Tyrosinase Inhibition Kinetics of Anisic Acid

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Anisic acid (*p*-methoxybenzoic acid) was characterized as a tyrosinase inhibitor from aniseed, a common food spice. It inhibited the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) catalyzed by tyrosinase with an IC_{50} of 0.60 mM. The inhibition of tyrosinase by anisic acid is a reversible reaction with residual enzyme activity. This phenolic acid was found to be a classical noncompetitive inhibitor and the inhibition constant K_I was obtained as 0.603 mM. Anisic acid also inhibited the hydroxylation of L-tyrosine catalyzed by tyrosinase. The lag phase caused by the monophenolase activity was lengthened and the steady-state activity of the enzyme was decreased by anisic acid.

Key words: Tyrosinase Inhibitory Activity, Anisic Acid, Noncompetitive Inhibition Kinetics

Introduction

Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase (PPO) (Zawistowski *et al.*, 1991; Whitaker, 1995; Mayer and Harel, 1998), is a copper containing mixed-function oxidase widely distributed in microorganisms, animals and plants. This oxidase catalyzes two distinct reactions of melanin synthesis, the hydroxylation of a monophenol and the conversion of an *o*-diphenol to the corresponding *o*-quinone (Robb, 1984). The hydroxylation of L-tyrosine, the initial step in melanin synthesis, is of considerable importance since it is also the initial step in catecholamine synthesis. Alterations in melanin synthesis occur in many disease states. Melanoma specific anticarcinogenic activity is also known being linked with tyrosinase activity (Prezioso *et al.*, 1992). Melanins are also found in the mammalian eye and brain. Tyrosinase may play a role in neuromelanin formation in the human brain and could be central to dopamine neurotoxicity as well as contribute to the neurodegeneration associated with Parkinson's disease (Xu *et al.*, 1997). Tyrosinase is also responsible for browning in plants and considered to be deleterious to the color quality of plant derived foods and beverages. This unfavorable darkening from enzymatic oxidation generally results in a loss of nutritional and economic values 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 medicinal (Mosher *et al.*, 1983) and cosmetic (Maeda and Fukuda, 1991) products in relation to hyperpigmentation. Hence, tyrosinase inhibitors should have broad applications.

By bioassay-guided fractionation using mushroom tyrosinase, anisaldehyde (*p*-methoxybenzaldehyde) was characterized as the principal inhibitor from the *n*-hexane extract of the seeds of *Pimpinella anisum* L. (Umbelliferae) (Kubo and Kinst-Hori, 1998), commonly known as aniseed and widely used as a food spice. In our continuing search for tyrosinase inhibitors from plants (Kubo, 1997), the ethyl acetate fraction of the same plant was found to show the inhibitory activity and was subjected to further characterization.

Material and Methods

General

General procedures were the same as in the previous works (Kubo *et al.*, 1994; Kubo and Kinst-Hori, 1998).

Chemicals

Anisic acid, *p*-ethoxybenzoate, *p*-propoxybenzoate, *p*-butoxybenzoate, 2-methoxybenzoic acid, 2,4-dimethoxybenzoic acid, and L-DOPA were purchased from Aldrich Chemical Co. (Milwaukee, WI). Benzoic acid, sodium dithionite, L-tyrosine, and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO).

Enzyme assay

The mushroom tyrosinase (EC 1.14.18.1) used for the bioassay was purchased from Sigma Chemical Co., and was used without further purification. Although mushroom tyrosinase differs somewhat from other sources (van Gelder *et al.*, 1997), this fungal source was used for the entire experiment because it is readily available. The preliminarily assay was tested at 6.67 μg/ml, unless otherwise specified. All the samples were first dissolved in DMSO and used for the experiment at 30 times dilution. The final concentration of DMSO in the test solution is 3.3 %. Tyrosinase functions both as a monophenolase and as an *o*-diphenolase. In the current experiment, L-DOPA was used as a substrate and hence, the activity studied is *o*-diphenolase inhibitory activity, unless otherwise noted. Tyrosinase catalyzes a reaction between two substrates, a phenolic compound and oxygen, but the assay was carried out in air-saturated solutions. The enzyme activity was monitored by dopachrome formation at 475 nm ($\varepsilon = 3700\ \text{M}^{-1}\ \text{cm}^{-1}$) accompanying the oxidation of L-DOPA. One unit (U) of the enzymatic activity was defined as the amount of enzyme increasing 0.001 absorbance at 475 nm in the current experimental condition.

The diphenolase assay was performed as previously described (Kubo and Kinst-Hori, 1998) with slight modifications (Chen *et al.*, 2000). First, 0.3 ml of a 5.0 mM L-DOPA aqueous solution was mixed with 0.6 ml of 0.25 M phosphate buffer (pH 6.8) and 1.9 ml water, incubated at 30 °C for 10 min. Then, 0.1 ml of the sample solution and 0.1 ml of the aqueous solution of the mushroom tyrosinase (0.2 mg/ml in 0.1 M phosphate buffer, pH 6.8) were added in this order to the mixture. This solution was immediately monitored for the formation of dopachrome by measuring the linear increase in optical density at 475 nm. The reaction was carried out under a constant temperature of 30 °C. Absorption measurements were recorded using a Spectra MAX plus Microplate spectrophotometer.

The monophenolase assay was performed with L-tyrosine as a substrate at 30 °C. To the 3 ml assay system containing 50 mM phosphate sodium buffer (pH 6.8), 2 mM L-tyrosine, different concentrations of anisic acid and 0.1 ml of the enzyme (1.0 mg/ml in 0.1 M phosphate buffer, pH 6.8) was added, and the solution was immediately monitored by the optical density at 475 nm, similar to the diphenolase assay.

Measurement of oxygen consumption

The reaction mixture consisted of 0.6 ml of 0.25 M phosphate buffer (pH 6.8), 1.9 ml of water and 0.1 ml of 6 mM anisic acid or anisaldehyde DMSO solution was first incubated at 30 °C for 5 min. Then, 0.1 ml of 0.05 M phosphate buffer solution of mushroom tyrosinase (20 μg) was added and oxygen consumption was measured with an OBH 100 oxygen electrode and an oxygraph equipped with a water-jacket chamber of YSI 5300 (all from Yellow Springs Instruments Co., Yellow Springs, OH) maintained at 30 °C for 20 min. A catalytic amount (0.01 mM) of L-DOPA was added after 5 min. The oximeter was calibrated with air (100 %) and sodium dithionite (0 %).

Results and Discussion

The effect of anisic acid on the oxidation of L-DOPA catalyzed by mushroom tyrosinase was first studied. Anisic acid showed a dose-dependent inhibitory effect on this oxidation. As anisic acid increased, the enzyme activity was rapidly decreased but not completely suppressed. As the concentration of anisic acid reached to 2 mM, the remaining enzymatic activity was about 24 %. The inhibitory concentration leading to 50 % activity lost (IC_{50}) was estimated to be 0.60 mM. This IC_{50} is nearly comparative to that of benzoic acid, a well-documented tyrosinase inhibitor (Conrad *et al.*, 1994).

Subsequently, the inhibition mechanism of mushroom tyrosinase by anisic acid during the oxidation of L-DOPA was elucidated. The relationship of enzyme activity in the presence of different concentrations (0, 0.1, 0.2, 0.3, and 0.4 mM) of anisic acid. The results were a family of straight lines,

which passed through the origin. Increasing the concentration of anisic acid resulted in the descending of the slopes of the lines, indicating that the inhibition of anisic acid on the enzyme was a reversible reaction course. The presence of anisic acid did not bring down the amount of the efficient enzyme, but resulted in the inhibition and the descending of the enzyme activity.

The inhibition kinetics of tyrosinase by anisic acid was investigated. The oxidation of L-DOPA catalyzed by tyrosinase follows Michaelis-Menten kinetics. The kinetic parameters for this enzyme obtained from a Lineweaver-Burk plot show that K_m is equal to 0.645 mM and V_m is equal to 138 mM/min. It should be noted however that tyrosinase catalyzes a reaction between two substrates, a phenolic compound and oxygen, but the assay was carried out in air-saturated aqueous solutions. Therefore, K_m and V_m values determined in this condition were only apparent, and the effect of oxygen concentration on these parameters is unknown.

The kinetic behavior of the oxidation of L-DOPA by mushroom tyrosinase at different concentrations of anisic acid, indicates that anisic acid is a classical noncompetitive inhibitor to the enzyme (Fig. 1). It can decrease the apparent value of V_m with no effect on that of K_m. This behavior is observed that anisic acid can combine with both free enzyme and the enzyme-substrate complex, and there are same binding intensity between anisic acid and both of the enzyme forms.

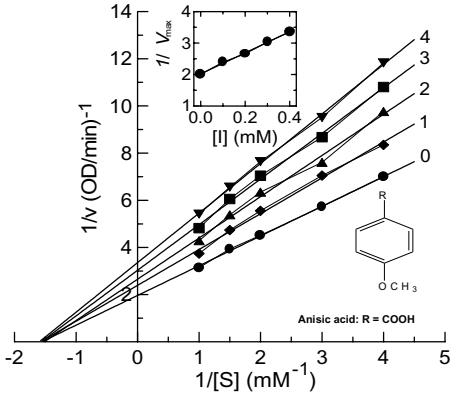

Fig. 1. Lineweaver-Burk plots for inhibition of anisic acid on mushroom tyrosinase for the catalysis of L-DOPA at 30 °C. Concentration of anisic acid for curves 0–4 was 0, 0.1, 0.2, 0.3, and 0.4 mM, respectively. The inset represents the plot of $1/V_{max}$ *versus* the anisic acid concentration for determining the inhibition constants K_I. The line was drawn using linear least squares fit. Chemical structure of anisic acid and its related phenolics.

The equilibrium constants for inhibitor binding with free enzyme (E), K_I, and with enzyme-substrate (ES) complex, K_{IS}, are the same in quantity, which can be obtained from the second plot of the "Intercept" versus concentration of inhibitor, which are linears (Fig. 1).

In addition, *p*-ethoxybenzoic acid, *p*-propoxybenzoic acid, and *p*-butoxybenzoic acid were also tested for their mushroom tyrosinase inhibitory activity emphasizing their inhibition type for comparison. As a result, the oxidation of L-DOPA catalyzed by mushroom tyrosinase was inhibited by these *p*-alkoxybenzoates and the inhibitory activity decreased with increasing carbon chain length. Interestingly, the inhibition type also changed with increasing carbon chain length as noncompetitive, mixed type, and, presumably to competitive inhibitors. Neither 2-methoxybenzoic acid and nor 2,4-dimethoxybenzoic acid exhibited any inhibitory activity up to 5 mM.

Tyrosinase contains functions both as a monophenolase and an *o*-diphenolase. The discussion so far described is based on the experiment using L-DOPA as a substrate. Therefore, the activity mentioned is *o*-diphenolase inhibitory activity of mushroom tyrosinase. On the other hand, the lag time is known for the oxidation of monophenol substrates such as L-tyrosine to L-DOPA. This lag time can be extended by monophenolase inhibitors such as tropolone (Kahn and Andrawis, 1985) and galangin (Kubo and Kinst-Hori, 1998). Anisic acid was noted to inhibit the monophenolase of mushroom tyrosinase. The time course of the oxidation of L-tyrosine (substrate) in the presence of different anisic acid concentrations is shown in Fig. 2. At each concentration of anisic acid, the lag period of L-tyrosine oxidation by tyrosinase was lengthened with increasing the concentration of this inhibitor. The steady-state rate was also influenced. As the concentration of anisic acid increased, the steady-state activity of the enzyme was rapidly decreased. As the concentration of anisic acid reached to 4 mM, the remaining enzymatic activity was about 20 %. The inhibitory concentration leading to 50 % activity lost (IC_{50}) was estimated to be 2.20 mM.

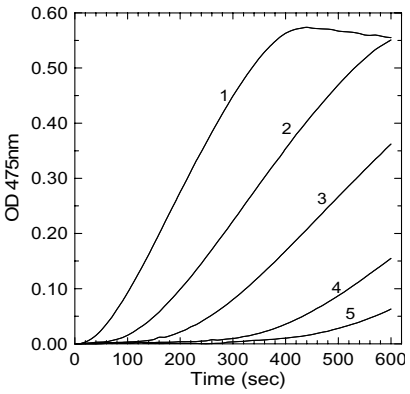

Fig. 2. Effects of anisic acid on the activity of mushroom tyrosinase for the oxidation of L-tyrosine at 30 °C. The reaction media (3 ml) contained 0.5 mM of tyrosine in 50 mM sodium phosphate buffer (pH 6.8), mushroom tyrosinase 33.3 μg/ml. The concentration of anisic acid for curves 1–6 was 0, 0.4, 1.0, 2.0, 3.0 and 4.0 mM, respectively.

In the different concentration of L-tyrosine, the lag period of the oxidation reaction was lengthened by anisic acid in different degree, as shown in Fig. 3. When the concentration of tyrosine was low, the lag period was lengthened by anisic acid by a greater degree. Increasing the concentration of monophenol resulted that the degree of lengthening lag period became weaker, indicating that the substrate offers marked protection of the enzyme against inhibition by anisic acid. It also can be seen from reducing the decreasing the steady-state activity of the enzyme in the presence of anisic acid.

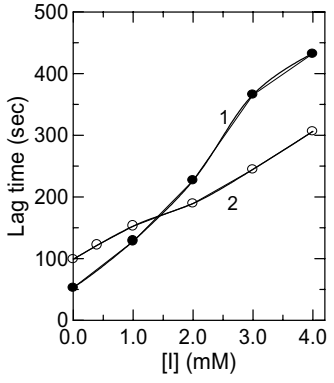

Fig. 3. Effect of monophenol concentration on lengthening the lag time of mushroom tyrosinase by anisic acid. The concentrations of L-tyrosine for curves 1 and 2 were 0.5 and 2.0 mM, respectively.

Table I. Kinetic and inhibition constants of anisic acid and anisaldehyde.

Constants	Anisic acid	Anisaldehyde
IC_{50}	0.60 mM	0.40 mM
K_M	0.645 mM	0.664 mM
V_m	0.138 mM/min	0.125 mM/min
Inhibition	reversible	reversible
Inhibition type	noncompetitive	noncompetitive
K_I	0.603 mM	0.376 mM

Anisaldehyde isolated from the same source was previously reported as a noncompetitive inhibitor (Kubo and Kinst-Hori, 1998), its inhibition mode was also reinvestigated. The result obtained by the current kinetic study confirmed that the inhibition mode of anisaldehyde is the same as before. The kinetic constants obtained are summarized in Table I. In addition, anisaldehyde did not inhibit the hydroxylation of L-tyrosine (monophenolase activity) the same as previously reported (Kubo and Kinst-Hori, 1998). This result is different from that of anisic acid being described above. In brief, anisic acid inhibits monophenolase activity but anisaldehyde does not.

As above mentioned, tyrosinase is known to catalyze a reaction between two substrates, a phenolic compound and oxygen. The discussion so far described is based on the experiment using mainly L-DOPA as a substrate. As the need arises, L-tyrosine was also assayed to see if the inhibitors characterized were effective on monophenolase activity. Consequently, the enzyme activity was monitored by dopachrome formation at 475 nm. On the other hand, the fact that oxygen is the other substrate needs to be considered. Hence, the enzyme activity was also monitored by oxygen consumption to examine it from a different angle. The mixture consisted of tyrosinase (20 μg) and 0.2 mM of the samples was incubated and oxygen consumption was measured for 20 min. The arrow indicates the time when catalytic amount (0.01 mM) of L-DOPA was added. The results obtained are shown in Fig. 3. As expected, neither anisic acid nor anisaldehyde consumed oxygen at all even after adding L-DOPA. The results are consistent with the data observed by monitoring dopachrome formation.

The current kinetic study of tyrosinase inhibition found that anisic acid is a noncompetitive in-

hibitor and leads to reversible inhibition with residual enzyme activity. The results obtained are consistent with those of the previous report (Kubo and Kinst-Hori, 1998). The kinetic behaviors of the *p*-alkoxybenzoic acids tested are similar to anisic acid.

According to previous papers (Conrad *et al.*, 1994), as long as a free carboxyl group exists in the aromatic ring, hydroxyphenolic acids such as *para*- and *meta*-hydroxybenzoic acid bind to the binuclear copper active center presumably with the more acidic carboxylic group. These hydroxyphenolic acids are coordinated to one copper as if monophenol substrates and are positioned over the binuclear active site, and inhibit the enzyme competitively (Wilcox *et al.*, 1985). However, this may not be the case for anisic acid. Although the precise explanation how these benzoic acid derivatives interact with the enzyme on a molecular basis is still largely unknown, anisic acid may disrupt the tertiary structure of the enzyme through intermolecular hydrogen bonding. This is more likely as a non-competitive inhibitor, in part, reducing the affinity of the substrates with the enzyme (Jackman *et al.*, 1992). This indicates that the carboxylic group is involved in the hydrogen-bonding interactions. Thus, anisic acid may disrupt this interaction.

The observation that neither 2-methoxybenzoic acid nor 2,4-dimethoxybenzoic acid exhibited any inhibitory activity supports the postulate because of the steric hindrance. The low conformational stabilities of native proteins make them easily susceptible to denaturation by altering the balance of the weak nonbonding forces that maintain the native conformation.

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