPA and quercetin.

4′-O-glycoside form could hinder their approach to the active site of tyrosinase, resulting in less inhibitory activity than that of licochalcone A. As to the diphenolase activity, L-DOPA, an *o*-diphenol, is the substrate. The presence of both 4′-hydroxyl and 3′-hydroxyl groups in ring B (shown in the bold line in **Figure 4**) of molecules such as quercetin (*7*) is needed in order to make the structure of the inhibitor molecule resemble L-DOPA, leading to its displacement of L-DOPA from the active site of the cofactor in a lock-and-key model. The absence of a 3′-hydroxyl group in the ring B for licuraside, isoliquiritin, and licochalcone A explains the absence of inhibitory effect of these molecules toward the diphenolase activity of tyrosinase. As to liquiritin, it is a flavanone glycoside, with a structure different from either the L-DOPA-like quercetin (flavonols) or the L-tyrosine-like chalones licuraside, isoliquiritin, and licochalcone A. Such structural dissimilarity could be one of the reasons it lacks of inhibitory activity toward tyrosinase.

Inhibition Mechanism of Licorice Flavonoids on Mushroom Tyrosinase. It was reported that some flavonoids inhibited tyrosinase activity by the chelation of active sites. Kim (*20*) proposed that the inhibition of catechin toward tyrosinase is probably due to the occurrence of chelation reactions between the vicinal 3′,4′-dihydroxyl group of catechin and the Cu^{2+} on tyrosinase. The structure of 3-hydroxy-4-keto moiety in quercetin also can chelate with copper (*7*). For the compounds tested in this study, neither the 3′,4′-dihydroxyl group nor the 3-hydroxy-4-keto group is present in their structures. So the inhibitions of licuraside, isoliquiritin, and licochalcone A toward tyrosinase likely involve mechanisms other than Cu chelation. The supposition was confirmed by experimental results. Unlike catechin, in the UV/vis spectra of licuraside, isoliquiritin, and licochalcone A, no bathochromic shift, which is characteristic of Cu clelation (*7*), was observed in any of the three sample solutions in the presence of added Cu^{2+}.

Figures **5**−**7** show respectively the dependence of enzyme activity on the concentrations of different inhibitors, including licuraside, isoliquiritin, and licochalcone A. In the figures, the abscissa, [E], is the concentration of tyrosinase, and the ordinate, *V*, is the change of the absorbency with time, which reflects the activity of tyrosinase. The plots for all the licorice flavonoids studied result in a family of straight lines, all passing through the origin. Increasing the concentrations of the inhibitors resulted in a decreasing slope of the lines, suggesting the reversibility of their inhibition reactions with the enzyme.

The inhibitory kinetics of licorice flavonoids was studied by reacting varying concentrations of them with L-tyrosine. The Lineweaver−Burk plots of 1/*V* versus 1/[S] result in a family of straight lines passing through the same point on the vertical axis, as illustrated respectively in Figures **8**−**10** for the three flavonoids. In the figures, the abscissa 1/[L-tyrosine] is the reciprocal of the concentrations of L-tyrosine, whereas the ordinate 1/*V* is the reciprocal of the change of the absorbency with time, which reflects the reciprocal of tyrosinase activity.

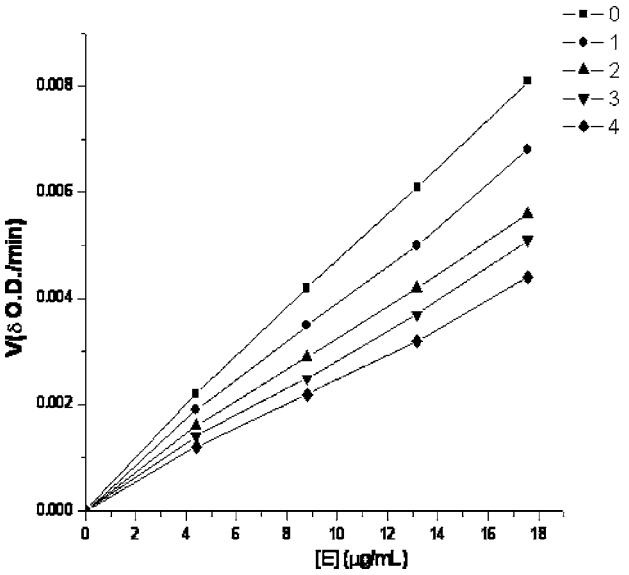

Figure 5. Effect of isoliquiritin concentrations on the monophenolase activity of tyrosinase. Concentrations of isoliquiritin for curves 0–4 are 0, 0.014, 0.029, 0.045, 0.059 mmol/L, respectively.

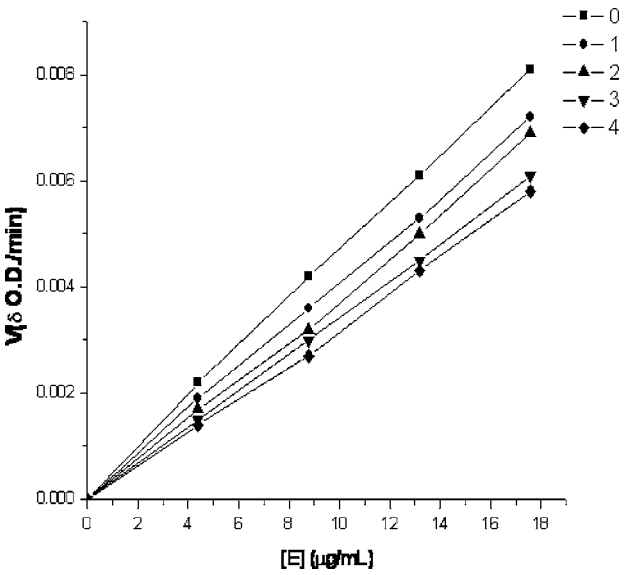

Figure 6. Effect of licuraside concentrations on the monophenolase activity of tyrosinase. Concentrations of licuraside for curves 0–4 are 0, 0.023, 0.046, 0.093, 0.14 mmol/L, respectively.

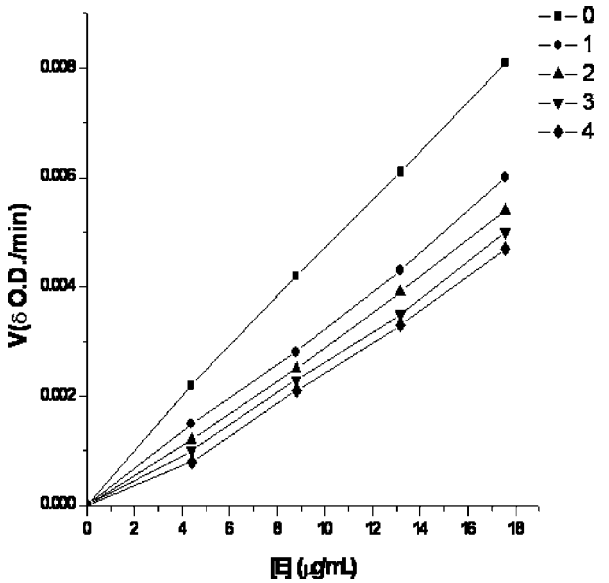

Figure 7. Effect of concentrations of licochalcone A on the monophenolase activity of tyrosinase. Concentrations of licochalcone A for curves 0–4 are 0, 0.014, 0.029, 0.044, 0.058 mmol/L, respectively.

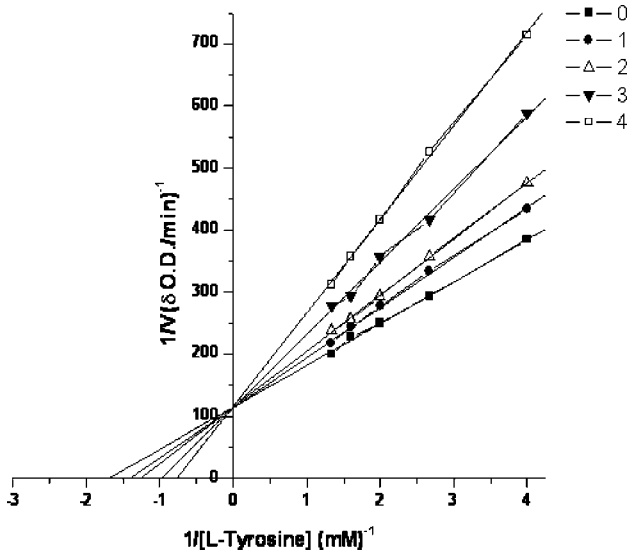

Figure 8. Lineweaver–Burk plots for the inhibition of licuraside on the monophenolase activity of tyrosinase. The mushroom tyrosinase concentration was 32.7 units/mL. Concentrations of licuraside for curves of 0–4 were 0, 0.023, 0.046, 0.093, 0.14 mM, respectively.

The results indicate that they all are competitive inhibitors, with their inhibitory activity toward tyrosinase decreaseing with increasing concentration of the substrate.

The inhibition kinetics was also analyzed by Dixon plots, which were obtained by plotting $1/V$ versus [I] with varying concentrations of substrate. Dixon plots give a family of straight lines passing through the same point at the second quadrant (plots not shown). The secondary plots of the slopes of the Dixon plots versus 1/[S] for licuraside, isoliquiritin, and licochalcone A are shown in **Figure 11**. In the figure, the abscissa 1/[L-tyrosine] is the reciprocal of the concentrations of L-tyrosine. In the ordinate, the term $K_m/V_{max}K_i$[L-tyrosine] is plotted, in which K_m is the Michaelis constant, V_{max} is the maximum velocity of the reaction, K_i is the inhibition constant, and [L-tyrosine] is the concentrations of L-tyrosine. The plots for the three flavonoids are all straight lines passing through the origin, which again strongly suggest that the three licorice flavonoids are competitive inhibitors for the monophenolase activity of tyrosinase (**Figure 11**). These inhibitors bind at the same site as the substrate and bind only to the free enzyme. As stated earlier, most competitive inhibitors closely resemble the molecular structure of the substrate. Thus, it is expected that licuraside, isoliquiritin, and licochalcone A can displace L-tyrosine from the active site because of their structural resemblance, as was shown earlier in **Figure 3**.

In the formation of melanin pigments, three types of tyrosinase (meta-, oxy-, and deoxytyrosinases) with different binuclear copper structures of the active site are involved (*21*, *22*). Kinetic studies on the steady state of the activity in the catalytic pathway show lower catalytic efficiency of tyrosinase toward monophenols than *o*-diphenols (*21*). The monophenolase activity is time dependent, with a characteristic lag time at the initial stage (*23*). Thus, the activity increases slowly initially, followed by accelerated rise afterward, until the cumulated amount of *o*-phenols reaches its equilibrium. The lag time is the time

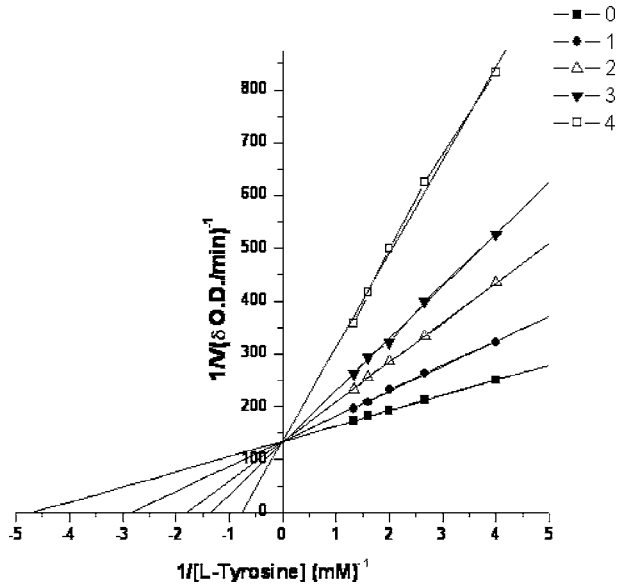

Figure 9. Lineweaver–Burk plots for the inhibition of isoliquiritin on the monophenolase activity of tyrosinase. The mushroom tyrosinase concentration was 32.7 units/mL. Concentrations of isoliquiritin for curves of 0–4 were 0, 0.015, 0.030, 0.059, 0.089 mM, respectively.

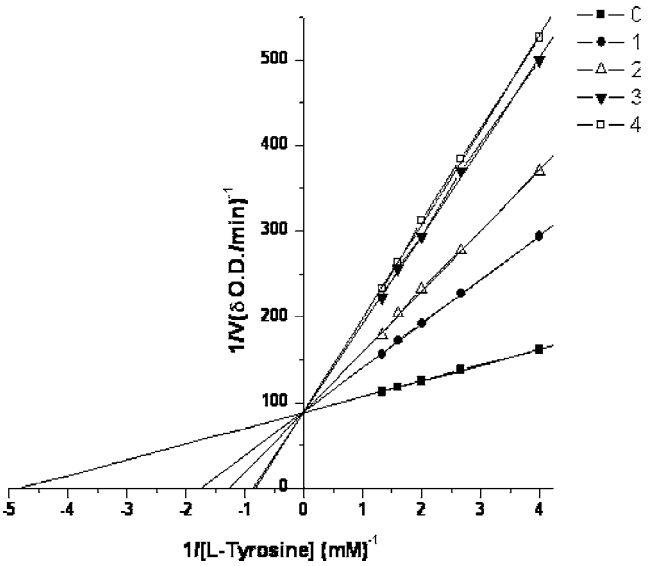

Figure 10. Lineweaver–Burk plots for the inhibition of licochalcone A on the monophenolase activity of tyrosinase. The mushroom tyrosinase concentration was 32.7 units/mL. Concentrations of licochalcone A for curves of 0–4 were 0, 0.015, 0.029, 0.058, 0.088 mM, respectively.

required to reach the steady-state concentration of *o*-diphenol. This amount of *o*-diphenol is necessary to drain the E_{oxy} from E_{met}−tyrosine complex in the catalytic cycle (*21*). The length of the lag time depends on various factors, such as the substrate and enzyme concentrations and the presence or absence of a hydrogen donor (*24*). The lag time can be shortened or abolished by the presence of reducing agents (hydrogen donors), especially *o*-diphenols (*7*). It was reported that L-DOPA at very low concentrations was the most effective reducing agent for eliminating the lag time (*23*, *24*).

In the present study, it was found that L-tyrosine was oxidized by the monophenolase almost without lag time when isoliquiritin and licochalcone A are present. However, licuraside showed less lag time suppression effect (5 min) than isoliquiritin and licochalcone A, while the lag time of the control sample (no inhibitor) is 6.5 min (**Figure 12**). The results thus indicate that

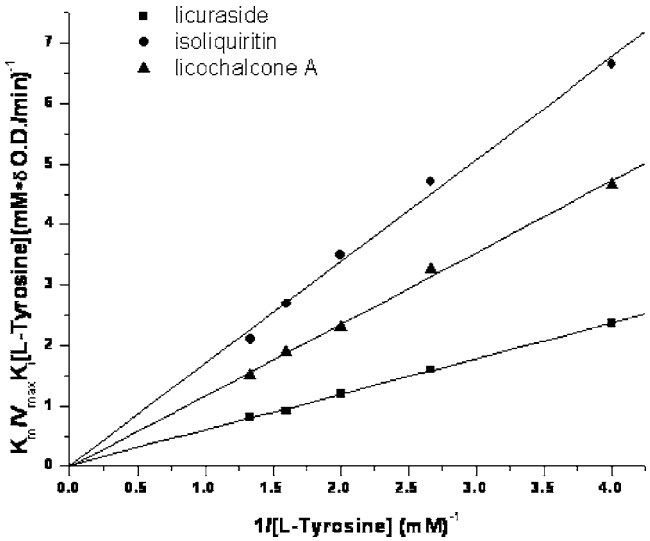

Figure 11. The plots of the slopes ($K_m/V_{max}K_i$[L-tyrosine]) of Dixon plots versus 1/[L-tyrosine] for licuraside, isoliquiritin, and licochalcone A.

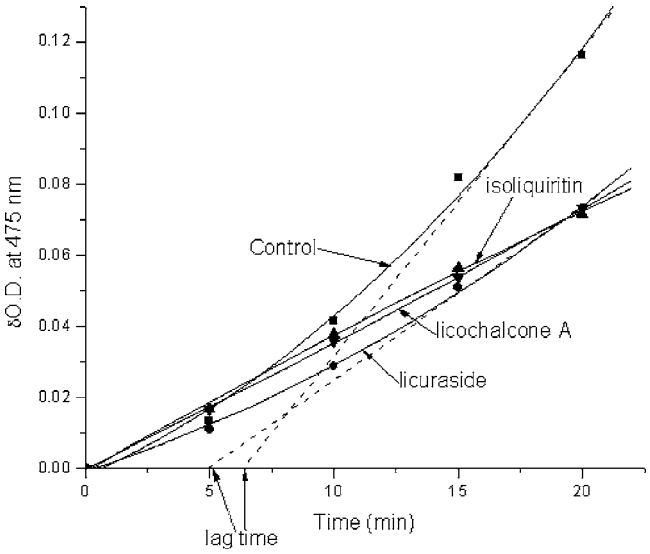

Figure 12. Monophenolase inhibition activities of licuraside, isoliquiritin, and licochalcone A. Experimental conditions: 0.375 mM L-tyrosine, 32.7 units/mL tyrosinase, 23.18 μM licuraside, 29.74 μM isoliquiritin, and 29.17 μM licochalcone A. ---, linear regression fit of steady-state rate.

these chalcones behave as cofactors, and their lag time suppression effect possibly owes to the reducing character of the molecules. The DPPH• radical scavenging activity of licochalcone A was the highest among the three chalcones and showed faster reaction rate than the other two chalcones. The radical scavenging activity of licuraside was higher than that of isoliquiritin. But the lag time of isoliquiritin was much shorter than that of licuraside in inhibiting monophenolase. It could be that it is more difficult for licuraside to donate hydrogen to tyrosinase than for isoliquiritin because of the higher steric hindrance involved in the former case.

Conclusions. In this study, five flavonoids were successfully isolated and purified from licorice. The inhibitory potency and capacity of these flavonoids toward monophenolase activity of mushroom tyrosinase were studied in detail. The results indicated that licuraside, isoliquiritin, and licochalcone A were all competitive inhibitors for monophenolase activity. No inhibitory activity was observed for liquiritin, whereas liquiritigenin activated the monophenolase activity as a cofactor. No significant inhibitory effects were observed for licuraside,

isoliquiritin, and licochalcone A on diphenolase activity with L-DOPA as the substrate. The different effects of these inhibitors on the monophenolase and diphenolase activities may lie on the different substrates involved in the two reactions. Licuraside, isoliquiritin, and licochalcone A were able to reduce to different extents the lag time in the inhibitory process as cofactors due likely to their different reducing characters. The results suggest that the three licorice flavonoids have the potential to be further developed as effective antibrowning agents for foodstuff or skin-whitening agents in cosmetics. The crude licorice extract itself, which contains these active species, could also be used in these applications, but then care must be taken to remove one of the flavonoids, i.e., liquiritigenin, because it shows the opposite effect of activation rather than inhibition of enzymatic activities.

ACKNOWLEDGMENT

We thank Dr. Qiaoe Wang for providing purified licochalcone A. We gratefully acknowledge Dr. Zhiwei Deng at the Analytical Center of Beijing Normal University for NMR analysis of the samples and for help with the interpretation of the NMR spectral data. We also thank Dr. Jincan Shen for recording the mass spectra. We are grateful to Biological Department of Xiamen University for providing us Microplate spectrophotometer for enzyme assay.

LITERATURE CITED

(1) Whitaker, J. R. Polyphenol oxidase. In *Food Enzymes, Structure and Mechanism*; Wong, D. W. S., Ed.; Chapman & Hall: New York, 1995; pp 271−307.

(2) Va'mos-Vigya'zo', L. Polyphenol oxidase and peroxidase in fruits and vegetables. *CRC Crit. Rev. Food Sci. Nutr*. **1981**, *15*, 49−127.

(3) McEvily, J. A.; Iyengar, R.; Otwell, W. S. Inhibition of enzymatic browning in foods and beverages. *Crit. Rev. Food Sci. Nutr*. **1992**, *32*, 253−273.

(4) Frenk, E. Treatment of melasma with depigmenting agents. In *Melasma: New Approaches to Treatment*; Martin Dunitz Ltd.: London, 1995; pp 9−15.

(5) Dooley, T. P. Topical skin depigmentation agents: Current products and discovery of novel inhibitors of melanogenesis. *J. Dematol. Treat*. **1997**, *7*, 188−200.

(6) Wu, L. C.; Chen, Y. C.; Ho, J. A.; Yang, C. S. Inhibitory effect of red koji extracts on mushroom tyrosinase. *J. Agric. Food Chem.* **2003**, *51*, 4240−4246.

(7) Kubo, I.; Kinst-Hori, I.; Chaudhuri, S. K.; Kubo, Y.; Sánchez, Y.; Ogura, T. Flavonols from *Heterotheca inuloides*: Tyrosinase inhibitory activity and structural criteria. *Bioorgan. Med. Chem.* **2000**, *8*, 1749−1755.

(8) No, J. K.; Soung, D. Y.; Kim, Y. J.; Shim, K. H.; Jun, Y. S.; Rhee, S. H.; Yokozawa, T.; Chung, H. Y. Inhibition of tyrosinase by green tea components. *Life Sci.* **1999**, *65*, 241−246.

(9) Yokota, T.; Nishio, H.; Kubota, Y.; Mizoguchi, M. The inhibitory effect of glabridin from licorice extracts on melanogenesis and inflammation. *Pigment Cell Res.* **1998**, *11*, 355−361.

(10) Nerya, O.; Vaya, J.; Musa, R.; Izrael, S.; Ben-Arie, R.; Tamir, S. Glabrene and isoliquiritigenin as tyrosinase inhibitors from licorice roots. *J. Agric. Food Chem.* **2003**, *51*, 1201−1207.

(11) Wang, Q. E.; Lee, S. C. F.; Wang, X. R. Isolation and purification of inflacoumarin A and licochalcone A from licorice by high-speed counter-current chromatography. *J Chromatogr. A* **2004**, *1048*, 51−57.

(12) Miyazawa, M.; Oshima, T.; Koshio, K.; Itsuzaki, Y.; Anzai, J. Tyrosinase inhibitor from black rice bran. *J. Agric. Food Chem.* **2003**, *51*, 6953−6956.

(13) Gadow, A. V.; Joubert, E.; Hansmann, C. F. Comparison of the antioxidant activity of aspalathin with that of other plant phenols of rooibos tea (*Aspalathus linearis*), R-tocopherol, BHT, and BHA. *J. Agric. Food Chem.* **1997**, *45*, 632−638.

(14) Brand-Williams, W.; Cuvelier, M. E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *Lebensm. Wiss. Technol.* **1995**, *28*, 25−30.

(15) Blois, M. S. Antioxidant determinations by the use of a stable free radical. *Nature.* **1958**, *181*, 1199−1200.

(16) Liu, Q.; Liu, Y. L. Studies on chemical constituents of *Glycyrrhiza eurycarpa* P. C. Li. *Acta Pharma. Sinica.* **1989**, *24*, 525−531.

(17) Liu, Q.; Liu, Y. L. Studies on chemical constituents of *Glycyrrhiza eurycarpa* P. C. Li (II). *Acta Botan. Sinica.* **1991**, *33*, 314−322.

(18) Chen, J. S.; Wei, C.; Rolle, R. S.; Otwell, W. S.; Balaban, M. O.; Marshall, M. R. Inhibitory effect of kojic acid on some plant and crustacean polyphenol oxidases. *J. Agric. Food Chem.* **1991**, *39*, 1396−1410.

(19) Nerya, O.; Musa, R.; Khatib, S.; Tamir, S.; Vaya, J. Chalcones as potent tyrosinase inhibitors: The effect of hydroxyl positions and numbers. *Phytochemistry.* **2004**, *65*, 1389−1395.

(20) Kim, Y. J.; Chung, J. E.; Kurisawa, M.; Uyama, H.; Kobayashi, S. New tyrosinase inhibitors, (+)-catechin-aldehyde polycondensates. *Biomacromolecules* **2004**, *5*, 474−479.

(21) Sánchez-Ferrer, A.; Rodríguez-López, J. N.; García-Cánovas, F.; García-Carmona, F. Tyrosinase: A comprehensive review of its mechanism. *Biochim. Biophys. Acta* **1995**, *1247*, 1−11.

(22) Seo, S. Y.; Sharma, V. K.; Sharma, N. Mushroom tyrosinase: Recent prospects *J. Agric. Food Chem.* **2003**, *51*, 2837−2853, 2837.

(23) Fenoll, L. G.; Rodríguez-López, J. N.; García-Sevilla, F.; García-Ruiz, P. A.; Varón, R.; García-Cánovas, F.; Tudela, J. Analysis and interpretation of the action mechanism of mushroom tyrosinase on monophenols and diphenols generating highly unstable *o*-quinone. *Biochim. Biophys. Acta* **2001**, *1548*, 1−22.

(24) Cooksey, C. J.; Garratt, P. J.; Land, E. J.; Pavel, S.; Ramsden, C. A.; Riley, P. A.; Smit, N. P. M. Evidence of the indirect formation of the catecholic intermediate substrate responsible for the autoactivation kinetics of tyrosinase. *J. Biol. Chem.* **1997**, *272*, 26226−26235.

Received for review May 30, 2005. Revised manuscript received July 27, 2005. Accepted July 28, 2005. The project was supported by the key project of the Natural Scientific Foundation of China (20235020).

JF051258H