Flavonols from Saffron Flower: Tyrosinase Inhibitory Activity and Inhibition Mechanism†

Isao Kubo* and Ikuyo Kinst-Hori

Department of Environmental Science, Policy and Management, University of California, Berkeley, California 94720-3112

A common flavonol, kaempferol, isolated from the fresh flower petals of *Crocus sativus* L. (Iridaceae) was found to inhibit the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) catalyzed by mushroom tyrosinase with an ID_{50} of 67 μg/mL (0.23 mM). Interestingly, its 3-*O*-glycoside derivatives did not inhibit this oxidation. The inhibition kinetics analyzed by a Lineweaver−Burk plot found kaempferol to be a competitive inhibitor, and this inhibitory activity presumably comes from its ability to chelate copper in the enzyme. This copper chelation mechanism can be applicable for all of the flavonols as long as their 3-hydroxyl group is free. However, quercetin, kaempferol, and galangin each affect the oxidation of L-tyrosine in somewhat different ways.

Keywords: *Crocus sativus; kaempferol; flavonols; tyrosinase inhibitory activity; competitive inhibitor; copper chelator*

INTRODUCTION

The spice saffron, which consists of the flower stigmas of *Crocus sativus*, is widely used as coloring and flavoring for food. In the production of 1 kg of saffron, more than 160,000 flowers of *C. sativus* are needed. In comparison with the stigma, the flower petal is almost completely neglected in commercial terms, although it is available in far greater tonnage and there is considerable potential for its exploitation. In a previous paper, we reported that kaempferol 3-*O*-sophoroside (**1**) was efficiently isolated in quantities from the methanol extract of the flower petals of *C. sativus* by rotation locular countercurrent chromatography (RLCC) together with a minute amount of kaempferol (**2**) without employing any solid packing material (Kubo et al., 1988). It appears that kaempferol 3-*O*-sophoroside and kaempferol are available in large quantity from the flower petals of *C. sativus*, because the latter is easily obtainable from the former by hydrolysis. We also reported the isolation of kaempferol and its 3-*O*-glucoside, together with quercetin and its 3-*O*-glucoside, as antioxidants from Mexican medicinal plants (Haraguchi et al., 1997).

Tyrosinase (EC 1.14.18.1) is a copper-containing enzyme that catalyzes two distinct reactions of melanin synthesis, the hydroxylation of a monophenol and the conversion of an *o*-diphenol to the corresponding *o*-quinone. In these oxidation reactions three different forms of binuclear copper in the active site are involved (Lerch, 1981; Wilcox et al., 1985). Tyrosinase is also known as a polyphenol oxidase (PPO) (Mayer, 1987; Whitaker, 1995), and the browning of some fruits, vegetables, and crustaceans due to tyrosinase causes a significant decrease in their nutritional and market values. This unfavorable darkening from enzymatic oxidation of phenols has therefore been of great concern (Friedman, 1996), and tyrosinase inhibitors should have broad applications. In addition, tyrosinase inhibitors have also become increasingly important in medicinal (Mosher et al., 1983) and cosmetic (Maeda and Fukuda, 1991) products in relation to hyperpigmentation. The flavonols isolated from the crocus flower petals and their congeners were studied for their tyrosinase inhibitory activity.

MATERIALS AND METHODS

General. All of the procedures used were the same as previously described (Kubo et al., 1994, 1995; Kubo and Yokokawa, 1992). UV−visible (240−540 nm) spectra were recorded in 0.067 M phosphate buffer (pH 6.8) by a Hitachi 100−80 spectrophotometer. The bathochromic shift of flavonols (0.05 mM) was monitored by adding 0.125 mM of $CuSO_4$.

Isolation of Flavonoids. The procedures employed for isolation of flavonoids, kaempferol, and its 3-*O*-sophoroside from the MeOH extract of the flower petals of *C. sativus* were previously described (Kubo et al., 1988). Their final purification was achieved by recycle HPLC using an ODS C_{18} column.

Plant Material. The dried flower stigmas of *C. sativus*, known as spice saffron, were purchased in the San Francisco Bay area. The above chromatographic method was applied for its MeOH extract, but neither kaempferol nor its 3-*O*-sophoroside was detected.

Chemicals. Quercetin and its 3-*O*-glycosides were available from our previous work (Kubo et al., 1994). Kaempferol and its 3-*O*-glucoside were generous gifts from Dr. M. Takasaki and Dr. M. Kozuka. 3-Hydroxyflavone was kindly provided by Prof. T. Kamikawa. Rutin, L-DOPA, L-tyrosine, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). Kojic acid, galangin, and $MgSO_4$ ($7H_2O$) were obtained from Aldrich Chemical Co. (Milwaukee, WI). $CaSO_4$ ($2H_2O$) and $CuSO_4$ ($5H_2O$) were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). The flavonols purchased and provided as gifts were used as received for comparison.

Enzyme Assay. The mushroom tyrosinase used for the bioassay was purchased from Sigma. Although mushroom tyrosinase differs somewhat from other sources (van Gelder et al., 1997), this fungal source was used for the present experiment because of its ready availability. The samples were

* To whom correspondence should be addressed. Phone: (510) 643-6303. Fax: (510) 643-0215. E-mail: ikubo@uclink4.berkeley.edu.

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Table 1. Tyrosinase Inhibitory Activity of Flavonols and Kojic Acid[a]

compound	ID_{50} (mM)	mode of inhibition
kaempferol (**2**)	0.23	competitive
quercetin (**4**)	0.07	competitive
kojic acid (**7**)	0.014	mixed[b]
buddlenoid A (**8**)	0.39[c]	*d*
buddlenoid B (**9**)	0.44[c]	*d*
galangin (**10**)	*e*	*d*

[a] With respect to L-DOPA. [b] Chen et al., 1991. [c] Kubo and Yokokawa, 1992. [d] Not tested. [e] Unable to establish.

first dissolved in DMSO and were used in the experiments at 30 times dilution. Unless otherwise specified, all of the samples tested were preliminarily assayed at 167 μg/mL. It should be noted however that several flavonols tested, such as kaempferol and galangin, are hardly soluble in the water-based test solution at this concentration. Although tyrosinase catalyzes a reaction between two substrates, a phenolic compound and oxygen, the assay was carried out in air-saturated solutions. Therefore, after several minutes, dopachrome formation reaches a plateau because all of the available oxygen in cuvette is consumed. The enzyme activity was monitored by dopachrome formation at 475 nm up to the appropriate time (usually not exceeding 10 min, unless otherwise specified). The extent of inhibition by the addition of samples (Table 1) is expressed as the percentage necessary for 50% inhibition (ID_{50}).

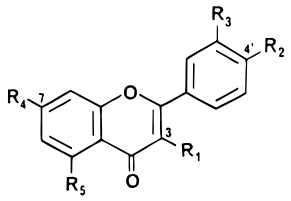
The assay was performed as previously described (Masamoto et al., 1980) with slight modifications. First, 1 mL of 2.5 mM L-DOPA or L-tyrosine solution was mixed with 1.8 mL of 0.1 M phosphate buffer (pH 6.8) and incubated at 25 °C for 10 min. Then, 0.1 mL of each sample solution and 0.1 mL of 1380 units/mL tyrosinase in aqueous solution (added last) were added in this order to the mixture. This solution was immediately monitored for the formation of dopachrome by measuring the linear increase in optical density at 475 nm.

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 samples solution, and 0.1 mL of the aqueous solution of the mushroom tyrosinase (138 units). The mixture was preincubated at 25 °C for 5 min. Then, 0.4 mL of 6.3 mM L-DOPA was added, and the reaction was monitored at 475 nm for 2 min.

To check if flavonols can chelate copper in the enzyme, the UV−visible spectra (240−540 nm) was measured. The mixture, consisting of 1.8 mL of 0.1 M phosphate buffer (pH 6.8), 1.0 mL of water, 0.1 mL of the samples (0.05 mM) solution, and 0.1 mL of the aqueous solution of the mushroom tyrosinase (138 units), was incubated at 25 °C for 30 min, and then the spectra were recorded.

RESULTS AND DISCUSSION

In a preliminary screening, kaempferol was found to inhibit the oxidation of L-DOPA catalyzed by mushroom tyrosinase (Kubo et al., 1994). A more detailed bioassay with the purified kaempferol was conducted, and this common flavonol was found to exhibit a concentration-dependent inhibitory effect on the oxidation. It should be noted however that kaempferol is not very soluble in the water-based test solution. Therefore, the ID_{50} value was obtained by using three concentrations in which this flavonol was soluble. The addition of 5.2 and 10.4 μg/mL of kaempferol to the assay system containing L-DOPA caused the inhibition of tyrosinase by 16% and 25%, respectively. The inhibition was linearly elevated to 34% when 20.8 μg/mL of kaempferol was added. On the basis of this observation, the ID_{50} of kaempferol was estimated to be 67 μg/mL (0.23 mM). This inhibitory activity was not diminished by adding excess Mg^{2+} or Ca^{2+}. Furthermore, this flavonol itself did not serve as a substrate at all. In contrast to kaempferol (**2**), its 3-*O*-glycoside analogues, kaempferol 3-*O*-sophoroside (**1**) and



1 R_1=OGlc^2-Glc, R_2=R_4=R_5=OH, R_3=H
2 R_1=R_2=R_4=R_5=OH, R_3=H
3 R_1=OGlc, R_2=R_4=R_5=OH, R_3=H
4 R_1=R_2=R_3=R_4=R_5=OH
5 R_1=OGlc, R_2=R_3=R_4=R_5=OH
6 R_1=OGlc^6-Rha, R_2=R_3=R_4=R_5=OH
8 R_1=R_2=R_3=R_5=OH, R_4=OGlc^6-*p*-coumaroyl
9 R_1=R_2=R_5=OH, R_3=OCH_3, R_4=OGlc^6-*p*-coumaroyl
10 R_1=R_4=R_5=OH, R_2=R_3=H
11 R_1=OH, R_2=R_3=R_4=R_5=H
12 R_1=H, R_2=OGlc, R_3=R_4=R_5=OH
13 R_1=H, R_2=R_3=R_5=OH, R_4=OGlc

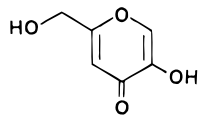


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Figure 1. Chemical structures of flavonols and kojic acid.

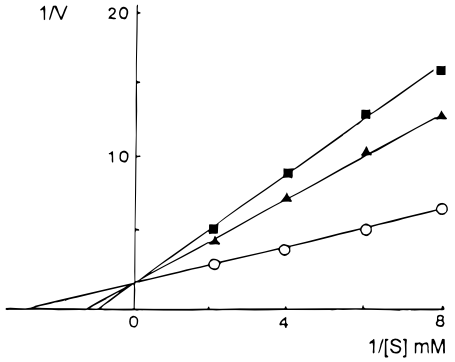
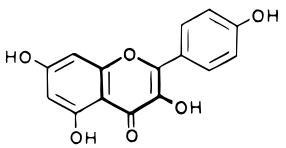


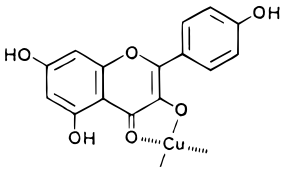
Figure 2. Lineweaver−Burk plots of mushroom tyrosinase and L-DOPA without (○) and with [(▲) 0.03 mM and (■) 0.05 mM] kaempferol. 1/*V*: 1/Δ475 nm/min.

kaempferol 3-*O*-glucoside (**3**) (see Figure 1 for structures), did not exhibit any inhibitory activity for the L-DOPA oxidation up to 1 mg/mL. These 3-*O*-glycosides are neither inhibitors nor substrates. The results obtained indicate that the free hydroxyl group at the C-3 position plays an important role in eliciting tyrosinase inhibitory activity. This finding can be supported by similar observations between quercetin (**4**) and its 3-*O*-glycosides, isoquercitrin or quercetin 3-*O*-glucoside (**5**) and rutin or quercetin 3-*O*-rutinoside (**6**) (Kubo et al., 1994). That is, quercetin inhibited the oxidation of L-DOPA catalyzed by mushroom tyrosinase with an ID_{50} of 22 μg/mL (0.07 mM), but its 3-*O*-glycoside derivatives (**5** and **6**) did not inhibit this oxidation up to 1 mg/mL. Interestingly, these 3-*O*-glycosides were not oxidized as substrates either, though both possess the catechol moiety in their molecules.

In addition, the inhibitory kinetics of kaempferol were analyzed by a Lineweaver−Burk plot as shown in Figure 2. The three lines, obtained from the uninhibited enzyme and from the two different concentrations of kaempferol, intersected on the vertical axis. The result



1a



1b

Figure 3. Chemical structure of kaempferol showing 3-hydroxy-4-keto moiety (**1a**) and chelation copper (**1b**).

demonstrates that kaempferol exhibited competitive inhibition for the oxidation of L-DOPA catalyzed by mushroom tyrosinase. Furthermore, a preincubation experiment with the enzyme in the presence of 0.07 mM kaempferol and in the absence of the substrate resulted in an increase of inhibitory activity from 24% to 58%. It should be noted however that the enzyme preincubated in the experiment was mostly *met*-tyrosinase, known as the resting form of the enzyme. The binuclear copper active site in the *met*-tyrosinase is bicupric and unable to bind oxygen, and kaempferol is not supposed to bind with it as a monophenol. The observed difference is not entirely clear but, at least in part, can be explained by postulating that kaempferol chelates copper in the *met*-tyrosinase and inhibits the enzyme activity. This was supported by the previous reports that some flavonols chelate copper (Thompson et al., 1976; Hudson and Lewis, 1983). It appears that, as shown with a bold line, a portion of the structure **1a** (3-hydroxy-4-keto moiety) (see Figure 3) in kaempferol is clearly superimposable with kojic acid (**7**). The inhibition exerted by kojic acid is well established as coming from its ability to chelate copper in the enzyme (Kahn et al., 1995; Beelik, 1956). The structural similarity suggests that kaempferol also can chelate copper in the enzyme. In the UV−visible spectrum of kaempferol, the absorption maximum of 362 nm was shifted to 404 nm by adding excess Cu^{2+}, as shown in Figure 4. This characteristic bathochromic shift indicates that the chelate formation involves the 4-carbonyl and 3-hydroxyl groups (Mabry et al., 1970). The fact that the same bathochromic shift was not observed with its 3-glycoside derivatives (**1** and **3**) further supports the above conclusion. As a result, the chelation **1b** is probably the main inhibition mechanism of kaempferol. This consequently explains why kaempferol was not oxidized by the enzyme as a substrate.

The experiment was extended to see if the flavonols actually can chelate copper in the enzyme, and we first attempted to utilize their bathochromic shift for this purpose. However, kaempferol did not show the same bathochromic shift after incubation with mushroom tyrosinase at 25 °C for 30 min. Instead, kaempferol showed the noticeable shift to a short wavelength as shown in Figure 5. The result indicates that this flavonol can chelate copper in the enzyme. However, kaempferol in the resulting complex can no longer be a planar structure and should be somehow twisted. Needless to add, a similar result was also observed with

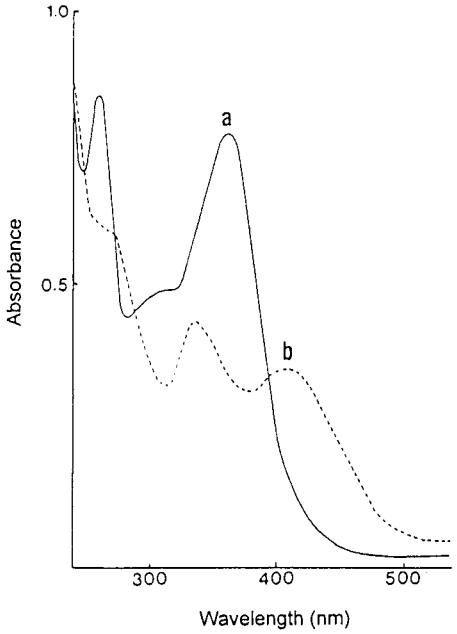


Figure 4. UV−visible spectrum of kaempferol (0.05 mM): (a) without and (b) with 0.125 mM $CuSO_4$.

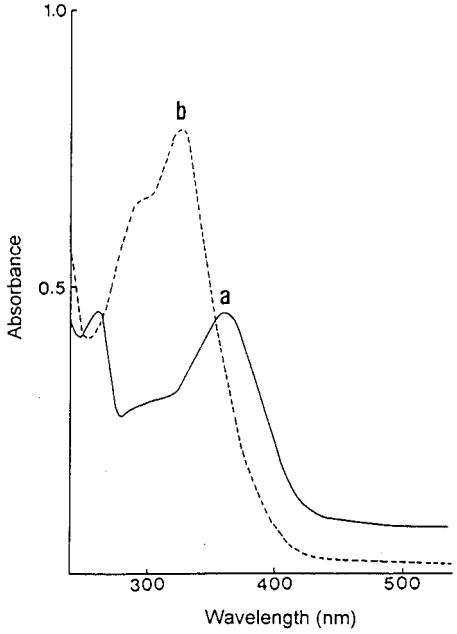


Figure 5. UV−visible spectrum of kaempferol (0.05 mM): (a) without and (b) with 46 units/mL tyrosinase.

quercetin. In contrast to kaempferol and quercetin, their 3-*O*-glycosides (**1**, **3**, **5**, and **6**) did not show this shift, indicating that they do not chelate copper in the enzyme and as a result do not inhibit the enzyme activity.

Interestingly, the inhibitory activity exerted by kaempferol is somehow different from that with kojic acid, which was previously reported to affect through the *oxy*-form of tyrosinase (Cabanes et al., 1994). It should be noted however that the result reported was obtained against tyrosinase isolated from frog epidermis and that this tyrosinase may be different from mushroom tyrosinase. Therefore, we tested kojic acid to determine if the above result is also the case against mushroom tyrosinase. In a similar preincubation experiment, kojic acid seemed to affect through the *oxy*-form. Although this difference may indicate a certain interaction between inhibitors and the tertiary structure

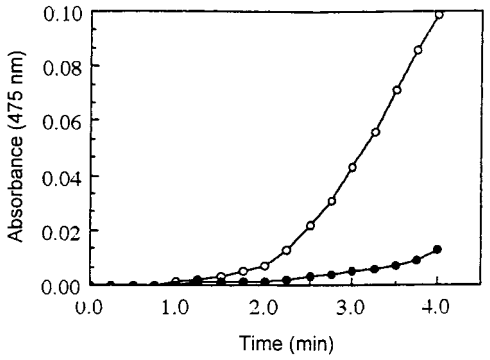


Figure 6. Inhibitory effect on the rate of hydroxylation of L-tyrosine by mushroom tyrosinase: (○) without and (●) with galangin (0.02 mM).

of the enzyme, this remains to be solved because the structure of mushroom tyrosinase has not yet been established.

We have previously reported the two tyrosinase inhibitory flavonols, buddlenoids A (**8**) and B (**9**), isolated from the aerial part of a Bolivian medicinal plant *Buddleia coriacea* REMY (Loganiaceae). The sugar moiety in these flavonols is located at the 7-position and does not block their chelate formation site in the molecules. Their inhibition mechanism could not be established because of their limited availability (Kubo and Yokokawa, 1992). It appears now however that these flavonol derivatives should be copper chelators and thus competitive inhibitors on the basis of the above-mentioned findings. The copper chelation mechanism found with kaempferol in this study can be applicable for all of the flavonols, as long as their 3-hydroxyl group is free. To further support this finding, a simpler flavonol, galangin (**10**), was also assayed. This flavonol is hardly soluble in the water-based test solution, and the appropriate data could not be obtained equivocally. Nevertheless, it seemed to show inhibitory activity almost comparable to that of kaempferol at the concentrations (<0.05 mM) in which it was soluble. In addition, it was also attempted to assay the simplest compound in the flavonol series, 3-hydroxyflavone (**11**), since this flavonoid was reported to show a similar bathochromic shift (Hudson and Lewis, 1983), but it was not soluble in the water-based test solution.

Tyrosinase contains a strongly coupled binuclear copper active site and functions both as a monophenolase (monophenol + O_2 → *o*-diphenol + H_2O) and as an *o*-diphenolase (2 *o*-diphenol + O_2 → 2 *o*-quinone + 2 H_2O) (Sánchez-Ferrer et al., 1995). The discussion so far described is on the basis of the experiment using L-DOPA as a substrate. Therefore, the activity aforementioned is *o*-diphenolase inhibitory activity of mushroom tyrosinase. It should be noted that the lag time is known for the oxidation of monophenolic substrates such as L-tyrosine to L-DOPA. This lag can be shortened or abolished by the presence of reducing agents known as cofactors, especially *o*-diphenols such as L-DOPA and catechin. It therefore appears that quercetin (*o*-diphenol) can be expected as an alternative cofactor to initiate this hydroxylase (monophenolase) activity but kaempferol and galangin cannot be. In fact, L-tyrosine was oxidized by the enzyme without the lag phase in the presence of quercetin. Thus, quercetin completely abolished this lag phase. Needless to add, kaempferol and galangin did not suppress this lag time. It appears that quercetin activates monophenolase activity as a cofactor but inhibits *o*-diphenolase activity and that kaempferol and galangin do not activate monophenolase activity but inhibit *o*-diphenolase activity. As expected from these results, quercetin 3-*O*-glycosides (**5** and **6**) also suppressed the lag phase but not completely, though these 3-*O*-glycosides behave as neither inhibitors nor substrates.

In comparison, the lag time can be extended by monophenolase inhibitors such as tropolone (Kahn and Andrawis, 1985). Kaempferol did not extend the lag time when L-tyrosine was used as a substrate. However, galangin significantly lengthened this lag phase, as shown in Figure 6. It is therefore apparent that galangin inhibits monophenolase activity but kaempferol does not, though both inhibit diphenolase activity of mushroom tyrosinase. The reason for this difference remains to be clarified. As far as the oxidation of L-tyrosine catalyzed by mushroom tyrosinase is concerned, quercetin, kaempferol, and galangin affect mushroom tyrosinase in different ways. As described above, the hydroxyl group at the 3-position in flavonols relates to the activity but may not be essential because other types of flavonoids, such as luteolin 4′-*O*-glucoside (**12**) and luteolin 7-*O*-glucoside (**13**), lacking this 3-hydroxyl group still showed inhibitory activity (Kubo et al., 1995). The inhibition mechanisms of other types of flavonoids will be described separately.

In this experiment, kaempferol has been characterized as a tyrosinase inhibitor in the flower petals of *C. sativus*, which are not eaten. We tried to isolate the same flavonols from the flower stigmas of *C. sativus* known as saffron, but the result was negative. However, kaempferol and its 3-*O*-glycoside derivatives have been identified in various vegetables, fruits, and beverages such as French beans (Hempel and Boehm, 1996), onions (Park and Lee, 1996), asparagus (Kartnig et al., 1985), blueberries (Kader et al., 1996), red raspberry juice (Rommel and Wrolstad, 1993), grapefruit juice (Ha et al., 1995), teas (Hertog et al., 1993), and honey (Ferreres et al., 1991). In addition to this tyrosinase inhibitory activity, kaempferol and its 3-*O*-glycoside derivatives are known to exhibit antioxidant activity (Torel et al., 1986; Larson, 1988; Jovanovic et al., 1994; Vinson et al., 1995; Haraguchi et al., 1997). Furthermore, kaempferol was reported to have antitumor promoting activity in mouse skin carcinogenesis (Yasukawa et al., 1990), anti-inflammatory and antiulcer activities (Goel et al., 1988), and inhibitory activity of HIV protease (Brinkworth et al., 1992). Kaempferol 3-*O*-sophoroside was also reported to have analgesic activity (Palanichamy and Nagarajan, 1990). It is therefore clear that kaempferol and its analogues are worthy of further studies as potential tyrosinase inhibitors.

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