Competitive Inhibition of Mushroom Tyrosinase by 4-Substituted Benzaldehydes

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A kinetic study of the inhibition of mushroom tyrosinase by 4-substituted benzaldehydes showed that these compounds behave as classical competitive inhibitors, inhibiting the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) by mushroom tyrosinase (*o*-diphenolase activity). The kinetic parameter (K_I) characterizing this inhibition was evaluated for all of the seven compounds assayed. Cuminaldehyde showed the most potent inhibitory activity ($K_I = 9\ \mu M$). It also inhibited the oxidation of L-tyrosine by mushroom tyrosinase (*o*-monophenolase activity) in a competitive manner. The corresponding kinetic parameter for this inhibition was evaluated ($K_I = 0.12$ mM).

Keywords: *Tyrosinase; monophenolase; diphenolase; cuminaldehyde; competitive inhibitors*

INTRODUCTION

Tyrosinase (monophenol monooxygenase E:C:1.14.18.1), also known as polyphenol oxidase (PPO) (*1*), is a copper-containing monooxygenase widely distributed in nature. It is responsible for the synthesis of melanin in animals and browning in plants. The enzyme catalyzes two distinct reactions involving molecular oxygen: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of the *o*-diphenols to *o*-quinones (diphenolase activity). Quinones are highly reactive compounds and can polymerize spontaneously to form high-molecular-weight compounds or brown pigments (melanins), or react with amino acids and proteins that enhance the brown color produced (*2*).

Browning can cause deleterious changes in the appearance and organoleptic properties of food products, resulting in shorter shelf life and decreased market value. The unfavorable browning of raw fruits, vegetables, and beverages is a major problem in the food industry and is believed to be one of the main causes of quality loss during postharvest handling and processing. Because of the undesirable effects of enzymatic browning, tyrosinase inhibitors should have a range of applications and, hence, a considerable number are known. In addition, tyrosinase inhibitors have become increasingly important in medicinal (*3*) and cosmetic products (*4*) in relation to hyperpigmentation.

A widely used method in the food and beverage industries to control browning is the addition of reducing agents (sulfites, ascorbic acid, etc.), which chemically reduce the *o*-quinones to the less reactive, colorless diphenols. However, these compounds can also have adverse health effects (specially sulfites) and are also relatively reactive compounds which may react with other components in the food system, resulting in unwanted effects (*5*).

Another important group of browning inhibitors is constituted by compounds structurally analogous to phenolic substrates. These generally show competitive inhibition toward these substrates, although such inhibition may vary depending on the enzyme source and the substrate used (*6*, *7*). Among this group, L-mimosine (*8*), tropolone (*9*), kojic acid (*10*), and 4-substituted resorcinols (*11*) have been described as competitive slow-binding inhibitors, according to the classification of reversible enzyme inhibitors established by Morrison (*12*). 4-Hexylresorcinol has been claimed to be the most effective inhibitor for use in the food industry, and it has been recognized as safe for use in the prevention of shrimp melanosis (*13*, *14*) and for browning control of fresh and hot-air-dried apple slices as well as potatoes, avocados, and apple and grape juices (*15*).

Although a large number of naturally occurring tyrosinase inhibitors have already been described (*16*), their individual activity is either not potent enough to be considered of practical use or safety regulations concerning food additives limit their in vivo use. There is, therefore, a constant search for tyrosinase inhibitors that can be obtained then by laboratory synthesis (*17*) or extraction from plants (*18*, *19*). Cuminaldehyde has been identified as a potent mushroom tyrosinase inhibitor in ether extracts from cumin, a common food spice. Several closely related compounds, derived from benzaldehyde, have been tested for tyrosinase inhibitory capacity. Despite their close structural similarity, these analogues did not behave as better inhibitors than cuminaldehyde; in fact, anisaldehyde exhibited the strongest inhibitory activity, although its ID_{50} was 6-fold less effective than that of cuminaldehyde (*19*).

All these benzaldehyde-selected inhibitors have been studied and described as noncompetitive PPO inhibitors and their ID_{50} values have been determined. However, ID_{50} is not considered a valid parameter for some kinds of tyrosinase inhibitors (*11*) and, furthermore, this kinetic parameter has only been evaluated as regards the diphenolase activity of tyrosinase. The aims of the present paper, therefore, are the kinetic study of the inhibition of monophenolase and diphenolase activities of tyrosinase by 4-substituted benzaldehyde derivates

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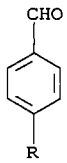

(1) R= H
(2) R= CH_2-CH_3
(3) R= C-$(CH_3)_3$
(4) R= CH-$(CH_3)_2$
(5) R= OCH_3
(6) R= OCH_2-CH_2-CH_3
(7) R= OCH_2-CH_2-CH_2-CH_3

Figure 1. Chemical structures of (1) benzaldehyde and its related derivatives: (2) 4-ethylbenzaldehyde, (3) 4-*tert*-butylbenzaldehyde, (4) 4-isopropyl benzaldehyde or cuminaldehyde, (5) 4-methoxybenzaldehyde or anisaldehyde, (6) 4-propoxybenzaldehyde, and (7) 4-butoxybenzaldehyde.

and an evaluation of the kinetic constants (K_I) characterizing the system.

The results shown in this paper demonstrate that all the 4-substituted benzaldehyde inhibitors tested behave as tyrosinase competitive inhibitors, in contrast to previous results of studies listed in the bibliography.

MATERIALS AND METHODS

Reagents. Mushroom tyrosinase (5350 units/mg), L-3,4-dihydroxyphenylalanine (L-DOPA), and L-tyrosine were purchased from Sigma Quimica (Madrid, Spain). Benzaldehyde, 4-ethylbenzaldehyde, 4-*tert*-butylbenzaldehyde, cuminaldehyde, anisaldehyde, 4-propoxybenzaldehyde, and 4-butoxybenzaldehyde were obtained from Aldrich (Madrid, Spain). All other chemicals were of analytical grade and supplied by Merck (Barcelona, Spain). All buffers were prepared with water purified by a MilliQ water purification system (Millipore Iberica, Madrid,Spain).

Enzyme Assays. *o*-Monophenolase and *o*-diphenolase activities of mushroom tyrosinase were determined by spectrophotometrically measuring the rate of dopachrome formation at 475 nm (ϵ = 3700 M^{-1} cm^{-1}) (*20*) by using a Uvikon 940 spectrophotometer. One unit of enzymatic activity was defined as the amount of enzyme transforming 1 μmol of L-tyrosine or L-DOPA/min. The temperature was controlled at 25 °C using a Haake D1G circulating bath with a heater/cooler and was checked using a Cole-Palmer digital thermometer with a precision of ±0.1 °C. The compositions of the assay media for monophenolase and diphenolase activities of tyrosinase are given in the legends of the figures. The reaction was started by addition of the enzyme. All the inhibitors tested were dissolved in ethanol and the final concentration of ethanol in the reaction medium was 10%. Controls, without inhibitor, containing ethanol at that concentration were routinely carried out.

Other Methods. Protein concentration was determined according to the dye-binding method of Bradford (*21*), using bovine serum albumin (BSA) as a standard.

RESULTS AND DISCUSSION

Cuminaldehyde (Figure 1), obtained by fractionation of ether extracts of cumin, was identified as a potent mushroom tyrosinase inhibitor by Kubo and Kinst-Hori (*19*), who classified it as a noncompetitive inhibitor of the enzyme diphenolase activity. They determined an I_{50} value of 0.05 mM by using L-DOPA as substrate, and reported that cuminaldehyde inhibited the monophenolase activity of tyrosinase although no kinetic study of this inhibitory effect was carried out.

To study in depth tyrosinase inhibition by cuminaldehyde, we studied its effect on the monophenolase and diphenolase activities of the enzyme, and determined the kinetic parameters (K_I).

When the diphenolase activity of mushroom tyrosinase was assayed by using L-DOPA as substrate, the reaction immediately reached a steady-state rate (Figure 2, curve a). The presence of cuminaldehyde in the assay medium decreased the *o*-diphenolase activity (Figure 2, curve b). In addition, tyrosinase also shows monophenolase activity. When the enzymatic reaction was started by the action of tyrosinase on L-tyrosine, a marked lag period, characteristic of monophenolase activity, was observed simultaneously with the appearance of the first stable product, dopachrome (Figure 2, curve c). The system reached a constant rate after the lag period, which was estimated by extrapolation of the linear portion of the product accumulation curve to the abscissa (*20*). As can be seen from curve d in Figure 2, cuminaldehyde also slowed the rate of dopachrome formation when tyrosine was used as substrate, behaving, therefore, as an inhibitor of the monophenolase activity of tyrosinase. The lag period depends on both enzyme and substrate concentrations in the reaction medium and can be shortened, or even abolished, by the presence of catalytic amounts of transition metal ions or, especially, *o*-diphenols (*22*). However, the lag phase is known to be extended by some monophenolase inhibitors such as tropolone (*7*) or cumic acid (*19*). Nevertheless, other inhibitors such as kampferol (*23*) or α,β-unsaturated aldehydes ((2E)-alkenals) (*24*) did not lengthen the lag phase. Cuminaldehyde lengthened this lag phase, as can be seen in Figure 2 (curves c and d), especially when the inhibitor concentration was higher than 0.2 mM (data not shown).

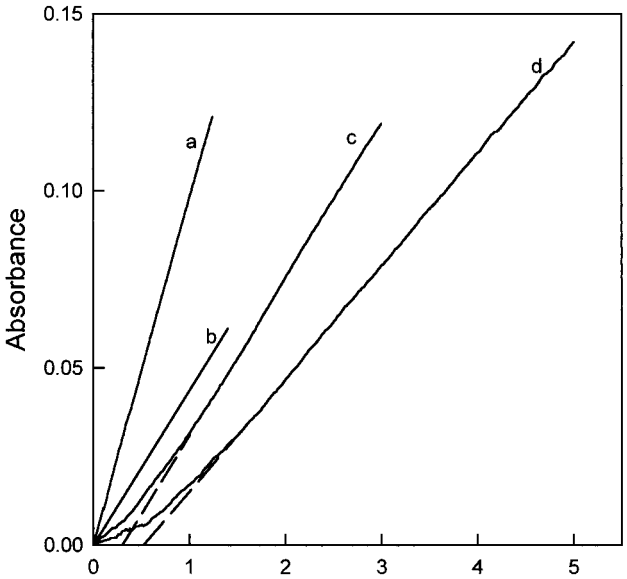

Figure 2. Progress curves for the inhibition of mushroom tyrosinase by cuminaldehyde. Curves a and b show the effect of the inhibitor on the *o*-diphenolase activity of the enzyme. The reaction media contained L-DOPA 1.5 mM in 70 mM sodium phosphate buffer, pH 6.5 (curve a). Curves c and d show the effect of cuminaldehyde on the monophenolase activity of the enzyme. The reaction media contained L-tyrosine 0.1125 mM in 70 sodium phosphate buffer, pH 6.5. Cuminaldehyde concentrations were (a) 0, (b) 0.05 mM, (c) 0, and (d) 0.15 mM. The reaction was started by the addition of the enzyme (15 μg/mL).

To obtain further information on the type of inhibition exerted by cuminaldehyde on mushroom tyrosinase, *o*-diphenolase activities (V) were measured as a function of L-DOPA concentration for several concentrations of the inhibitor. A plot of 1/V vs inhibitor concentration (Figure 3) shows that cuminaldehyde produced a simple competitive inhibition in contrast to the noncompetitive

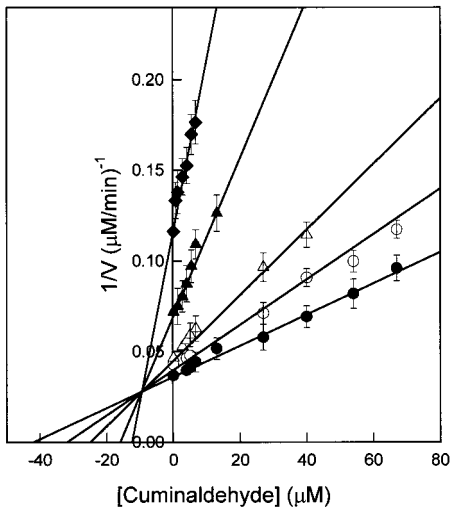

Figure 3. Dixon plots for the inhibition of the *o*-diphenolase activity of mushroom tyrosinase by cuminaldehyde. The reaction media (1.0 mL) contained L-DOPA in 70 mM sodium phosphate buffer (pH 6.5), the indicated concentration of inhibitor, and mushroom tyrosinase (15 μg/mL). L-DOPA concentrations were (●) 1.5, (○) 1, (△) 0.5, (▲) 0.26, and (◆) 0.15 mM.

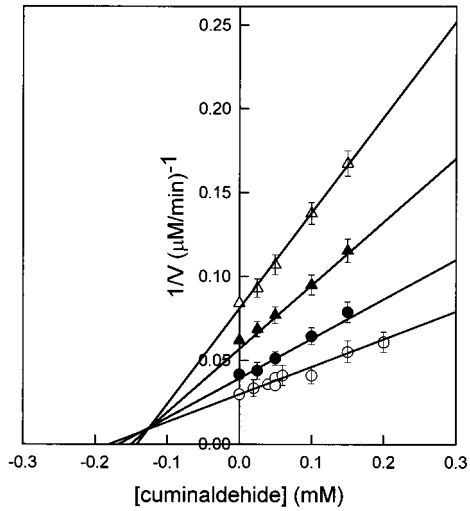

Figure 4. Dixon plots for the inhibition of the *o*-monophenolase activity of mushroom tyrosinase by cuminaldehyde. The reaction media (1.0 mL) contained L-tyrosine in 70 mM sodium phosphate buffer (pH 6.5), the indicated concentration of inhibitor, and mushroom tyrosinase (35 μg/mL). L-Tyrosine concentrations were (○) 0.45, (●) 0.225, (△) 0.15, (▲) 0.1125, and (◆) 0.075 mM.

inhibition reported in the literature (*19*). The K_I value estimated from this Dixon plot was 9 μM.

To ascertain whether cuminaldehyde also behaved as a competitive inhibitor as regards the monophenolase activity of mushroom tyrosinase, the rate of dopachrome accumulation was measured as a function of L-tyrosine concentration for several concentrations of inhibitor. The Dixon plot obtained (Figure 4) shows that cuminaldehyde is also a competitive inhibitor of the monophenolase activity of tyrosinase. The K_I value obtained for this plot was 0.12 mM.

The aldehyde group is generally known to react with biologically important nucleophilic groups such as sulfhydryl, amino, or hydroxyl groups. The formation of a Schiff base with a primary amino group in the enzyme has been proposed as being responsible for the inhibitory effect of cuminaldehyde. It is known that the formation

Table 1. Values of the Kinetic Constants (K_I) for the Inhibition of the Diphenolase Activity of Mushroom Tyrosinase by Benzaldehyde and Related Compounds

compounds tested	K_I (mM)
group I	
4-methoxybenzaldehyde (anisaldehyde)	1.5
4-propoxybenzaldehyde	1.3
4-butoxybenzaldehyde	1
group II	
benzaldehyde	0.03
4-ethylbenzaldehyde	0.04
4-*tert*-butylbenzaldehyde	0.02
4-isopropylbenzaldehyde (cuminaldehyde)	0.009

of a Schiff base is largely governed by factors affecting the stability of the carbon−nitrogen double bond. And it has been suggested that the more stable the Schiff base is, the more potent the inhibitory activity. More specifically, the more hydrophobic the electron donor group at the *para* position in benzaldehyde, the stronger the inhibition (*19*). Taking into account this hypothesis and the strong tyrosinase inhibitory activity of cuminaldehyde, several closely related compounds were tested as tyrosinase inhibitors. All these compounds are derived from benzaldehyde by substitution at the *para* position of an electron-donating group (Figure 1). When the inhibition kinetics of these compounds were analyzed by Dixon plots (not shown) and the corresponding values of K_I were determined (Table 1), the results showed that their inhibitory activities were no greater than that of cuminaldehyde (Table 1) and that the benzaldehyde derivatives tested behaved as competitive inhibitors of the *o*-diphenolase activity of mushroom tyrosinase. These inhibitors could be divided into two groups according to their K_I values and to the nature of the substituent at the *para* position. One group would include anisaldehyde (metoxybenzaldehyde), propoxybenzaldehyde, and butoxybenzaldehyde (group I). These compounds are worse inhibitors than benzaldehyde and the rest of the analogues tested (group II). For instance, butoxybenzaldehyde has a K_I value of 1 mM, which is about 50- and 100-fold higher than that of 4-*tert*-butylbenzaldehyde and cuminaldehyde, respectively. The difference in inhibitory activity between these two groups could be explained by the lower electron-donating capacity of the methoxy-, propoxy-, and butoxy-substituents at the *para* position. However, as can be seen in Table 1, there is no significant difference in the K_I values between the inhibitors of the same group. Nevertheless, according to their increasing inductive effect, the order of inhibitory activity for the compounds in group II (except cuminaldehyde) would be: 4-*tert*-butylbenzaldehyde> 4-ethylbenzaldehyde >benzaldehyde. For that reason, the inductive effect of the substituent group is probably not the only factor to explain the inhibition of tyrosinase activity: steric effects should also probably be considered. It has been proposed that the isopropyl group in cuminaldehyde is better embraced than the methoxy group in anisaldehyde, by means of the hydrophobic protein pocket close to the binuclear active site, which would explain its greater inhibitory activity (*25*). Therefore, several factors must influence the binding stability of the enzyme and the inhibitor, as well as the stability of the Schiff base formed.

In addition, preincubation of the enzyme with the inhibitor in the absence of the substrate did not decrease the enzyme activity significantly, suggesting that these

benzaldehyde derivatives are inhibitors rather than inactivators of the enzyme (*7*).

CONCLUSION

The results obtained in this paper clearly show that benzaldehyde and its 4-substituted derived analogues can be classified as competitive inhibitors of mushroom tyrosinase. Despite their close structural similarity, cuminaldehyde showed a higher inhibitory activity than the rest of benzaldehyde derivatives. Cuminaldehyde also behaved as a competitive inhibitor of the monophenolase activity of mushroom tyrosinase.

The reason for the inhibitory activity has been attributed to the formation of a Schiff base between the aldehyde group and a primary amino group of the enzyme. This Schiff base would be stabilized by the inductive effect of the electron-donating group at the *para* position. However, the inductive effect cannot be the only explanation for the inhibition of tyrosinase because benzaldehyde itself showed similar inhibitory activity to the other 4-substituted derivatives. Moreover, the K_I value for benzaldehyde was even smaller than the K_I values obtained for methoxy-, propoxy-, and butoxy-benzaldehydes.

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