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Tyrosinase Inhibitors of *Pulsatilla cernua* Root-Derived Materials

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The inhibition of mushroom tyrosinase by *Pulsatilla cernua* root-derived materials was evaluated. The bioactive components of *Pulsatilla cernua* root were characterized by spectroscopic analyses as 3,4-dihydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid, which exhibited potent antityrosinase activity. The ID_{50} values of 3,4-dihydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid were 0.97 and 0.33 mM, respectively. The compounds isolated from *Pulsatilla cernua* roots exhibited noncompetitive inhibition against oxidation of L-DOPA by mushroom tyrosinase. This activity was compared with that of three cinnamic acid derivatives and four well-known tyrosinase inhibitors. The ID_{50} of 4-hydroxy-3-methoxycinnamic acid exhibited superior activity relative to anisaldehyde, anisic acid, benzoic acid, benzaldehyde, cinnamic acid, and cinnamaldehyde; but antityrosinase inhibitors and cinnamic acid derivatives, except for cinnamyl alcohol, were slightly more effective than 3,4-dihydroxycinnamic acid. In the case of benzaldehyde and cinnamaldehyde, the aldehyde group is, apparently, a key group in eliciting potent inhibitory activity, whereas anisaldehyde is more effective than anisic acid. Methoxy substitutions, such as 2-methoxycinnamic acid, 3-methoxycinnamic acid, and 4-methoxycinnamic acid, enhanced inhibition of tyrosinase activity. As a naturally occurring tyrosinase inhibitor, 3,4-dihydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid may be useful as new agents to inhibit the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) by mushroom tyrosinase.

KEYWORDS: ***Pulsatilla cernua*; 3,4-dihydroxycinnamic acid; 4-hydroxy-3-methoxycinnamic acid; cinnamic acid; tyrosinase inhibitory activity; noncompetitive inhibition**

INTRODUCTION

Tyrosinase (EC 1.14.18.1) is a multifunctional enzyme that catalyzes both the hydroxylation of monophenols, such as tyrosine to *o*-diphenols by monophenol monooxygenase, and the oxidation of *o*-diphenols to *o*-quinones (EC 1.10.3.1, catechol oxidase). In insects, several functions of this enzyme have been reported in the generation of *o*-diphenols and quinones for pigmentation, wound healing, parasite encapsulation, and sclerotization (*1*−*6*). Because these functions may be the important reactions that are effectively utilized in various types of developmental and defensive processes in insects, the enzyme may be an alternative target site for the control of insect pests. In the food industry, tyrosinase, which is also known as polyphenol oxidase (PPO), is responsible for enzymatic browning reactions in damaged fruits during post-harvest handling and processing. Browning is caused by the oxidation of phenolic compounds containing two *o*-dihydroxy groups to the corresponding *o*-quinone. This reaction produces undesirable changes in color, flavor, and nutritive value of the product. The degree of browning among different fruit cultivars is variable because of differences in phenolic content and tyrosinase activity (*7*−*9*). Control of enzymatic browning during processing is important in fruit pulp manufacturing. The prevention and control of enzymatic browning has been reviewed (*10*). In addition, tyrosinase inhibitors are becoming important constituents of cosmetic products that relate to hyperpigmentation (*11*). Therefore, there is a concerted effort to search for naturally occurring tyrosinase inhibitors from plants, because plants constitute a rich source of bioactive chemicals and many of them are largely free from harmful adverse effects (*12*, *13*).

In our continuing investigation of natural product insect control agents, we have examined tyrosinase inhibitors from plants (*14*, *15*). In preliminary screening, using mushroom tyrosinases, methanol extracts of *P. cernua* roots were found to inhibit the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) (*14*, *15*). This plant species is important not only as an herbicide, but in East Asia it is considered to have medicinal properties, such as antiinflammatory, antimutagenic, antitumorogenic, and antimicrobial activities (*16*−*20*). However, little work has been carried out on the effects of *P. cernua* root-derived materials against mushroom tyrosinase activity, despite its excellent pharmacological action (*16*−*20*). Tyrosinase inhibitors isolated from *P. cernua* roots may be a good source for

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lead compounds as alternatives for the nonnatural tyrosinase inhibitors currently used. The importance of finding effective tyrosinase inhibitors led us to further investigate natural compounds.

On the basis of an earlier report, the active components of *P. cernua* roots were isolated and characterized by spectroscopic analyses to develop new and safer agents of tyrosinase inhibitors. Additionally, the tyrosinase activities of commercially available cinnamic acid derivatives (*21*) are also presented in relation to the results obtained.

MATERIALS AND METHODS

Chemicals. Anisaldehyde, anisic acid, benzaldehyde, cinnamaldehyde, cinnamic acid, cinnamyl alcohol, L-DOPA, 2-methoxycinnamic acid, 3-methoxycinnamic acid, and 4-methoxycinnamic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). Benzoic acid and L-tyrosine were purchased from Sigma (St. Louis, MO). Note that the ID_{50} value of benzaldehyde varied with time. This can be explained by its gradual auto-oxidation to benzoic acid. Hence, the data reported were observed only with fresh benzaldehyde.

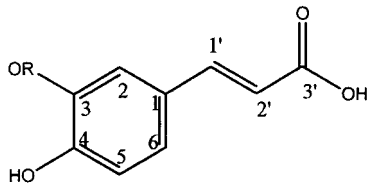
Isolation and Identification. The roots (4.1 kg) of *P. cernua* (Family Ranunculaceae) were oven dried at 60 °C for 3 days, finely powdered, extracted twice with methanol (10 L) at room temperature, and filtered (Toyo filter paper No. 2, Japan). The combined filtrate was concentrated in vacuo at 35 °C to yield about 9.2% (based on the dry weight of the root). The extract (20 g) was sequentially extracted with hexane (2.9 g), chloroform (6.6 g), ethyl acetate (2.1 g), and water-soluble (8.4 g) portions for subsequent bioassay. The organic solvent portions were concentrated to dryness in vacuo at 35 °C; and the water portion was freeze dried.

The ethyl acetate fraction (10 g) was chromatographed on a silica gel column (Merck 70−230 mesh, 500 g, 70 cm × 5.5 i.d.) and successively eluted with a stepwise gradient of ethyl acetate/methanol (0, 5, 10, 15, 20, and 25%). The active 5% fraction was chromatographed on a silica gel column and eluted with a stepwise gradient of chloroform/methanol (0, 10, 20, 30, 40, and 50%). The active 30% fraction was collected and analyzed by TLC (hexane/ethyl acetate, 3:1). Fractions with identical Rf values were combined. This was chromatographed on a Sephadex LH-20 column (Pharmacia 25−100 mesh, 200 cm × 3.5 i.d.) and eluted with methanol/chloroform (4:1). For further separation of the biologically active substance(s), a Waters Delta Prep 4000 HPLC was used. The column was a 300 mm × 39 i.d. Bondapak C_{18} (Waters) using a stepwise gradient of methanol/water (30, 40, 50, 60, 70, 80, 90, and 100%) at a flow rate of 1 mL/min and detection at 254 nm. Finally, one potent active principle (**1**) was isolated in the 30% methanol/water fraction, and the other major (**2**) potent active principle was isolated in the 60% methanol/water fraction.

Structural determination of the active isolate was based on spectroscopic analysis. ^1H (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded with a Bruker AM-500 spectrometer (Rheinspettem, Germany), and chemical shifts are given in ppm. UV spectra were obtained on a Waters 490 spectrometer (Milford, MA), and mass spectra were obtained on a JEOL JMS-AX 302 WA spectrometer (Tokyo, Japan).

Bioassay. Mushroom tyrosinase (EC 1.14.18.1) used for the bioassay was purchased from Sigma Chemical Co. (St. Louis, MO). Although it differs somewhat from other sources, this fungal source was used for the experiment because it is readily available. Because the mode of inhibition depends on the structures of both the substrate and inhibitor, L-DOPA was used as the substrate in these experiments, unless otherwise specified. Therefore, the inhibitors discussed in this paper are inhibitors of diphenolase activity of mushroom tyrosinase, and their effect on the enzyme was determined by spectrophotometry (dopachrome formation at 475 nm). The preliminary assays were tested at 167 μg/mL, unless otherwise specified. All the samples were first dissolved in dimethyl sulfoxide (DMSO) and employed at 30 times dilution, as previously described in Masamoto et al. (*21*). Thus, 1 mL of a 2.5 mM L-DOPA solution was mixed with 1.8 mL of 0.1 M phosphate buffer (pH 6.8), and incubated for 10 min at room temperature. Then, 0.1 mL of the sample solution and 0.1 mL of the aqueous solution of mushroom tyrosinase (130 units, added last) were added to the mixture to immediately measure the initial rate of linear increase in optical density at 475 nm. Inhibition by the addition of samples is expressed as a percentage necessary to give a 50% inhibition (ID_{50}).

The preincubation mixture consisted of 1.8 mL of 0.1 M phosphate buffer (pH 6.8), 0.6 mL of water, 0.1 mL of the sample solution (equivalent amount of ID_{50}), and 0.1 mL of the aqueous solution of mushroom tyrosinase (130 units). The mixture was preincubated at room temperature 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.



1, 3,4-dihydroxycinnamic acid, R = -H

2, 4-hydroxy-3-methoxycinnamic acid, R = $-CH_3$

Figure 1. Structures of 3,4-dihydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid.

Table 1. ID_{50} and Mode of Inhibition of *Pulsatilla Cernua* Root-Derived Components and Other Related Components

compound	ID_{50} (mM)	type of inhibition[a]
3,4-dihydroxycinnamic acid	0.97	noncompetitive
4-hydroxy-3-methoxycinnamic acid	0.33	noncompetitive
2-methoxycinnamic acid	0.34	noncompetitive
3-methoxycinnamic acid	0.35	noncompetitive
4-methoxycinnamic acid	0.34	noncompetitive
cinnamyl alcohol	ND[b]	ND
cinnamic acid	0.70	mixed
cinnamaldehyde	0.97	noncompetitive
anisic acid	0.68	noncompetitive
anisaldehyde	0.38	noncompetitive
benzoic acid	0.71	competitive
benzaldehyde	0.83	noncompetitive

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

RESULTS AND DISCUSSION

In our routine screening, using mushroom tyrosinase, we observed that methanol extracts of *P. cernua* roots showed significant inhibition of L-DOPA oxidation. In fractionation, guided by tyrosinase inhibitory activity, chloroform and ethyl acetate fractions from methanol extracts showed, respectively, moderate and strong inhibitory activity against mushroom tyrosinase. Little or no activity was present in the hexane, butanol, and water fractions. However, two active compounds were isolated. Structural determination of the isolates was made by spectroscopic analysis (including EI-MS and NMR) and by direct comparison with authentic reference compounds, and they were characterized as 3,4-dihydroxycinnamic acid ($C_9H_8O_4$, MW 180.2) and 4-hydroxy-3-methoxycinnamic acid ($C_{10}H_{10}O_4$, MW 194.2) (**Figure 1**).

Bioassays with pure 3,4-dihydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid showed a dose-dependent inhibitory effect L-DOPA oxidation by mushroom tyrosinase, and the ID_{50} values were established as 0.97 mM and 0.33 mM, respectively (**Table 1**). These results indicate that 4-hydroxy-3-methoxycinnamic acid is approximately three times more effective than 3,4-dihydroxycinnamic acid as an antityrosinase agent. Furthermore, the inhibition kinetics of 3,4-dihydroxy-

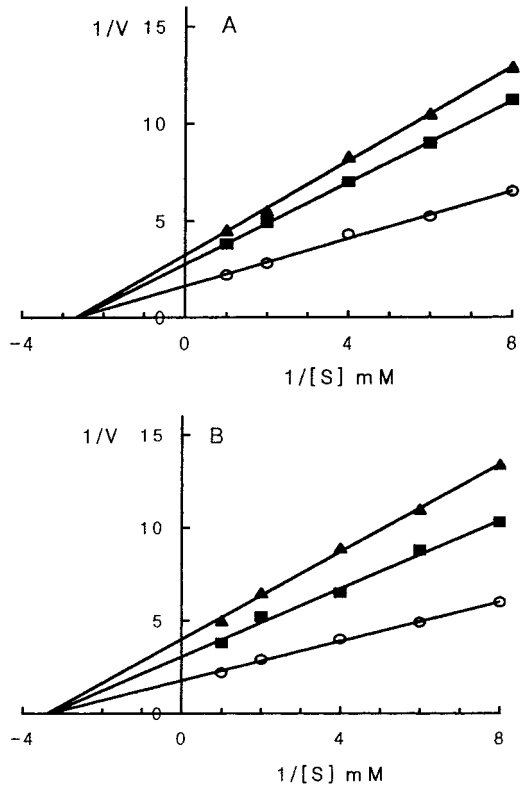


Figure 2. Lineweaver–Burk plots of mushroom tyrosinase and L-DOPA: (A) without (○) and with 3,4-dihydroxycinnamic acid [(■) 0.6 mM and (▲) 1.0 mM] 1/V: 1/475 nm/min; and (B) without (○) and with 4-hydroxy-3-methoxycinnamic acid [(■) 0.15 mM and (▲) 0.22 mM] 1/V: 1/475 nm/min.

cinnamic acid and 4-hydroxy-3-methoxycinnamic acid were analyzed by a Lineweaver−Burk plot as shown **Figure 2**. The three lines, obtained from the uninhibited enzyme and from the two different concentrations of 3,4-dihydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid, intersected on the horizontal axis. This result indicates that 3,4-dihydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid exhibited a noncompetitive inhibition for L-DOPA oxidation by mushroom tyrosinase. In testing the inhibition of tyrosinase activity by polyphenol esterase obtained from the biomass of *Aspergillus niger*, the ID_{50} was more enhanced upon adding 3,4-dihydroxycinnamic acid than that of polyphenol esterase only (*22*). However, with this type of inhibition, our results are not consistent with previous reports that polyphenol esterase of *A. niger* exhibited an uncompetitive inhibition upon adding 3,4-dihydroxycinnamic acid. It is not easy correlate our data precisely with those of earlier reports because of differences in substrate characteristics and additives. Nevertheless, 3,4-dihydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid isolated from *P. cernua* roots may be useful in controlling/mediating antityrosinase activity.

Finding potent tyrosinase inhibitory activity with 3,4-dihydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid led us to evaluate other cinnamic acid derivatives and well-known tyrosinase inhibitors such as anisaldehyde, anisic acid, benzaldehyde, and benzoic acid (*23*, *24*). There was a significant difference in the inhibitory effect on the tyrosinase activity among the treatments. In a comparison of their ID_{50} values, 4-hydroxy-3-methoxycinnamic acid exhibited superior activity compared to that of anisic acid, benzoic acid, benzaldehyde, cinnamic acid, and cinnamaldehyde, and it was slightly higher than anisaldehyde (ID_{50} 0.38 mM). In tests with 3,4-dihydroxycinnamic acid, the following were slightly more effective, as shown in **Table 1**: anisic acid, benzoic acid, benzaldehyde, cinnamic acid, and cinnamaldehyde. However, anisaldehyde was 3-fold more effective than 3,4-dihydroxycinnamic acid. Benzoic acid, benzaldehyde, cinnamic acid, and cinnamaldehyde were consistent with previous reports wherein the aldehyde group is apparently a key group in eliciting potent inhibitory activity (*24*, *25*), but anisaldehyde is more effective than anisic acid. The inhibition kinetics indicated that benzoic acid, cinnamic acid, and cinnamaldehyde exhibited a competitive inhibition for L-DOPA oxidation by mushroom tyrosinase (**Table 1**). Of the compounds tested on the mode of inhibition, anisaldehyde and anisic acid exhibited noncompetitive inhibition. No activity was observed for cinnamyl alcohol.

To test the effect of methoxy substitutions, 2-methoxycinnamic acid, 3-methoxycinnamic acid, and 4-methoxycinnamic acid were also evaluated for their inhibitory activity against mushroom tyrosinase (**Table 1**). The ID_{50} values of 4-hydroxy-3-methoxycinnamic acid, 2-methoxy cinnamic acid, 3-methoxy cinnamic acid, and 4-methoxy cinnamic acid are approximately 0.33−0.35 mM, but the ID_{50} values of 3,4-dihydroxycinnamic acid and cinnamic acid are 0.97 and 0.70, respectively (**Table 1**). The methoxy derivatives of cinnamic acid gave results that agreed with those of previous reports (*21*). That is, the methyl substitution decreased activity (*24*). Furthermore, in the inhibition kinetics, 3,4-dihydroxycinnamic acid, 4-hydroxy-3-methoxycinnamic acid, 2-methoxycinnamic acid, 3-methoxycinnamic acid, and 4-methoxycinnamic acid exhibited a noncompetitive inhibition for L-DOPA oxidation by mushroom tyrosinase, whereas cinnamic acid displayed mixed inhibition (**Table 1**). It should be noted that further studies are required to understand the effect of methoxy substitution and the mode of inhibition on tyrosinase inhibitory activity.

Safety is a primary consideration for tyrosinase inhibitors, especially for those used in food and cosmetic products, which may be regularly utilized in unregulated quantities. 3,4-Dihydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid occur in apple, coffee, lettuce, and celery in amounts ranging from 2.4 to 23.9 mg/g, which is higher than the concentration needed to protect from enzymatic browning (*26*). Many investigations have demonstrated that 3,4-dihydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid have antitumor (*27*), antimutagenic (*28*, *29*), antioxidative (*30*), and photoprotective effects (*31*). On the basis of our limited data and some earlier findings, the inhibitory action of 3,4-dihydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid against tyrosinase activity may be an indication of at least one of the biological actions of *P. cernua* roots.

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