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Tyrosinase Inhibitory *p*-Coumaric Acid from Ginseng Leaves

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By bioassay-guided fractionation using mushroom tyrosinase, *p*-coumaric acid was characterized as the principal tyrosinase inhibitor from the fresh leaves of *Panax ginseng* (Araliaceae). It inhibited the oxidation of L-tyrosine more strongly than that of L-3,4-dihydroxyphenylalanine (L-DOPA) by this enzyme. On the basis of this finding, various related phenylpropanoid analogues were also tested in order to gain new insights into their structural criteria. Copyright © 1999 John Wiley & Sons, Ltd.

Keywords: Panax ginseng; *p*-coumaric acid; mushroom tyrosinase inhibitory activity; noncompetitive inhibition; monophenolase.

INTRODUCTION

Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase (PPO) (Mayer, 1987; Whitaker, 1981), is a copper containing enzyme found in microorganisms, animals and plants. As part of our continuing investigation of naturally occurring alternative insect control agents (Kubo, 1993), we search for tyrosinase inhibitors from plants (Kubo, 1997). Because tyrosinase is a key enzyme in the insect moulting process (Andersen, 1979), its inhibitors might ultimately provide clues to control insect pests. 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. A few antimelanogenic reagents, such as monobenzone and hydroquinone, are clinically useful. Tyrosinase is responsible not only for melanization in animals but also browning in plants. The latter case is considered to be deleterious to the colour quality of plant derived foods and beverages and results in a loss of nutritional value (Friedman, 1996). Hence, tyrosinase inhibitors should have broad applications.

Ginseng, the root of *Panax ginseng* (Araliaceae) is a very well known medicine of plant origin and about 25 000 tonnes per year are produced in South Korea alone. In comparison with the root, the leaf is almost completely neglected in commercial terms, although it is available in far greater tonnage and there is considerable potential for its exploitation. In our continuing search for tyrosinase inhibitors from plants (Kubo, 1997), the crude methanol extract of the fresh leaves of *P. ginseng* showed inhibitory activity on the oxidation of L-DOPA by mushroom tyrosinase and was subjected to further fractionation.

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MATERIALS AND METHODS

Chemicals. *p*-Coumaric acid, cinnamic acid, caffeic acid, dihydrocoumaric acid, *p*-methoxycinnamic acid, L-DOPA, L-tyrosine, cinnamaldehyde, *p*-methoxycinnamaldehyde, salicylic acid, gentisic acid and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St Louis, MO). $MgSO_4(7H_2O)$ was obtained from Aldrich Chemical Co. (Milwaukee, WI). $CaSO_4(2H_2O)$ was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Dihydrocinnamaldehyde was provided by Professor Tadao Kamikawa, Kinki University, Osaka, Japan. Methyl coumarate was isolated from the dried flowers of *Trixis michuacana* var *longifolia* (Compositae).

Extraction and identification. The fresh leaves (500 g) of *P. ginseng* were obtained from Moo-Joo Kun, Jun-La, Korea and extracted with MeOH ($\times 3$) at ambient temperatures. After concentration of solvent, the water based suspension was partitioned with *n*-hexane and EtOAc. Subsequent bioassay indicated the EtOAc extract to be active. Repeated column chromatography (CC; SiO_2) gave 12 mg of *p*-coumaric acid, identical in all respects including spectroscopic data to an authentic sample. Identification of phenolic acids in the methanol extract was achieved by HPLC using a C_{18} reversed phase column.

The dried flowers of *T. michuacana* var *longifolia* were generous gifts from Professor Tetsuya Ogura, Universidad Autonoma de Guadalajara, Guadalajara, Mexico. Methyl coumarate was isolated from the dried flowers by repeated CC (SiO_2) and identified by comparison with those of the published spectroscopic data (Daayf *et al.*, 1997). It should be noted that the MeOH extract of the

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dried flower of *T. michuacana* var *longifolia* showed potent tyrosinase inhibitory activity and quercetin was isolated in a large quantity as the principle inhibitor.

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 this experiment because it is readily available. Tyrosinase catalyses a reaction between two substrates, a phenolic compound and oxygen, but the assay was carried out in air-saturated solutions. All the samples were first dissolved in DMSO and used for the actual experiment at 30 times dilution. The preliminary assay was tested at 167 μg/mL. The enzyme activity was monitored by dopachrome formation at 475 nm up to the appropriate time (usually not longer than 10 min).

The assay was performed as previously described (Masamoto *et al.*, 1980). Thus, 1 mL of 2.5 mM L-DOPA or L-tyrosine solution was mixed with 0.1 mL of the sample solution and 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 aqueous solution of mushroom tyrosinase (138 units) was added last to the mixture. This solution was immediately monitored for the formation of dopachrome by measuring the linear increase in optical density at 475 nm up to the appropriate time (usually not longer than 10 min). The extent of inhibition by the addition of samples is expressed as the percentage necessary for 50% inhibition (ID_{50}).

The pre-incubation 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 (138 units). The mixture was pre-incubated 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.

RESULTS

Characterization of tyrosinase inhibitory *p*-coumaric acid

The methanol extract of the leaves of *P. ginseng* was suspended in water and extracted with ethyl acetate, which in subsequent bioassay was shown to be the active fraction. After repeated chromatographic methods *p*-coumaric acid (**1**) was characterized as the principal inhibitor by spectroscopic methods. This phenolic acid was previously identified in the root of *P. ginseng* together with caffeic acid, salicylic acid and gentisic acid (Wee *et al.*, 1989).

The bioassay with the purified **1** showed a dose-dependent inhibitory effect on the oxidation of L-DOPA by mushroom tyrosinase and the ID_{50} was established as 600 μg/mL (3.65 mM). The inhibition kinetics of this oxidation were analysed by a Lineweaver–Burk plot which indicated that **1** is a mixed type inhibitor as shown in Fig. 1. In addition, pre-incubation of the enzyme in the presence of 3.65 mM of **1** and in the absence of L-DOPA did not decrease the enzyme activity significantly. This result suggests that **1** is an inhibitor rather than an inactivator of the enzyme (Kahn and Andrawis, 1985). In addition, *p*-coumaric acid itself was not oxidized as a substrate and the inhibitory activity was not suppressed by adding excess Mg^{2+} or Ca^{2+}. As for benzoic acid, **1** can be classified as a HA-type inhibitor, which is a substrate analogue in that the carboxylate is conjugated into an aromatic ring, producing a planar structure (Duckworth and Coleman, 1970; Wilcox *et al.*, 1985). It should be noted that *p*-coumaric acid has been reported to inhibit several tyrosinases (Pifferi *et al.*, 1974; Conrad *et al.*, 1994) and also to be oxidized as a substrate by apple tyrosinase (Cheynier and Moutounet, 1992). It is not easy to relate our data precisely to those of earlier reports because of differences in test methods, substrates and sources of the enzyme.

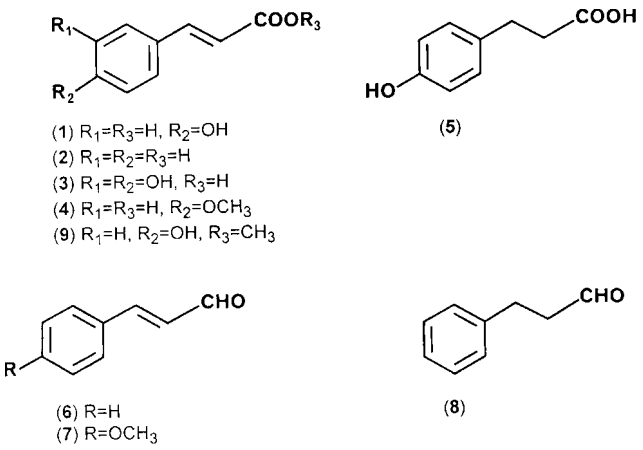

Figure 1. Chemical structure of *p*-coumaric acid and its related phenylpropanoids.

Tyrosinase inhibitory phenylpropanoids and their structural criteria

In order to clarify the mode of inhibition of **1** at a molecular level, several closely related congeners such as cinnamic acid (**2**), caffeic acid (**3**), *p*-methoxycinnamic acid (**4**), *p*-dihydrocoumaric acid (**5**) (*p*-hydroxyphenylpropionic acid), cinnamaldehyde (**6**), *p*-methoxycinnamaldehyde (**7**), and dihydrocinnamaldehyde (**8**) (phenylpropionaldehyde) were also examined for comparison. Their ID_{50} values and modes of inhibition are listed in Table 1, of those additional seven tested, **4** showed the most potent inhibitory activity with an ID_{50} of 43.8 μg/mL (0.27 mM) followed by **2** with an ID_{50} of 106.7 μg/mL (0.72 mM). The result obtained indicates that the aldehydes (**6,7**) were slightly less potent inhibitors than the corresponding phenolic acids (**2,4**) as previously reported (Conrad *et al.*, 1994). On the other hand, both **3** and **5** were oxidized by the enzyme as

Table 1. Tyrosinase inhibitory activity of *p*-coumaric acid and related compounds[a]

Compounds tested	ID_{50} (mM)	Mode of inhibition
p-Coumaric acid	3.65	Mixed
Cinnamic acid	0.72	Mixed
p-Methoxycinnamic acid	0.27	Mixed
Cinnamaldehyde	0.98	Noncompetitive
p-Methoxycinnamaldehyde	2.35	Noncompetitive

[a] With respect to the oxidation of L-DOPA by mushroom tyrosinase.

substrates. The compounds substituted in the ring position *para* to the carboxyl were reported to bind marginally better than those with no substituents (Winkler *et al.*, 1981). However, this was not the case for **1** since its ID_{50} was about 5-fold lower than that of **2**, although the ID_{50} of **4** was about 3-fold higher than that of **2**. This can be explained by the fact that the protein pocket contributes to the stability of binding of the conjugated ligands by interacting with planar aromatic portions of the molecule (Eickman *et al.*, 1978). The polar hydroxyl group, which is exposed on the other side of the molecule in **1**, may not be embraced by the hydrophobic protein pocket but the nonpolar methoxy group may well be. It should be noted however, that *p*-hydroxycinnamic acid (*p*-coumaric acid) was reported to inhibit the oxidation of 4-methylcatechol by apple tyrosinase to a greater extent than cinnamic acid (Janovitz-Klapp *et al.*, 1990).

The two aldehydes tested, cinnamaldehyde (**6**) and *p*-methoxycinnamaldehyde (**7**), also inhibited the oxidation of L-DOPA: ID_{50}s were 129.4 μg/mL (0.98 mM) and 314.9 μg/mL (2.35 mM), respectively. In addition, pre-incubation of the enzyme in the presence of 0.98 mM of **6** and in the absence of L-DOPA did not decrease the enzyme activity significantly. The reason for the tyrosinase inhibitory activity exerted by **6** and **7**, at least in part, can be explained as follows: aldehydes are generally protein-reactive compounds and many readily react with biologically important nucleophilic groups, such as sulfhydryl, amino or hydroxyl. 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. As noncompetitive inhibitors, **6** and **7** bind at different sites from the substrate on the enzyme, and thus may form a Schiff base with a primary amino group in the enzyme rather than binding to the active site. Their activity could be in part, based on the assumption that the enzyme [**E**] is complexed with an inhibitor [**I**]. The resulting complex [**EI**] is inactive (Kubo and Kinst-Hori, 1998). This was supported by the fact that dihydrocinnamaldehyde (**8**) did not exhibit any activity since it does not form a stable Schiff base.

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 + $2H_2O$) (Sánchez-Ferrer *et al.*, 1995). The discussion so far is, however, on the basis of the experiment using L-DOPA as a substrate and therefore, the activity described is the *o*-diphenolase inhibitory activity of mushroom tyrosinase. It should be noted that a lag time is known for the oxidation of monophenolic substrates such as L-tyrosine, and this lag can be shortened or abolished by the presence of reducing agents (cofactors), especially *o*-diphenols (such as L-DOPA and caffeic acid). This means that caffeic acid (**3**) behaves as a cofactor and substrate (Cheynier and Moutounet, 1992), similar to L-DOPA. In fact, L-tyrosine was oxidized by the enzyme without the lag phase in the presence of **3**.

Monophenolase inhibitory activity

Inhibitors of monophenolase activity are known to extend the lag phase on the other hand. Interestingly, *p*-coumaric acid (**1**) significantly lengthened this lag phase, as illustrated in Fig. 3, indicating that it is a monophenolase inhibitor. In fact, this activity of **1** was observed even at a concentration of 18.2 μg/mL (0.11 mM) which is about 33-fold lower than the ID_{50} of its *o*-diphenolase inhibitory activity. This indicates that **1** binds not only to the *oxy* form but also the *met* form of the coupled binuclear copper site, preferentially with the more acidic carboxylic group (Conrad *et al.*, 1994). The latter assumption can be supported by the fact that its methyl ester (**9**) isolated from the dried flowers of *Trixis michuacana* var *longifolia* (Compositae), a Mexican medicinal plant, was oxidized as a substrate. It should be noted, however, that esterification of **1** decreased the inhibitory activity, but did not destroy it, indicating **9** is a substrate inhibitor. More specifically, this ester inhibits the oxidation of L-DOPA by mushroom tyrosinase at higher concentrations, but behaves as a substrate at lower (<167 μg/mL) concentrations. The latter can be observed as enhancement at 475 nm as a result. This suggests that monophenolase inhibitory activity seems to be characteristic of HA-type inhibitors, and as expected, the other two HA-type inhibitors tested (**2,4**) also extended the lag phase at concentrations of their ID_{50}s of *o*-diphenolase inhibitory activity. However, the monophenolase inhibitory activity of **1** is much more potent compared with its *o*-diphenolase inhibitory activity. It should be added that the two aldehydes tested (**6,7**) did not extend the lag time at all when L-tyrosine was used as a substrate.

The methanol extract of the fresh leaves of *P. ginseng* inhibited the oxidation of L-DOPA by mushroom tyrosinase as described above, but the lag phase was not observed when L-tyrosine was used as a substrate (Fig. 4). As already described *p*-coumaric acid has been characterized as a monophenolase inhibitor, so this may indicate that a cofactor coexists in the plant. Since caffeic acid was previously isolated from the root of *P. ginseng*, this diphenolic acid is also likely to be present in the leaves. As expected, HPLC analysis identified caffeic acid together with salicylic acid in minute amounts. Salicylic acid was previously reported as a noncompetitive inhibitor of mushroom tyrosinase with respect to the oxidation of L-DOPA (Kubo *et al.*, 1995), though it did not extend the lag phase when L-tyrosine was used as a substrate.

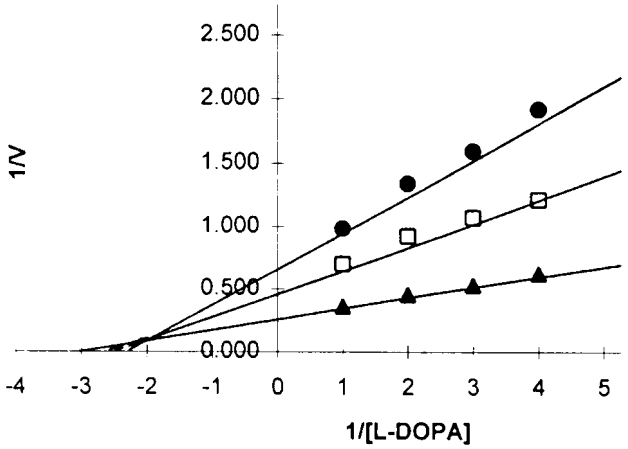

Figure 2. Lineweaver–Burk plots of mushroom tyrosinase and L-DOPA without (▲) and with *p*-coumaric acid [(□): 1.8 mM, (●): 3.6 mM]. 1/V: 1/ 475 nm/min.

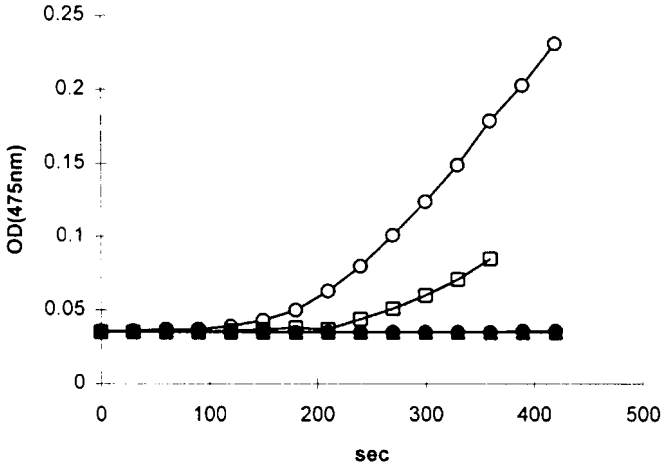

Figure 3. Effect of *p*-coumaric acid on the rate of L-tyrosine hydroxylation by mushroom tyrosinase: (○) 0.1 mM L-tyrosine, [(□) 0.11 mM, (●) 0.22 mM, (▲) 3.6 mM] *p*-coumaric acid.

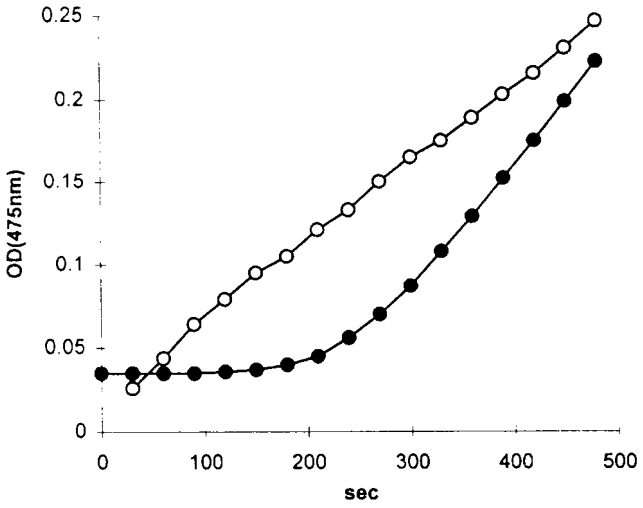

Figure 4. Cofactor effect on L-tyrosine hydroxylation by mushroom tyrosinase, without (●) and with (○) ginseng leaves extract.

DISCUSSION

It appears that the phenylpropanoids described above inhibit mushroom tyrosinase in different ways. *p*-Coumaric acid (**1**), cinnamic acid (**2**), and *p*-methoxycinnamic acid (**4**) act as HA-type inhibitors and inhibit both monophenolase and *o*-diphenolase activity. The polar hydroxyl group at the *para* position in **1** increases monophenolase inhibitory activity while decreasing *o*-diphenolase inhibitory activity compared with those of **2**. In contrast, the lipophilic methoxy group at the *para* position in **4** increases the *o*-diphenolase inhibitory activity but decreases the monophenolase inhibitory activity compared with those of **2**. Similar results were also observed for *p*-hydroxybenzoic acid, benzoic acid and anisic acid (data not shown). *p*-Coumaric acid and *p*-hydroxybenzoic acid bind to the binuclear copper active centre preferentially with the more acidic carboxylic group and compete with the substrate (Winkler *et al.*, 1981). As a result, these HA-type inhibitors did not serve as substrates at all. Obviously, monophenolase inhibitors are important since L-tyrosine is an important substrate, but our current knowledge is very limited. As far as mushroom tyrosinase is concerned, *p*-coumaric acid is a potent monophenolase inhibitor, though its *o*-diphenolase inhibitory activity is moderate. However, as the major active principle, *p*-coumaric appears to be responsible for the activity of the methanol extract observed in the preliminary assay.

In the case of *p*-dihydrocoumaric acid (**5**), it was easily oxidized as a substrate. It appears that its carboxylate is no longer conjugated into the aromatic ring indicating that this conjugation is important for eliciting the activity. The structure of **5** resembles *p*-cresol which has been most frequently employed as an experimental substrate (Passi and Nazzaro-Porro, 1981; Behbahani *et al.*, 1993). In addition, esterification of *p*-coumaric acid decreased the inhibitory activity, but did not destroy it. In general, esterification of the carboxylic group decreased the inhibitory strength (Pifferi *et al.*, 1974) and, more importantly, the ester is no longer an HA-type inhibitor but a substrate inhibitor. On the other hand, cinnamaldehyde (**6**) and *p*-methoxycinnamaldehyde (**7**) inhibited the oxidation of L-DOPA by forming a Schiff base but did not inhibit monophenolase activity. The results obtained so far may hint to their interaction with the enzyme but this remains unclear since the structure of mushroom tyrosinase used for this study has not yet been established.

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. Compared with many other tyrosinase inhibitors, *p*-coumaric acid is almost tasteless, colourless, and odourless, and therefore can be a superior food additive, used particularly to inhibit the oxidation of L-tyrosine. In addition, substituted derivatives of cinnamic acid are predominant phenolic acids present in foods of plant origin (Shahidi and Naczk, 1995). On the other hand, cinnamaldehyde is known as generally recognized as safe (GRAS) (Feron *et al.*, 1991)

Acknowledgements

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