Flavonoids as Mushroom Tyrosinase Inhibitors: A Fluorescence Quenching Study

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Flavonoids, a group of naturally occurring antioxidants and metal chelators, can be used as tyrosinase inhibitors due to their formation of copper−flavonoid complexes. Thus, to investigate the underlying inhibition mechanism, a large group of flavonoids from several major flavones and flavonols were tested using fluorescence quenching spectroscopy. In addition, large differences in the tyrosinase inhibitory activities and chelating capacities according to the location of the hydroxyl group(s) in combination with the A and B rings in the flavonoids were confirmed. Accordingly, the major conclusions from this work are as follows: (i) The tyrosinase inhibitory activity is not only dependent on the number of hydroxyl groups in the flavonoids, (ii) the enzyme is primarily quenched by the hydroxyl group(s) of A and B rings on the ether side of the flavonoids, and (iii) the tyrosinase inhibitory activity of 7,8,3′,4′-tetrahydroxyflavone is supported by a virtual model of docking with the mushroom tyrosinase, which depicts the quenching of the enzyme. The results also demonstrated that the dihydroxy substitutions in the A and B rings are crucial for Cu^{2+}-chelate formation, thereby influencing the tyrosinase inhibitory activity.

KEYWORDS: Tyrosinase inhibitor; flavonoids; fluorescence quenching; copper chelator

INTRODUCTION

Over 4000 structurally unique flavonoids, i.e., diphenylpropanes, have already been identified in plant sources (*1*). These low molecular weight substances are phenylbenzopyrones (phenylchromones) with an assortment of structures based on a common three-ring nucleus comprised of two benzene rings (A and B) linked through a heterocyclic pyran or pyrone (with a double bond) ring in the middle. Some flavonoids have been found to possess antilipoperoxidant (*2*), antitumoral (*3−5*), antiplatelet (*6*), antiischemic (*7*), antiallergic, and antiinflammatory (*8*, *9*) activities. There have also been reports of flavonoids inhibiting the activities of an array of enzymes, including lipoxygenase (*10*, *11*), cyclooxygenase (*10*, *11*), monooxygenase (*12*), xanthine oxidase (*13*), mitochondrial succinoxidase and NADH-oxidase (*14*), and protein kinases (*15*, *16*). This inhibition of enzymes by certain flavonoids may be due to the interaction of the flavonoid with free radicals generated at the active site of the enzymes (*17−19*) or with metal ion(s) in the catalytic domain of the enzymes (*20*, *21*).

Copper-containing metalloenzymes are able to reversibly bind and activate dioxygen, which plays a vital role in biological catalysis (*22*). The dicopper proteins, hemocyanin, tyrosinase, and catechol oxidase, have been revealed to contain a coupled dinuclear metal center (classified as type 3). Spectroscopic studies and sequence alignments suggest that the tyrosinase catalytic dicopper site is similar to that of hemocyanin, which has already been structurally characterized by X-ray crystallography (*22*, *23*). The active sites of tyrosinase, catechol oxidase, and hemocyanin contain two copper atoms. Each of these, CuA and CuB, are coordinated by three histidines (**Figure 1A**). Extended X-ray absorption fine structure data show similar Cu−Cu distances for both proteins and have revealed that the coppers have three histidine ligands (*22*). The two copper-binding regions show the highest conservation throughout all type 3 copper proteins. In particular, the region binding CuB is highly conserved, whereas the CuA-binding region shows more sequence variety and has been held responsible for the differing functions of tyrosinase, catechol oxidase, and hemocyanin. Both copper-coordinating regions are shown in **Figure 1B** (*24*). A sequence alignment of the mushroom tyrosinase, octopus hemocyanin, and sweet potato catechol oxidase revealed that these enzymes exhibited a sequence identity of about 40% with all six metal ligands conserved. Therefore, the dimetal center of catechol oxidase may serve as a structural model for the catalytic dicopper center of tyrosinase. Thus, on the basis of the high structural similarity of the active site among dicopper enzymes, in principle, they should be inhibited by similar

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936 *J. Agric. Food Chem.,* Vol. 54, No. 3, 2006

Kim et al.
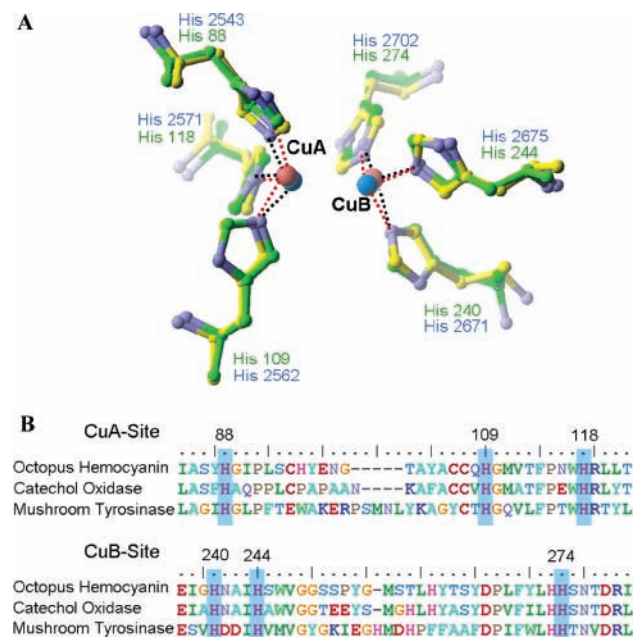
Figure 1. Catalytic site similarity of catechol oxidase and hemocyanin. (**A**) Superimposed structure of catalytic dinuclear copper site of hemocyanin (green) and catechol oxidase (cyan). (**B**) Sequence alignment of the CuA and CuB binding sites of catechol oxidase, octopus hemocyanin, and mushroom tyrosinase. Sequence identities of catechol oxidase and octopus hemocyanin for mushroom tyrosinase (from *Agaricus bisporus*) were 41.1 and 39.1%, respectively.

inhibitor analogues. The existence of these metal-containing regions then raises the issue of whether flavonoids could inhibit tyrosinase due to their innate metal-interacting characteristics. Therefore, the present study investigated the tyrosinase inhibitory activities of certain flavonoids, including mainly flavones, flavonols, and their hydroxyl-substituted derivatives.

There have already been several studies on the fluorescence quenching of proteins induced by drugs or other small molecules (*25*−*32*). Fluorescence quenching is a decrease in the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with quencher molecules. Fluorescence quenching can be dynamic, resulting from collisional encounters between the fluorophore and the quencher, or static, resulting from the formation of a ground-state complex between the fluorophore and the quencher (*33*). In both cases, molecular contact is required between the fluorophore and the quencher for fluorescence quenching to occur. The application of fluorescence quenching as a technique can also reveal the accessibility of the fluorophores to quenchers.

Accordingly, in this paper, the quenching of the intrinsic tryptophan fluorescence of mushroom tyrosinase was used as a tool to study the interaction of various different flavonoids with dicopper protein in an attempt to characterize the chemical associations taking place.

MATERIALS AND METHODS

Reagents. All of the flavonoids used in this study were contributed by the IDR Research Institute. The mushroom tyrosinase, L-tyrosine, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). The buffers used were 0.1 M phosphate buffer at pH 6.8 and 0.02 M Bis-Tris buffer at pH 6.0. DMSO was used to solubilize the flavonoids at a concentration of 10% v/v.
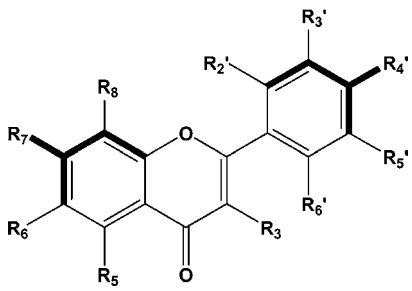
Preparation of Commercial Tyrosinase. The Sigma (2100 units/mg) tyrosinases were purified using the BioLogic Duo-Flow FPLC system (Bio-Rad Laboratories, Richmond, CA) with an anion exchanger

column (length 75 cm; diameter 5 cm) employing DEAE-Sepharose Fast Flow (Amersham Pharmacia Biotech, Buckingham, United Kingdom). The column was equilibrated with 20 mM Bis-Tris buffer at pH 6.0. A stepwise gradient of increasing sodium chloride (NaCl) concentrations was also applied (3 mL/min). The fractions eluted at 100 mM sodium chloride were collected, dialyzed, and concentrated using an ultrafiltration cell (Amicon, Beverly, MA). These concentrated enzyme solutions were then frozen and used as a source of the commercial enzyme. The monophenolase activities of tyrosinase on L-tyrosine were determined by measuring the dopachrome (2-carboxy-2,3-dihydro-indole-5,6-quinone) accumulation at 490 nm using a PowerWave X340 microplate reader (BIO-TEK Instrument Inc., United States). The inhibitory effects on the enzyme activity by the test samples are presented as the percentage of inhibition, $[1 - \text{sample OD}_{490}/\text{control OD}_{490}] \times 100$. The data were collected as the mean ± standard error ($n = 3$), and their significance was analyzed using Student's *t*-test. The temperature was controlled at 37 °C using a heater with a precision of ±0.1 °C. This temperature was chosen to increase the dopachrome stability. The IC$_{50}$ represented the concentration of the sample that inhibits 50% of the enzyme activity.

Fluorescence Quenching and Copper Interaction Study. The fluorescence intensities were recorded using an RF-5301PC spectro-fluorophotometer (Shimadzu, Japan) with an excitation wavelength (λ_{ex}) of 290 nm and excitation and emission slit widths of 5 nm. To determine the linear concentration range of the protein fluorescence, a series of tyrosinase solutions with increasing concentrations (0−25 μM) were prepared in a copper-containing buffer to saturate the enzyme. The maximum emission wavelength (λ_{em}) for the tyrosinase was 340 nm, and the linear range of the tyrosinase fluorescence was between 0 and 4.62 μM. Therefore, 0.77 μM tyrosinase was chosen as the concentration for the fluorescence-quenching experiments. A dilution series of flavonoid solutions (0.01−10.0 μM) was prepared in buffer:DMSO (9:1) solvent. For each data point, 0.25 mL of the appropriate flavonoid solution was added to 2.75 mL of a tyrosinase solution to final flavonoid concentrations ranging from 6.5 to 500 nM. The change in the fluorescence emission intensity was measured within 1 min of the addition of the flavonoid to the tyrosinase. The addition of a constant volume of the quencher to the protein solution avoided the complications due to dilution effects with titration type experiments. Each measurement was repeated in triplicate, and the means and standard deviations were calculated. The fluorescence quenching data were plotted as the fluorescence intensity against the flavonoid concentration. To evaluate the quenching effect of DMSO, the effect of diluting the tyrosinase by the titration buffer was evaluated, and the effect was compared to the dilution with DMSO. It was observed that DMSO had the same effect on tyrosinase fluorescence as the buffer dilution effect (data not shown). Thus, the effect of DMSO on the flavonoid interaction with tyrosinase was considered negligible at the amount used. Furthermore, no effect was observed on the tyrosinase fluorescence emission spectrum with a similar addition of DMSO (data not shown), suggesting no change in the tyrosinase conformation. Fluorescence quenching is described by the Stern−Volmer equation (*34*):

$$\frac{F_0}{F} = 1 + k_{\text{q}}\tau_0[Q] = 1 + K_{\text{SV}}[Q]$$

where F_0 and F are the fluorescence intensities before and after the addition of the quencher, respectively, k_{q} is the bimolecular quenching constant, τ_0 is the lifetime of the fluorophore in the absence of the quencher, $[Q]$ is the concentration of the quencher, and K_{SV} is the Stern−Volmer quenching constant. Hence, the above equation is applied to determine the K_{SV} using linear regression of a plot of F_0/F against $[Q]$. A linear Stern−Volmer plot is generally indicative of a single class of fluorophores, all equally accessible to the quencher. For the calculation of quenching constants, the data were plotted as a Stern−Volmer plot of F_0/F against $[Q]$, with the quenching constants calculated by linear regression. To investigate the interaction between the copper ions and the flavonoids, stock solutions of each flavonoid (1 mM) were prepared in methanol. Then, 25 μM solutions were prepared in a cuvette-containing phosphate buffer (10 mM, pH 7.4), and the absorption spectra were recorded between 240 and 600 nm. Scans with

Flavonoids as Tyrosinase Inhibitors

J. Agric. Food Chem., Vol. 54, No. 3, 2006   **937**

Table 1. Mushroom Tyrosinase Inhibitory Activities of the Flavonoids Used in the Present Study

compd no.	R3	R5	R6	R7	R8	R2'	R3'	R4'	R5'	R6'	IC50 (μM)
1				OH	OH		OH	OH			0.07
2	OH	OH		OH				OH			3.49
3		OH		OH		OH					8.51
4	OH			OH				OH			8.88
5				OH		OH					12.78
6				OH	OH			OH			12.95
7		OH		OH			OH	OH			17.48
8				OH			OH	OH			24.10
9				OH			OH				59.00
10				OH						OH	59.00
11		OH		OH				OH			74.01
12			OH						OH		98.33
13	OH	OH		OH		OH		OH			99.26
14				OH				OH			118.00
15		OH		OH							137.67
16	OH		OH			OH		OH			139.49
17		OH						OH	OH		174.67
18		OH	OH	OH				OH			174.68
19	OH							OH			177.00
20	OH			OH		OH		OH			181.67
21	OH							OH	OH		222.02
22			OH	OH							236.00
23	OH	OH		OH		OH		OH	OH		277.54
24	OH	OH	OH	OH		OH		OH			314.23
25		OH						OH			393.32

Figure 2. Emission spectra of tyrosinase at λ_{ex} = 290 nm showing the quenching effect of increasing the concentration of compound **1**. Inset: Stern–Volmer plot describing the tyrosinase quenching caused by association with compound **1**.

(Inset legend: F_o/F vs Compound 1 (nM); ● None, ○ 12.9 nM, ▼ 25.7 nM, ▽ 51.0 nM, ■ 75.9 nM, □ 100.5 nM)

5, 10, 25, 50, or 100 μM CuSO$_4$ were taken after 10 s using a DU800 spectrophotometer (Beckman Coulter Inc., CA), which were compared to the flavonoids alone.

Model Build and Molecular Docking. We first built a three-dimensional structure of the catalytic site of the catechol oxidase and hemocyanin with the aid of the published structures of reference proteins whose sequences have about a 40% similarity to *Agaricus bisporous* mushroom tyrosinase. Sweet potato catechol oxidase (*Ipomoea batatas*; PDB code, 1bt3) and octopus hemocyanin (*Octopus doflein*; PDB code, 1js8) were used as reference proteins in this study. The modeling of tyrosinase was initially performed using the HOMOLOGY module of the INSIGHT II (Version 2000, Accelrys Inc., San Diego, CA) and further refined using the Discover version 2.98 of the INSIGHT II.

RESULTS

Tyrosinase Inhibition. Table 1 shows the tyrosinase inhibitory activities (IC$_{50}$) of the flavonoids, which are classified according to the sites in the A and B rings attached to the hydroxyl group(s). In the case of IC$_{50}$ values for items less than 100 μM, the tyrosinase inhibitory activity requires that the flavonoids have hydroxyl groups at the R$_7$ and R$_{4'}$ sites in the A and B rings, respectively, which are exposed in the bold region in the flavonoid structural model. Compound **1**, the best tyrosinase inhibitor among the flavonoids tested, contains two hydroxyl groups (catechol) in each A and B ring. One possible explanation for this result is that one or more of the catechol regions are related to the interaction with the catalytic region containing dicopper ions. When comparing compounds **1** and **8** or **1** and **6**, the flavonoids possessing hydroxyl groups at the R$_{3'}$ and R$_8$ exhibit an increased inhibitory activity by 340- and 180-fold, respectively. In the case of the R$_5$ position, the hydroxyl group is important in raising the inhibitory activities, as shown from the comparisons between the two sets of compounds **3** and **5** and compounds **7** and **8**. However, the hydroxyl group at the R$_3$ position, which divides flavonoids into either a flavone or a flavonol, exhibits a 13-fold reduction in the inhibitory activity by flavonols when compared to compounds **4** and **14**, demonstrating that the flavone derivatives are better tyrosinase inhibitors than the flavonol derivatives when they contain the same hydroxyl groups in their structures.

Other researchers have reported that flavonoids are important plant constituents as antioxidants (*13*, *35*). Yet, relatively few flavonoids are known as tyrosinase inhibitors, except for kaempferol (**2**) and genistein, which are abundant flavonoids in plants. As such, the current study also demonstrates the binding affinity based on the fluorescence quenching of the tryptophan residue in the enzyme that shows interaction between the tyrosinase and the flavonoids.

Fluorescence Quenching. Tryptophan fluorescence has been frequently examined among the intrinsic aromatic fluorophores in tyrosinase molecules to obtain information about conformational changes. The interaction of compound **1** with tyrosinase and the conformational alteration in the tyrosinase are evaluated by measuring the intrinsic fluorescence intensity of the protein before and after the addition of compound **1**. The fluorescence emission spectra of the tyrosinase are collected with different concentrations of compound **1**, which exhibited the most inhibitory activity among the tested flavonoids, and are recorded from a range of 300−450 nm at λ_{ex} 290 nm. The tyrosinase has a strong fluorescence emission with a peak at 337 nm on excitation at 290 nm. **Figure 2** shows that the addition of compound **1** causes a dramatic change in the fluorescence emission spectra. In this study, the fluorescence intensities of the emission peaks are inversely decreased with an increasing concentration of compound **1**. Although the decline in the fluorescence intensity is caused by quenching, there is no significant λ_{em} shift with the accumulation of compound **1**, indicating that the tyrosinase bound to the flavonoids solely depends on the concentration. Moreover, the fact that the flavonoid masks the tryptophan of tyrosinase implies that the tyrosinase became disagglomerated and its structure was loosened.
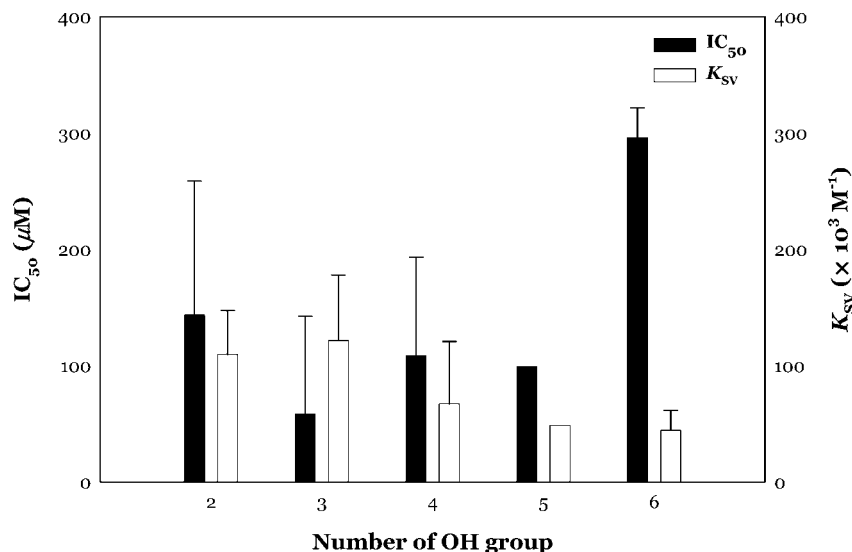
Figure 3. Tyrosinase inhibitory activities and Stern–Volmer quenching constants for flavonoids.

The Stern−Volmer plot inserted in **Figure 2** measures the Stern−Volmer quenching constants of compound **1**. A linear slope in a Stern−Volmer plot is generally indicative of a single class of fluorophores, which are all equally accessible to the quencher. In many cases, the fluorophore can be quenched both by collision and by complex formation with the same quencher. When this is the case, the Stern−Volmer plot exhibits an upward curvature, concave toward the *y*-axis at a high [Q] (*33*). As such, the present results, shown in **Figure 2**, suggest a good linear agreement with the Stern−Volmer plot in the quenching case of compound **1**. Therefore, it is concluded that compound **1** is a good quencher of the fluorophore in tyrosinase.

The hydroxyl group attached to the benzene ring in the stilbene structure is reportedly principal to inhibit tyrosinase and oxyresveratrol; one more hydroxy-substituted stilbene inhibits tyrosinase much more than resveratrol (*36*). However, a discrepancy is observed in the tyrosinase inhibitory activity of the flavonoids possessing numbers of hydroxyl groups. Therefore, whether the number of hydroxyl groups in the flavonoids affects the tyrosinase inhibitory activity and also which structural characteristics of the flavonoids inhibit the tyrosinase are investigated by analyzing the Stern−Volmer quenching constants. As shown in **Figure 3**, the enzyme inhibitory activity of the flavonoids with more than three hydroxyl groups is inversely proportional to their Stern−Volmer quenching constants. Consequently, it appears that an excessive increment of hydroxyl groups in the flavonoids hinders their inhibitory activity toward tyrosinase. This finding leads us to regard the location rather than the number of the hydroxyl groups as more important for the inhibitory activity of the flavonoid since the location of hydroxyl groups alters the binding affinity of flavonoid to tyrosinase. Thus, to examine which locations of the hydroxyl groups in the flavonoids affect the Stern−Volmer quenching constant, a comparison is made of the Stern−Volmer quenching constants of the flavonoids that are subdivided according to their deductive structural vicinity related to the binding affinity to tyrosinase.

The Stern−Volmer quenching constants and tyrosinase inhibitory activities (IC_{50}) are calculated in accordance with the hydroxyl group numbers on each side of the A and B rings and also subdivided into the ether (C−O−C) side and carbonyl (CO−C) side of the C ring. As shown in **Table 2**, the average IC_{50} of the flavonoids decreases with the number of hydroxyl groups along the ether side of the C (middle) ring. It is suggested

Table 2. Relationship between the Tyrosinase Inhibitory Activity and the Stern–Volmer Quenching Constant According to the Ratio of the Hydroxyl Group(s) at Different Localities in Flavonoids

| (no. of OH groups at $R_{2'}$, $R_{3'}$, $R_{4'}$, R_7, and R_8)/ (total no. of OH groups) × 100 (%)	average IC_{50} (μM)	average K_{SV} (×10³ M⁻¹)
33	222.02	59.66
50	191.24	71.68
67	66.52	121.67
100	37.82	131.87

that the hydroxyl groups of A and B rings on the ether side of the C ring in flavonoid also assist in the tyrosinase inhibitory activity. Meanwhile, the hydroxyl groups on the other side, the carbonyl group of the C ring, interfere with the interaction between the flavonoid and the tyrosinase. Thus, these hydroxyl groups do not contribute to the tyrosinase inhibitory activity. Moreover, the Stern−Volmer quenching constants gradually decrease with the number of hydroxyl groups on the same side. Consequently, it is deduced that (i) the tyrosinase inhibitory activity is not dependent on the number of hydroxyl groups in the flavonoids, (ii) the hydroxyl group(s) of A and B rings on the ether side of the C ring primarily quenches the enzyme, (iii) the tyrosinase inhibitory activity of the flavonoids is generated from their quenching the tyrosinase, and finally, (iv) the hydroxyl groups on the carbonyl side of the C ring in the flavonoids disturb the tyrosinase quenching, thus reducing the inhibitory activity. Because hydroxyl groups at specific locations in the flavonoids are found to quench the tyrosinase, it is also examined whether compound **1**, the best tyrosinase inhibitor among the flavonoids tested, actually chelates the copper ion, which has been verified as a metal constituent in the tyrosinase catalytic domain.

Flavonoids as Tyrosinase Inhibitors

J. Agric. Food Chem., Vol. 54, No. 3, 2006 **939**
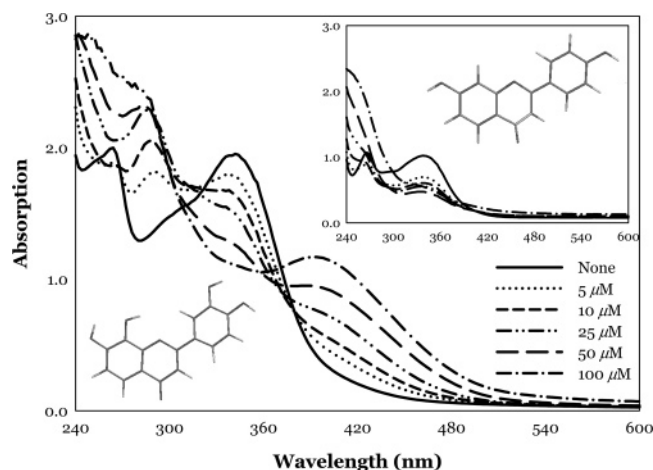
Figure 4. Absorption spectra for compound **1** and the Cu–compound **1** complex at pH 7.4. Inset: Absorption spectra for compound **14** and the Cu–compound **14** complex at the same pH.

Effect of Copper Ion on Tyrosinase Spectra. Flavonoids have been reported to act as an antioxidant due to their innate function in chelating metal ions (*37*). Therefore, the structural correspondence between flavonoids and a copper−flavonoid complex is considered based on collecting the spectra of compounds **1** and **14** treated with increasing concentrations of copper ion. The direct interaction of the flavonoids with the Cu^{2+} ions at pH 7.4 is assessed by UV−visible spectroscopy. The effect of the stepwise increments of the $CuSO_4$ concentration on the spectral characteristics of each flavonoid is described in terms of shifts in band I (320−385 nm) and band II (250−285 nm), related to the B and A ring absorptions, respectively (*38*). The results are shown in **Figure 4**. The flavone, compound **1** contains a 3′,4′-dihydroxy structure in the B ring, 7,8-dihydroxy structure in the A ring, and lacks a free 3-hydroxy group in the C ring. On interaction with the Cu^{2+} ions, new peaks (290 and 390 nm) are produced, which are assumed to be characteristic of a copper−flavone chelate. The interaction between compound **1** and the copper ion(s) shows various significant red shift peaks. For example, the peak at 264 nm for compound **1** is red-shifted to 290 nm, as the catechol group in the A ring in compound **1** binds to the copper ion. In addition, the spectral characteristics of band I involved in the B ring are shifted from 340 to 390 nm, indicating that the catechol group in the B ring participates in the formation of the chelate with the copper ion. However, compound **14** contains two monohydroxy structures in the A and B rings and exhibits a decreasing peak at 340 nm without any wavelength shift. This characteristic bathochromic shift indicates that the chelate formation involves a catechol structure in the A and B rings in compound **1** (**Figure 4**). As such, it is concluded that these inherent characteristics of flavonoids to form a chelate with the copper ion inhibit the tyrosinase by producing an interaction between the flavonoid and copper ion and the catalytic domain of the tyrosinase.

Molecular Docking Analysis. To understand the mechanism underlying the highly potent inhibition of the mushroom tyrosinase by flavonoids, the docking modes of compound **1** are examined in the catalytic sites of the tyrosinase. To obtain the complex structure between the mushroom tyrosinase and compound **1**, the structural information in relation to the active site region with phenylthiourea (PTU) bound to the dinuclear copper site from the X-ray crystal structure of a plant catechol oxidase (*I. batatas*; PDB code, 1bug) is used. Compound **1** was initially found as the best match to the PTU compound in the cocrystal structure. Compound **1** is relaxed using the CVFF force
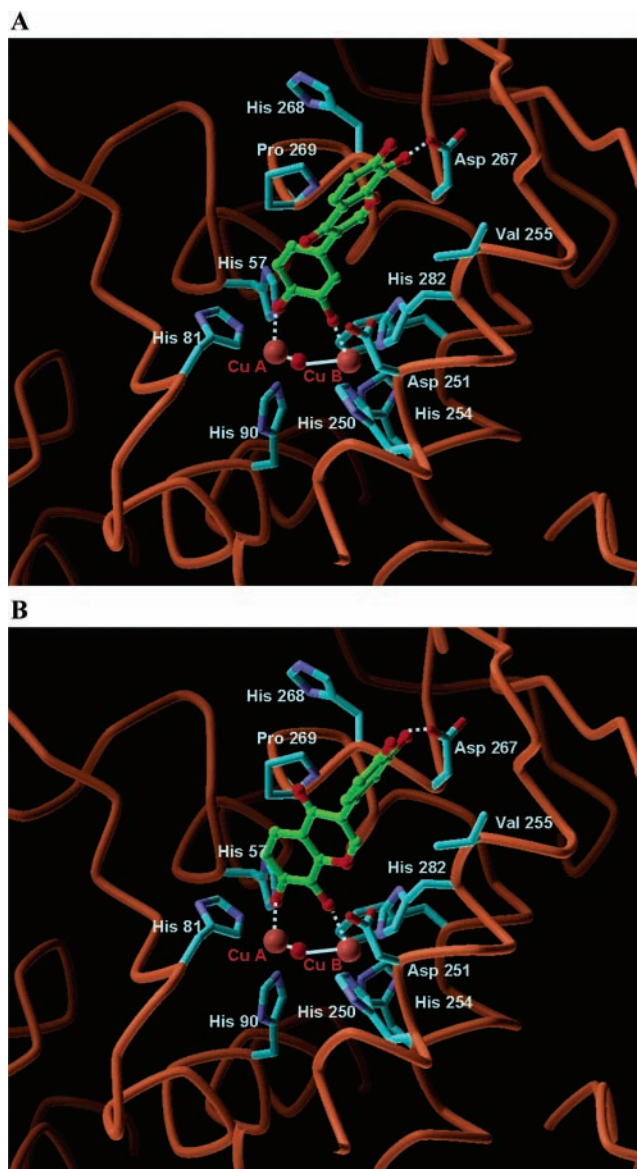
Figure 5. Suggested docking model for compound **1** with mushroom tyrosinase. The homology model of mushroom tyrosinase was constructed using the HOMOLOGY module of INSIGHT II (Version 2000, Accelrys Inc.) and crystal structures of catechol oxidase (PDB code, 1bt3) and octopus hemocyanin (PDB code, 1js8) used as the template molecules. (**A**) The dihydroxy phenyl (C ring) of compound **1** (green) chelates the catalytic dicopper center (cyan). (**B**) The dihydroxy phenyl (A ring) of compound **1** (green) chelates the catalytic dicopper center (cyan) of tyrosinase. Hydrogen bond interactions exist between the hydroxyl groups ($R_{3'}$ and R_8) of compound **1** and the Asp 267 in the vicinity of the tyrosinase catalytic site.

field, as integrated in the Discover version 2.98 of INSIGHT II. As shown in **Figure 5**, compound **1** and the mushroom tyrosinase docking model support the structure−activity relationships (SAR) of the assayed compounds. Inhibition by compound **1** of the mushroom tyrosinase is docked by 3′,4′-dihydroxy phenyl (B ring) chelating to the coppers in the active site. In addition, a hydrogen bond interaction is found to exist between the R_8 hydroxyl group in the A ring of compound **1** and the Asp 267 in the mushroom tyrosinase (**Figure 5A**). Alternatively, 3′,4′-dihydroxy phenyl (B ring) and the R_8 hydroxyl group in the A ring can be replaced by 7,8-dihydroxy phenyl of the A ring and 3′-hydroxyl group of the B ring,

respectively (**Figure 5B**). Consequently, this additional interaction, except for the Cu chelation of compound **1**, appears to strengthen the formation of a Cu—flavonoid chelate, resulting in potent tyrosinase inhibitory activity.

DISCUSSION

Tyrosinase is an enzyme that has several valuable catalytic functions, such as the hydroxylation of phenols into catechols, the dehydrogenation of catechols into *o*-quinones, and the conversion of tyrosine into melanin pigments. This enzyme is also known as a polyphenol oxidase and contains a type 3 copper center, a strongly coupled dinuclear copper active site in the catalytic domain. Interestingly, the inhibitory activity exerted by certain flavonoids and the number and position of the hydroxy substituents seem to play an important role in the inhibitory effect of flavonoid compounds on mushroom tyrosinase activity. It has been reported that a higher number of hydroxyl groups in the benzene ring may play a critical role in exerting the inhibitory effect on tyrosinase activity and that methylated or glycosylated flavonoids exhibit less inhibitory effects (*19*). The present results partially agreed with such former results on the inhibitory activities of flavonoids. However, the current study finds that the direction of the hydroxyl groups in flavonoids also influences the tyrosinase inhibitory activity. In particular, the catechol group apparently binds with the copper ions in the catalytic domain of tyrosinase, thereby inhibiting the tyrosinase activity. Although flavonoids possess a catechol group, their inhibitory activity toward tyrosinase can be lost or reduced due to steric hindrance with excessive hydroxyl moiety. As such, the relationship between the flavonoid structures and the inhibitory activity on tyrosinase is deduced.

Kubo et al. proposed that a flavone, quercetin, preferentially displaced L-DOPA from the active site of the cofactor due to its structural resemblance, as most competitive inhibitors closely resemble, at least in part, the structure of the substrate. In the case of quercetin, a portion of the structure including the B ring is analogous to L-DOPA. Consequently, they explained that a partial resemblance may overlap the lock-and-key model to tyrosinase, thereby influencing the inhibitory activity on tyrosinase (*20*). However, when comparing flavonoids similar to quercetin, their inhibitory activities (IC$_{50}$) on tyrosinase fluctuated from 20 to 300 μM, as shown in **Table 1**. These differences in the IC$_{50}$ values for the flavonoids were not related to a structural resemblance but rather related to the ability to form the chelate with copper ions. It is further demonstrated in the current study that the location of the hydroxyl groups in the benzene ring is of importance regarding the inhibitory activity of flavonoids. Meanwhile, flavonoid derivatives containing sugar hardly show any tyrosinase inhibitory activity, as the bulky sugar moiety hinders their approach to the active site in the enzyme.

Because the catechol groups in the A and B rings play an important role in chelating the copper ion, the Stern—Volmer quenching constants of various flavonoids are calculated. Moreover, the copper chelation by flavonoids is investigated to explain the association area between flavonoids and copper ions to produce tyrosinase inhibiting capacity.

Subsequently, the catalytic site of tyrosinase is proposed and built through homology modeling. The model is then used to support insights into understanding the interaction between an inhibitor and the dinuclear copper site of the mushroom tyrosinase. Although the enzyme-based assay results do not always reflect the molecular level interactions between the enzyme and the inhibitors due to their substituent positions, the docking model does provide some clues to the function of flavonoid derivatives as antityrosinase agents. Therefore, the tyrosinase-binding model with the potent inhibitor, compound **1**, can be useful for further virtual library screening or refinement of antityrosinase lead compounds. The molecular docking study implies that several inhibitory molecules preferentially bind to the catalytic site of tyrosinase. The resulting model shows a satisfactory structural similarity both in fitting and in predicting validation. The molecular docking analysis also supports the hypothesis that the dicopper catalytic site of tyrosinase is a preferential binding site for flavonoids, thereby explaining the variety of tyrosinase inhibitory activities by flavonoids substituted with several hydroxyl groups in **Table 1**.

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Flavonoids as Tyrosinase Inhibitors

J. Agric. Food Chem., Vol. 54, No. 3, 2006 **941**

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