Tyrosinase Inhibitors from Anise Oil

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Anisaldehyde characterized in the seeds of *Pimpinella anisum* L. (Umbelliferae), also known as aniseed, was found to inhibit the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) by mushroom tyrosinase (EC 1.14.18.1) with an ID_{50} of 43 μg/mL (0.32 mM). The inhibition kinetics analyzed by a Lineweaver−Burk plot established anisaldehyde to be a noncompetitive inhibitor for this oxidation. On the basis of this finding, various related analogues were also tested in order to gain new insights into their structural functions.

Keywords: *Anisaldehyde; aniseed; tyrosinase inhibitor; L-DOPA; noncompetitive inhibition; Schiff base formation*

INTRODUCTION

In our continuing investigation of naturally occurring alternative insect control agents (Kubo, 1993), we have searched for tyrosinase inhibitors from plants (Kubo, 1997). Because tyrosinase is one of the key enzymes in the insect-molting process (Andersen, 1979), tyrosinase inhibitors might ultimately provide clues to control insect pests. Tyrosinase, also known as polyphenol oxidase (PPO) (Mayer and Harel, 1979; Mayer, 1987), is a copper-containing oxygenase widely distributed in the phylogenetic scale. Three different forms of binuclear copper in the active site are involved in the reaction. 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. This unfavorable darkening from enzymatic oxidation of phenols generally results in a loss of nutritional value and has been of great concern (Friedman, 1996). 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.

In our earlier report, the flavor compounds of the anise obtained by steam distillation of the seeds of *Pimpinella anisum* were reported to exhibit a broad spectrum of antimicrobial activity (Kubo and Himejima, 1991). The same volatile fraction was also found to inhibit the L-DOPA oxidation by mushroom tyrosinase. Tyrosinase inhibitors isolated from regularly consumed condiments and flavors of foods and beverages, like anise, may be superior compared to nonnatural products. Owing to the importance of finding effective tyrosinase inhibitors, the flavor compounds were further investigated.

MATERIALS AND METHODS

Chemicals. Anisaldehyde, methyleugenol, pulegone, camphor, cymene, benzaldehyde, *p*-hydroxybenzaldehyde, anisyl alcohol, anisole, *p*-hydroxybenzyl alcohol, *p*-hydroxyanisole, and L-DOPA were purchased from Aldrich Chemical Co. (Milwaukee, WI). Anethole and L-tyrosine were obtained from Sigma Chemical Co. (St. Louis, MO). L-Ascorbic acid was purchased from ICN Biochemicals (Cleveland, OH). Stearic acid and β-terpinyl acetate were from our previous work (Kubo and Himejima, 1991). It should be noted that the ID_{50} value of benzaldehyde was variable with time. This variation can be explained by its gradual auto-oxidation to benzoic acid. Hence, the data reported was obtained with benzaldehyde from a fresh bottle.

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, this fungal source was used for the experiment because it is readily available. Since the mode of inhibition depends on the structure of both the substrate and inhibitor, L-DOPA was used as the substrate in this experiment, unless otherwise specified. Therefore, 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 assay was tested at 167 μg/mL, unless otherwise specified. All the samples were first dissolved in dimethyl sulfoxide (DMSO) and used for the experiment at 30 times dilution. The assay was performed as previously described (Mason and Peterson, 1965; Masamoto et al., 1980). 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 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

In our routine screening using mushroom tyrosinase, the anise oil obtained by steam distillation of the *n*-hexane extract of the seeds of *P. anisum* inhibited the L-DOPA oxidation. Among flavor compounds character-

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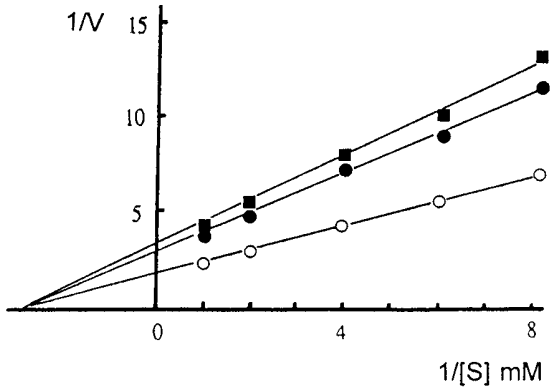
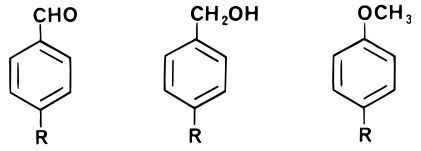


Figure 1. Lineweaver−Burk plots of mushroom tyrosinase and L-DOPA without (○) and with [(●) 0.13 mM, (■) 0.21 mM] anisaldehyde. $1/V$: $1/\Delta$ 475 nm/min.

ized in this distillate, anethole (**1**), anisaldehyde (**2**), anisketone (**3**), and methyleugenol (**4**) are four major aromatic compounds in decreasing concentrations (Kubo and Himejima, 1991). In a preliminary assay at the



2. R=OCH_3
5. R=H
6. R=OH

7. R=OCH_3
9. R=OH

8. R=H
10. R=OH

concentration of 167 μg/mL, only anisaldehyde showed significant (>60%) and distinct inhibitory activity of the L-DOPA oxidation by mushroom tyrosinase. It should be noted that anisketone was not tested because of its limited availability. The other two phenolics did not show inhibitory activity at this concentration. However, anethole is hardly soluble in the water based test solution at this concentration. Therefore, its appropriate data could not be obtained, but it is evident that neither exhibited superior inhibitory activity compared to anisaldehyde. Finding this potent tyrosinase inhibitory activity of the easily obtainable anisaldehyde led us to investigate it in more detail. In addition, on the basis of our continuing search for tyrosinase inhibitors from plants, the other flavor compounds characterized in the same distillate such as pulegone, camphor, cymene, β-terpinyl acetate, and stearic acid (Kubo and Himejima, 1991) did not inhibit this enzyme activity.

The bioassay with the pure anisaldehyde showed a dose-dependent inhibitory effect on the L-DOPA oxidation by mushroom tyrosinase. The addition of 20 μg/mL of anisaldehyde to the assay system containing 0.85 mM of L-DOPA solution caused the inhibition of tyrosinase by 40%. The inhibition was elevated to 75% when 270 μg/mL of anisaldehyde was added. The ID_{50} was established as 43 μg/mL (0.32 mM). The inhibition kinetics of anisaldehyde were analyzed by a Lineweaver−Burk plot as shown in Figure 1. The three lines, obtained from the uninhibited enzyme and from the two different concentrations of anisaldehyde, intersected on the horizontal axis. This result indicates that anisaldehyde exhibited a noncompetitive inhibitor for the oxidation of L-DOPA by mushroom tyrosinase. In addition, preincubation of the enzyme in the presence of 0.32 mM of anisaldehyde and in the absence of the substrate indicated that this compound is not a direct inactivator of the enzyme since it did not decrease the enzyme activity significantly. It should be noted, however, that the enzyme preincubated in this experiment was mostly *met* tyrosinase, known as the resting form of tyrosinase (Lerch, 1981). In addition, anisaldehyde was also tested for inhibition of monophenolase activity. This activity can be observed as a lag phase in oxidation of monophenolic substrates such as L-tyrosine. As a result, anisaldehyde did not prolong the lag phase indicating that it did not inhibit monophenolase activity.

Table 1. ID_{50} and Mode of Inhibition of the Anise Phenolics and Benzaldehydes

compound tested	ID_{50} (mM)	mode of inhibition
anisaldehyde (**2**)	0.32	noncompetitive
benzaldehyde (**5**)	0.82	noncompetitive
p-hydroxybenzaldehyde (**6**)	1.2	competitive
anise alcohol (**7**)	5.3	−[a]
anisole (**8**)	7.1	−
p-hydroxybenzyl alcohol (**9**)	0.65	−
p-hydroxyanisole (**10**)	0.15	−

[a] −, not tested.

On the basis of this potent tyrosinase inhibitory activity, several closely related congeners such as benzaldehyde (**5**), *p*-hydroxybenzaldehyde (**6**), anisyl alcohol (*p*-methoxybenzyl alcohol) (**7**), and anisole (**8**) were also tested for comparison. It should be noted that the latter two phenolic compounds were also characterized in the anise oil. The results are listed in Table 1. Although none of them exhibited superior activity compared to anisaldehyde, the simplest compound, benzaldehyde, showed the most potent inhibitory activity with an ID_{50} of 87 μg/mL (0.82 mM). This ID_{50} is slightly less than that of anisaldehyde, but almost comparable to that of a well-known tyrosinase inhibitor, benzoic acid (Duckworth and Coleman, 1970; Wilcox et al., 1985). *p*-Hydroxybenzaldehyde also showed inhibitory activity with an ID_{50} of 143 μg/mL (1.2 mM). Thus, inhibition was slightly enhanced by *p*-methoxy substitution and slightly decreased by *p*-hydroxy substitution compared to benzaldehyde. Beside the three aldehydes (**2**, **5**, and **6**), all the remaining compounds (**1**, **4**, **7**, **8**) did not show significant inhibitory activity. In addition, as the aldehyde group in anisaldehyde is reduced to the corresponding benzyl alcohol (**7**), the inhibitory activity was decreased. The results obtained indicate that the aldehyde group is apparently a key group in eliciting potent inhibitory activity. It should be noted that these aldehyde compounds (**2**, **5**, and **6**) themselves cannot be oxidized by the enzyme as substrates.

In addition, the two related phenolic analogues, *p*-hydroxybenzyl alcohol (**9**) and *p*-hydroxyanisole (*p*-methoxyphenol) (**10**), characterized in the anise oil, were also tested. As listed in Table 1, the ID_{50} of 19 μg/mL (0.15 mM) of *p*-hydroxyanisole showed even slightly more potent inhibitory activity than that of anisaldehyde. However, the above two monohydroxyphenolic compounds were oxidized as substrates by the enzyme with lag phase, although *p*-hydroxybenzyl alcohol is hardly soluble in the water based test solution so that the exact lag time was not measured. The lag periods were suppressed as soon as L-DOPA became available. Obviously, they are substrate inhibitors. The structure of *p*-hydroxybenzyl alcohol resembles *p*-cresol that has been most frequently employed as an experimental substrate (Passi and Nazzaro-Porro, 1981; Behbahani

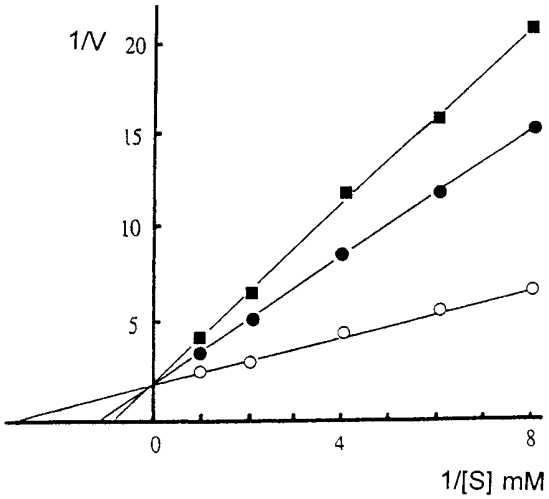


Figure 2. Lineweaver−Burk plots of mushroom tyrosinase and L-DOPA without (○) and with [(●) 0.6 mM, (■) 1.0 mM] *p*-hydroxybenzaldehyde. 1/*V*: 1/Δ 475 nm/min.

et al., 1993), and its oxidation sequence should be similar. In fact, a similar sequence of oxidation was described with *p*-hydroxyanisole (Passi and Nazzaro-Porro, 1981).

The inhibition kinetics by a Lineweaver−Burk plot established benzaldehyde to be a noncompetitive inhibitor, while *p*-hydroxybenzaldehyde a competitive inhibitor as shown in Figure 2. Thus, these two aldehydes affect the enzyme in different ways. The reason for this difference, at least in part, can be explained as follows. *p*-Hydroxybenzaldehyde binds to the binuclear copper active center with the phenolic hydroxy group and competes with the substrate. Interestingly, this monophenolic competitive inhibitor did not serve as a substrate at all. This result is consistent with a previous report (Passi and Nazzaro-Porro, 1981) which indicated that the rate of oxidation is dependent on the electronic properties of the substituent of para-substituted phenol. For example, phenols bearing electron-withdrawing groups such as an aldehyde group at the para position do not serve as substrates at all, but are competitive inhibitors. The aromatic ring is apparently sufficiently deactivated to prevent electrophilic attack by oxygen (Conrad et al., 1994). In addition, as a monophenolic competitive inhibitor, *p*-hydroxybenzaldehyde may only react with *oxy* tyrosinase in an ordered sequence (Eickman et al., 1978; Himmelwright et al., 1980; Winkler et al., 1981).

Aldehydes are, on the other hand, generally protein-reactive compounds. The aldehyde group in benzaldehyde is 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. As a noncompetitive inhibitor, benzaldehyde may form a Schiff base with a primary amino group in the enzyme rather than binding to the binuclear copper active center. The inhibitory activity exerted by benzaldehyde could be based on the assumption that the enzyme [**E**] is complexed with an inhibitor [**I**] and substrate [**S**]. The resulting complex [**ESI**] is inactive and nonproductive. The Schiff base is expected to be largely governed by those factors affecting the stability of the C=N bond. Hence, the introduction of an

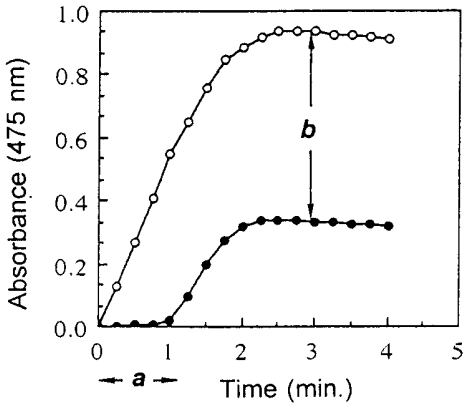


Figure 3. Inhibitory effect of 0.3 mM L-ascorbic acid on mushroom tyrosinase: (○) without L-ascorbic acid, (●) with L-ascorbic acid.

electron-donating methoxy group at the para position in anisaldehyde stabilizes its Schiff base more than that of benzaldehyde and thus the former increases the activity compared to the latter. The data so far obtained indicates that reaction with the other biologically important nucleophilic groups such as sulfhydryl or hydroxyl groups seems unlikely. However, participation of these groups cannot be entirely ruled out because of the limited evidence currently available.

Since the anise has long been used as a condiment, flavor in foods and beverages (Boelens, 1991), as well as oral hygiene products worldwide, its potential for human oral toxicity either is not serious or has been overlooked. On the other hand, L-ascorbic acid, known as vitamin C, is often used in combination with other tyrosinase inhibitors such as kojic acid (Chen et al., 1991), but little is known about the mutual effect of the combination. This knowledge is essential for a better understanding in preventing the enzymatic browning of fruits, vegetables, and crustaceans. Hence, anisaldehyde was tested in combination with L-ascorbic acid. The selection of L-ascorbic acid is not only a well-known tyrosinase inhibitor (Ros et al., 1993) but its effect on tyrosinase has been well studied because of its extensive use in food processing (Golan-Goldhirsh and Whitaker, 1984). Thus, the effect of L-ascorbic acid is known to reduce *o*-quinones back to the corresponding *o*-dihydroxyphenol (Ros et al., 1993). This can be observed as a lag period since the activity is measured by formation of a rather stable dopachrome, a nonenzymatically oxidized product of dopaquinone as shown in Figure 3. It should be noted, however, that our assay was carried out in air-saturated aqueous solutions. Therefore, after several minutes, dopachrome formation reached the plateau as all the available oxygen in the cuvette is consumed. The lag phase ***a*** demonstrates the time in which ascorbic acid reduces dopaquinone back to L-DOPA, indicating indirectly the amount of ascorbic acid consumed. In other words, ascorbic acid is oxidized to dehydroascorbic acid prior to L-DOPA. Similarly, the difference ***b*** is also proportional to the amount of oxygen in the cuvette used for oxidation of ascorbic acid. As illustrated in Figure 4, in the current combination experiment anisaldehyde was found to prolong the lag phase of ascorbic acid shown as ***c***. The reason for this remains unclear. Nevertheless, vitamin C in food may be protected from its oxidation by the combination of tyrosinase inhibitors if they inhibit quinone formation.

In addition to the broad antimicrobial activity of the

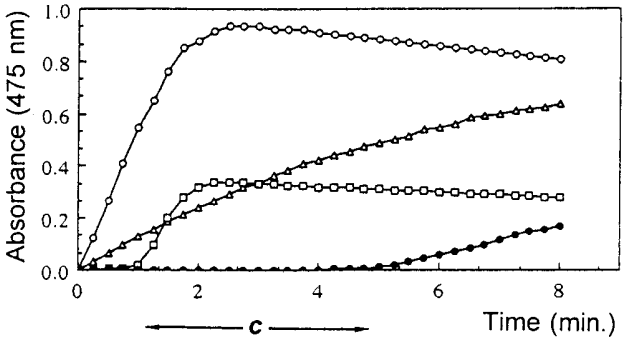


Figure 4. Combination effect of 0.3 mM L-ascorbic acid and 3 mM anisaldehyde: (○) without L-ascorbic acid and anisaldehyde; (□) with L-ascorbic acid, without anisaldehyde; (△) without L-ascorbic acid, with anisaldehyde; (●) with L-ascorbic acid and anisaldehyde.

anise flavor (Kubo and Himejima, 1991), its tyrosinase inhibitory activity can be considered for as an additional application. Furthermore, anisaldehyde, one of its main components, can be considered as a tyrosinase inhibitor. In the latter case, the strong odor of anisaldehyde may limit its practical application, but it can be mixed with other odorous substances to yield an agreeable odor like the anise itself. In an oral toxicity study, no deleterious effects were found as rats were fed diets containing anisaldehyde 1000 and 10 000 ppm for more than 15 weeks (Hagan et al., 1967). This previous report is favorable for its use. Nevertheless, both anise and anisaldehyde are listed as food flavor ingredients in *Fenaroli's Handbook of Flavor Ingredients* (Burdock, 1995).

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