Tyrosinase Inhibitors from Cumin

Isao Kubo* and Ikuyo Kinst-Hori

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

Cuminaldehyde (*p*-isopropylbenzaldehyde) was identified as a potent mushroom tyrosinase inhibitor from cumin, a common food spice. This benzaldehyde derivative was found to inhibit the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) by mushroom tyrosinase with an ID_{50} of 7.7 μg/mL (0.05 mM). Its oxidized analogue, cumic acid (*p*-isopropylbenzoic acid), was also characterized to inhibit this oxidation with an ID_{50} of 43 μg/mL (0.26 mM). These two inhibitors affect mushroom tyrosinase activity in different ways.

Keywords: *Cumin; cuminaldehyde; cumic acid; tyrosinase inhibitory activity; noncompetitive inhibition; Schiff base formation*

INTRODUCTION

Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase (PPO) (Zawistowski et al., 1991; Whitaker, 1995; Mayer and Harel, 1991), is a copper containing enzyme widely distributed in the phylogenetic scale. It is responsible for not only melanization in animals but also browning in plants. The latter case is considered to be deleterious to the color quality of plant derived foods and beverages. Tyrosinase catalyzes the oxidation of phenolic substrates to *o*-quinones, which are then polymerized to brown, red, or black pigments. This unfavorable darkening from enzymatic oxidation generally results in a loss of nutritional value and has been of great concern (Friedman, 1996). Similarly, the unfavorable browning caused by tyrosinase on the surface of seafood products has also been of great concern (Ogawa et al., 1984). In addition, tyrosinase inhibitors have become increasingly important in cosmetic (Maeda and Fukuda, 1991) and medicinal (Mosher et al., 1983) products in relation to hyperpigmentation. Hence, tyrosinase inhibitors should have broad applications, and the inhibitors isolated from regularly consumed condiments and flavors of foods and beverages may be superior compared to nonnatural products.

The seeds of *Cuminum cyminum* L. (Umbelliferae) are known as cumin, a product of the Mediterranean Basin (Boelens, 1991), and widely used as a food spice. In our continuing search for tyrosinase inhibitors from plants (Kubo, 1997), the ether extract of the seeds of *C. cyminum* was found to inhibit the oxidation of L-DOPA by mushroom tyrosinase and subjected to further fractionation.

MATERIALS AND METHODS

General. General procedures were the same as previous work (Kubo and Yokokawa, 1992; Kubo et al., 1995).

Plant Material. The seeds of *C. cyminum* were purchased at market places in Singapore and the San Francisco Bay area.

* Author to whom correspondence should be addressed (phone (510) 643-6303; fax (510) 643-0215; e-mail ikubo@uclink.berkeley.edu).

Extraction and Identification. The seeds (120 g) of *C. cyminum* were extracted with ether (×3) at ambient temperatures. Repeated column chromatographies on SiO_2 gave 18 mg of cuminaldehyde and 0.3 mg of cumic acid as active principles, identical in all respects including spectroscopic data to authentic samples. Identification of these two phenolics in the ether extract was also achieved by HPLC.

Chemicals. Cuminaldehyde, cumic acid, anisic acid, benzaldehyde, anisaldehyde, *p*-hydroxybenzaldehyde, and L-DOPA used for the assay were purchased from Aldrich Chemical Co. (Milwaukee, WI) and were used as received. L-Tyrosine, benzoic acid, vanillin, and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO) and were used as received.

Enzyme Assay. The mushroom tyrosinase (EC 1.14.18.1) used for the bioassay was purchased from Sigma Chemical Co. (St. Louis, MO). Although mushroom tyrosinase differs somewhat from other sources (van Gelder et al., 1997), this fungal source was used for the experiment because it is readily available. Their effect on the enzyme was determined by spectrophotometry (dopachrome formation at 475 nm) up to the appropriate time (usually not longer than 10 min). The preliminarily assay was tested at 167 μg/mL. All the samples were first dissolved in DMSO and used for the experiment at 30 times dilution.

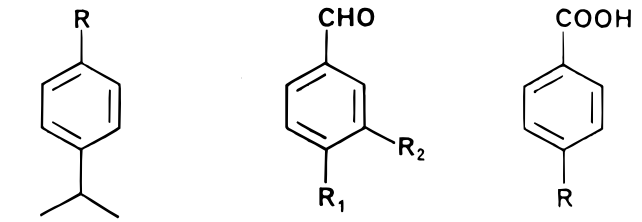
The assay was performed as previously described (Masamoto et al., 1980). Thus, 1 mL of a 2.5 mM L-DOPA or L-tyrosine solution was mixed with 1.8 mL of 0.1 M phosphate buffer (pH 6.8) and incubated at 25 °C for 10 min. Then, 0.1 mL of the sample solution and 0.1 mL of the aqueous solution of the mushroom tyrosinase (138 units, added last) were added to the mixture to immediately measure the initial rate of 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 (equivalent amount of ID_{50}), 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 solution was added, and the reaction was monitored at 475 nm for 2 min.

RESULTS AND DISCUSSION

Bioassay guided fractionation of the ether extract of cumin led to the isolation of two active principles. Cuminaldehyde (*p*-isopropylbenzaldehyde) (**1**), the principal inhibitor, and with cumic acid (*p*-isopropylbenzoic acid) (**2**) in minute amounts (Figure 1) were isolated

10.1021/jf980226+ CCC: $15.00
Published on Web 12/03/1998



1. R=CHO
2. R=COOH
3. $R_1=R_2=H$
5. $R_1=OH$, $R_2=H$
6. $R_1=OCH_3$, $R_2=H$
8. $R_1=OH$, $R_2=OCH_3$
4. R=H
7. $R=OCH_3$

Figure 1. Chemical structures of cuminaldehyde (**1**) and cumic acid (**2**) and their congeners (**3**−**8**).

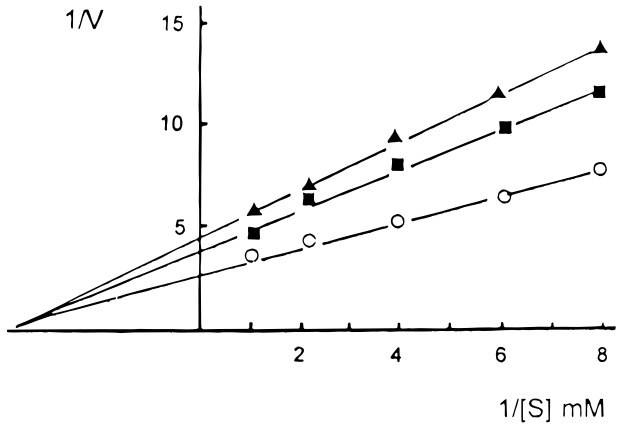


Figure 2. Lineweaver−Burk plots of mushroom tyrosinase and L-DOPA without (○) and with cuminaldehyde [(■) 0.02 mM and (▲) 0.03 mM]. 1/V: 1/475 nm/min.

after repeated column chromatographies on SiO_2 and identified by spectroscopic methods. The bioassay with pure cuminaldehyde showed a dose-dependent inhibitory effect on the oxidation of L-DOPA, and the ID_{50} was established as 7.7 μg/mL (0.05 mM) which is about 16-fold more potent than that of benzaldehyde (**3**). The inhibition kinetics analyzed by a Lineweaver−Burk plot indicated that cuminaldehyde is a noncompetitive inhibitor with respect to L-DOPA as shown in Figure 2. In addition, preincubation of the enzyme in the presence of 0.05 mM of cuminaldehyde and in the absence of L-DOPA did not decrease the enzyme activity significantly. The result likely suggests that cuminaldehyde is an inhibitor rather than an inactivator of the enzyme (Kahn and Andrawis, 1985). It should be noted however that most of the enzyme used is *met*-tyrosinase, known also as the resting form of the enzyme. The aldehyde group is generally known to react with biologically important nucleophilic groups such as sulfhydryl, amino, or hydroxyl groups. Formation of a Schiff base with a primary amino group in the enzyme is more likely, since the aromatic nucleus is known to stabilize it by conjugation (Schauenstein et al., 1977). The potent inhibitory activity exerted by cuminaldehyde could be in part based on the assumption that the enzyme [**E**] is complexed with cuminaldehyde [**I**] and the resulting complex [**EI**] is inactive.

The bioassay with the pure cumic acid also showed a dose-dependent inhibitory effect on the oxidation of L-DOPA. The ID_{50} was established as 43 μg/mL (0.26 mM) which is about 5-fold less effective than cuminaldehyde. This is not consistent with the previous reports that the aldehydes are less potent inhibitors than the

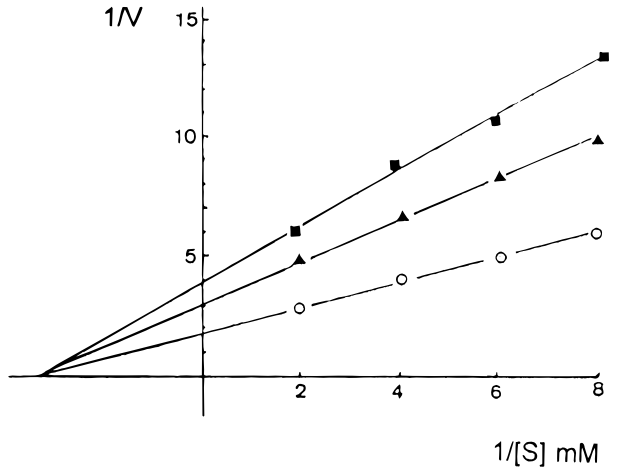


Figure 3. Lineweaver−Burk plots of mushroom tyrosinase and L-DOPA without (○) and with cumic acid [(▲) 0.11 mM and (■) 0.18 mM]. 1/V: 1/475 nm/min.

corresponding phenolic acids (Wilcox et al., 1985). However, it is not easy to relate our current data precisely to those of earlier reports because of differences in substrates, test methods, and sources of the enzyme. The inhibition kinetics of cumic acid were analyzed by a Lineweaver−Burk plot which indicated that cumic acid is a noncompetitive inhibitor for the oxidation of L-DOPA as shown in Figure 3. In addition, preincubation of the enzyme in the presence of 0.26 mM of cumic acid and in the absence of L-DOPA did not decrease the enzyme activity significantly. Similar to benzoic acid (**4**), a well documented tyrosinase inhibitor (Duckworth and Coleman, 1970; Pifferi et al., 1974; Gutteridge and Robb, 1975; Conrad et al., 1994), cumic acid binds to the coupled binuclear copper active site with the carboxylic group and can be classified as a HA-type inhibitor (Conrad et al., 1994), which is a substrate analogue in that the carboxylate is conjugated into an aromatic ring, producing a planar structure. As expected, this HA-type inhibitor did not serve as a substrate (Passi and Nazzaro-Porro, 1981; Conrad et al., 1994). The phenolic compounds substituted in the ring position para to the carboxyl was reported to bind marginally better than those with no substituents (Wilcox et al., 1985) and this is the case for cumic acid. The introduction of an electron-donating isopropyl group at the para position in cumic acid increased the inhibitory activity compared to benzoic acid. The ID_{50} of the former shows about 2.5-fold more effective than that of the latter.

Finding the potent tyrosinase inhibitory activity of cuminaldehyde and cumic acid led us to compare with several closely related congeners such as *p*-hydroxybenzaldehyde (**5**), anisaldehyde (**6**), anisic acid (**7**), and vanillin (**8**), in addition to benzaldehyde and benzoic acid. Despite their close structural similarity, these analogues did not show superior inhibitory activity than cuminaldehyde as shown in Table 1. Among them, anisaldehyde exhibited the most potent inhibitory activity, but its ID_{50} is still 6-fold less effective than that of cuminaldehyde. Vanillin (4-hydroxy-3-methoxybenzaldehyde), an important flavoring agent, showed almost no activity, so it was not further studied. The ID_{50}s of anisaldehyde and cuminaldehyde are about 2.5- and 16-fold more effective than that of benzaldehyde. It is known that the Schiff base is largely governed by those factors affecting the stability of the carbon−nitrogen

Table 1. ID_{50} and Mode of Inhibition of the Cumin and Related Phenolics

compds tested	ID_{50} (mM)	mode of inhibition[a]
cuminaldehyde	0.05	noncompetitive
cumic acid	0.26	noncompetitive
benzaldehyde	0.82	noncompetitive
benzoic acid	0.64	mixed
p-hydroxybenzaldehyde	1.2	competitive
anisaldehyde	0.32	noncompetitive
anisic acid	0.64	noncompetitive
vanillin	70.0	*b*

[a] With respect to L-DOPA. [b] Not tested.

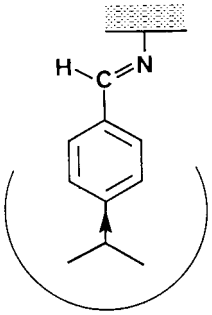


Figure 4. Isopropyl group at the para position in cuminaldehyde contributes not only to stabilize the Schiff base through the inductive effect (→) but also to embrace the formed Schiff base by the hydrophobic protein pocket (∪).

double bond. The electron-donating groups such as methoxy and isopropyl at the para position seem to contribute not only by the inductive effect but also to the stability to the binding sites in the enzyme. It appears that the isopropyl group in cuminaldehyde is better embraced by the hydrophobic protein pocket compared to the methoxy group in anisaldehyde (Tanford, 1980). More specifically, a more hydrophobic electron donor group at the para position in benzaldehyde makes a more potent inhibitor (Figure 4).

On the other hand, *p*-hydroxybenzaldehyde affects the enzyme in different ways. More specifically, this aldehyde binds to the coupled binuclear copper active site with the hydroxy group, rather than forming a Schiff base with the aldehyde group (Kubo and Kinst-Hori, 1998). This can be supported by the observation that the inhibition kinetics analyzed by a Lineweaver−Burk plot indicated that *p*-hydroxybenzaldehyde is a competitive inhibitor with respect to L-DOPA. This phenol with an electron-withdrawing aldehyde group in the para position did not serve as a substrate. The aromatic ring is apparently sufficiently deactivated to prevent electrophilic attack by oxygen (Conrad et al., 1994). It should be noted however that the ID_{50} of *p*-hydroxybenzaldehyde is 24-fold less effective than that of cuminaldehyde as far as ID_{50} is compared. In the case of vanillin, the additional methoxy group adjacent to the hydroxy group may hinder its approach to the coupled binuclear copper active site.

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) (Robb, 1984; Sánchez-Ferrer et al., 1995). The discussion so far described is on the basis of the experiment using L-DOPA as the substrate. Therefore, the inhibitory activity mentioned is with respect to *o*-diphenolase activity of mushroom tyrosinase. The lag time is known for the oxidation of monophenolic substrates such as

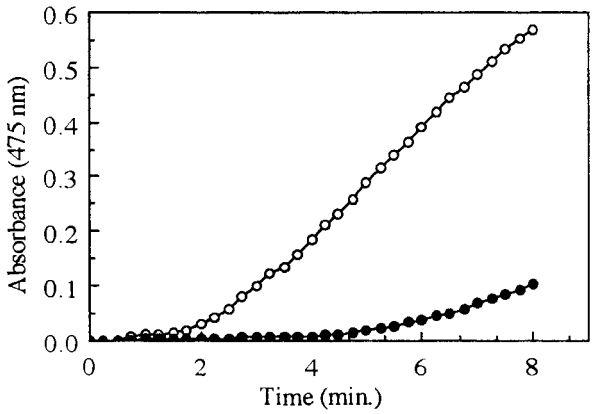


Figure 5. Inhibitory effect on the rate of hydroxylation of L-tyrosine by mushroom tyrosinase without (○) and with (●) cumic acid (0.5 mM).

L-tyrosine, and it can be shortened or abolished by the presence of cofactors, especially *o*-diphenols (such as L-DOPA and caffeic acid). In contrast, the lag phase is known to be extended by monophenolase inhibitors (Kahn and Andrawis, 1985; Sánchez-Ferrer et al., 1995). Cumic acid lengthened this lag phase, indicating that it inhibits monophenolase activity (Figure 5). In addition, benzoic acid and anisic acid also extend the lag time. This inhibition of the monophenolase activity seems a characteristic of HA-type inhibitors (Conrad et al., 1994). On the other hand, the aldehyde compounds tested (**1**, **3**, **6**) did not shorten the lag time at all. A number of tyrosinase inhibitors have been reported but mostly as diphenolase inhibitors. Although monophenols such as L-tyrosine are important substrates, our knowledge about monophenolase inhibitors is still limited. Accumulation of this knowledge may provide clues to better idea to control the enzyme activity. It appears that the two phenolics characterized from cumin, cuminaldehyde, and cumic acid, and their congeners (**3**−**7**) affect mushroom tyrosinase in different ways, and that the major active principle, cuminaldehyde, appears to be responsible for the high activity of the ether extract observed in the preliminary screening.

Safety is a primary consideration for tyrosinase inhibitors, especially for those in food and cosmetic products, which may be utilized in unregulated quantities on a regular basis. Cumin itself is used as a flavor for cheese, liquors, candies (Boelens, 1991) as well as curry. Cuminaldehyde is listed as a food flavor ingredient in *Fenaroli's Handbook of Flavor Ingredients* (Burdock, 1995). This aldehyde is not only one of the main flavor components of the cumin but also in a large number of essential oils such as cinnamon and bitter orange. It is used for chewing gum, soft candy, frozen dairy, and baked goods in amounts ranging from 27.2 to 38.5 ppm (Burdock, 1995) which is higher than the concentration needed to protect from enzymatic browning. The strong odor of cuminaldehyde may limit its practical application, but it can be mixed with other odorous substances to yield an agreeable odor like the cumin itself. In contrast, cumic acid is almost odorless, colorless, and tasteless and therefore can be a superior food additive, particularly to inhibit the oxidation of L-tyrosine by tyrosinase. For example, use of this somewhat water soluble phenolic acid to protect crustaceans against the oxidation of L-tyrosine caused by tyrosinase, black spotting in shrimp, seems to be an appropriate application.

ACKNOWLEDGMENT

We are indebted to Ms. J. Y. Lim for experimental assistance and Mr. K. S. Tan for assistance of obtaining the plant in Singapore.

LITERATURE CITED

Boelens, M. H. Spices and Condiments II. In *Volatile Compounds in Foods and Beverages*; Maarse, H., Ed.; Dekker: New York, 1991; pp 449−482.

Burdock, G. A. *Fenaroli's Handbook of Flavor Ingredients*, 3rd ed.; CRC Press: Boca Raton, FL, 1995; Vol. II, p 133.

Conrad, J. S.; Dawso, S. R.; Hubbard, E. R.; Meyers, T. E.; Strothkamp, K. G. Inhibitor binding to the binuclear active site of tyrosinase: temperature, pH, and solvent deuterium isotope effects. *Biochemistry* **1994**, *33*, 5739−5744.

Duckworth, H. W.; Coleman, J. E. Physicochemical and kinetic properties of mushroom tyrosinase. *J. Biol. Chem.* **1970**, *245*, 1613−1625.

Friedman, M. Food browning and its prevention: An overview. *J. Agric. Food Chem.* **1996**, *44*, 631−653.

Gutteridge, S.; Robb, D. The catecholase activity of *Neurospora* tyrosinase. *Eur. J. Biochem.* **1975**, *54*, 107−116.

Kahn, V.; Andrawis, A. Inhibition of mushroom tyrosinase by tropolone. *Phytochemistry* **1985**, *24*, 905−908.

Kubo, I. Tyrosinase inhibitors from plants. In *Phytochemicals for Pest Control*; Hedin, P., Hollingworth, R., Masler, E., Miyamoto, J., Thompson, D., Eds.; ACS Symposium Series 658; American Chemical Society: Washington, DC, 1997; pp 310−326.

Kubo, I.; Kinst-Hori, I. Tyrosinase inhibitors from anise oil. *J. Agric. Food Chem.* **1998**, *46*, 1268−1271.

Kubo, I.; Yokokawa, Y. Two tyrosinase inhibiting flavonol glycosides from *Buddleia coriacea*. *Phytochemistry* **1992**, *31*, 1075−1077.

Kubo, I.; Yokokawa, Y.; Kinst-Hori, I. Tyrosinase inhibitors from Bolivian medicinal plants. *J. Nat. Prod.* **1995**, *58*, 739−743.

Maeda, K.; Fukuda, M. *In vitro* effectiveness of several whitening cosmetic compounds in human melanocytes. *J. Soc. Cosmet. Chem.* **1991**, *42*, 361−368.

Masamoto, Y.; Iida, S.; Kubo, M. Inhibitory effect of Chinese crude drugs on tyrosinase. *Planta Med.* **1980**, *40*, 361−365.

Mayer, A. M.; Harel, E. Phenoloxidases and their significance in fruit and vegetables. In *Food Enzymology*; Fox, P. F., Ed.; Elsevier: London, 1991; pp 373−398.

Mosher, D. B.; Pathak, M. A.; Fitzpatrick, T. B. Vitiligo, etiology, pathogenesis, diagnosis, and treatment. In *Update: Dermatology in General Medicine*; Fitzpatrick, T. B., Eisen, A. Z., Wolff, K., Freedberg, I. M., Austen, K. F., Eds.; McGraw-Hill: New York, 1983; pp 205−225.

Ogawa, M.; Perdigao, N. B.; Santiago, M. E.; Kozima, T. T. On physiological aspects of black spot appearance in shrimp. *Bull. Jpn. Soc. Sci. Fish.* **1984**, *50*, 1763−1769.

Pifferi, P. G.; Baldassari, L.; Cultrera, R. Inhibition by carboxylic acid of an o-diphenol oxidase from *Prunusavium* fruits. *J. Sci. Food Agric.* **1974**, *25*, 263−270.

Robb, D. A. Tyrosinase. In *Copper Proteins and Copper Enzymes*; Lontie, R., Ed.; CRC Press: Boca Raton, FL, 1984; Vol. II; pp 207−240.

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

Schauenstein, E.; Esterbauer, H.; Zollner, H. In *Aldehydes in Biological Systems*; Pion: London, 1977.

Tanford, C. *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, 2nd ed.; John Wiley & Sons: New York, 1980.

van Gelder, C. W. G.; Flurkey, W. H.; Wichers, H. J. Sequence and structural features of plant and fungal tyrosinases. *Phytochemistry* **1997**, *45*, 1309−1323.

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

Wilcox, D. E.; Porras, A. G.; Hwang, Y. T.; Lerch, K.; Winkler, M. E.; Solomon, E. I. Substrate analogue binding to the coupled binuclear copper active site in tyrosinase. *J. Am. Chem. Soc.* **1985**, *107*, 4015−4027.

Zawistowski, J.; Biliaderis, C. G.; Eskin, N. A. M. Polyphenol oxidase. In *Oxidative Enzymes in Foods*, Robinson, D. S., Eskin, N. A. M., Eds.; Elsevier: London, 1991; pp 217−273.

Received for review March 6, 1998. Revised manuscript received October 13, 1998. Accepted October 15, 1998.

JF980226+